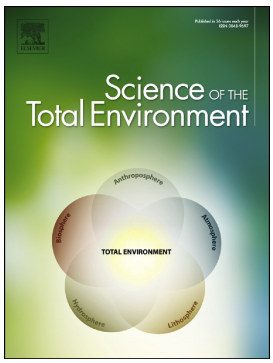

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Long-term nitrogen and phosphorus fertilization reveals that phosphorus limitation shapes the microbial community composition and functions in tropical montane forest soil

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Abstract

Microorganisms govern soil nutrient cycling. It is therefore critical to understand their responses to human-induced increases in N and P inputs. We investigated microbial community composition, biomass, functional gene abundance, and enzyme activities in response to 10-year N and P addition in a primary tropical montane forest, and we explored the drivers behind these effects. Fungi were more sensitive to nutrient addition than bacteria, and the fungal community shift was mainly driven by P availability. N addition aggravated P limitation, to which microbes responded by increasing the abundance of P cycling functional genes and phosphatase activity. In contrast, P addition alleviated P deficiency, and thus P cycling functional gene abundance and phosphatase activity decreased. The shift of microbial community composition, changes in functional genes involved in P cycling, and phosphatase activity were mainly driven by P addition, which also induced the alteration of soil stoichiometry (C/P and N/P). Eliminating P deficiency through fertilization accelerated C cycling by increasing the activity of C degradation enzymes. The abundances of C and P functional genes were positively correlated, indicating the intensive coupling of C and P cycling in P-limited forest soil. In summary, a long-term fertilization experiment demonstrated that soil microorganisms could adapt to induced environmental changes in soil nutrient stoichiometry, not only through shifts of microbial community composition and functional gene abundances, but also through the regulation of enzyme production. The response of the microbial community to N and P imbalance and effects of the microbial community on soil nutrient cycling should be incorporated into the ecosystem biogeochemical model.

Keywords: Nitrogen and phosphorus fertilization; microbial community composition; enzyme activity; functional gene abundance; nutrient cycling; tropical montane forest

Introduction

Tropical forests are characterized by high plant diversity, hold about 30% of terrestrial carbon (C) stocks, and play a key role in global nutrient cycles (Pan et al., 2011; Camenzind et al., 2014). However, tropical forests are disturbed and may be endangered by human activities, even if not directly felled (Dixon et al., 1994; Bardgett and van der Putten, 2014). Imbalanced N and P inputs are catastrophic, especially in the tropics, where the highly weathered soils are mostly poor in P (Camenzind et al., 2018), but continuously receive high N deposition (30–70 kg ha^{-1} y^{-1}) (Reay et al., 2008). Imbalanced N and P inputs may cause changes in ecosystem structure and functions, with unforeseeable effects on soil biogeochemical cycles (Huang et al., 2012). This highlights the urgent need to study the independent and interactive effects of N and P addition on tropical forest ecosystems.

The addition of N and P can influence the above- and belowground properties of forest ecosystems, such as plant growth, belowground C input, root physiology and exudation and soil biochemistry (Janssens et al., 2010; Carrara et al., 2018; Jiang et al., 2018a). All such changes ultimately affect microbial biomass, community composition, functional gene abundance, and microbial activities (Abarenkov et al., 2010; Bellemain et al., 2010; Zheng et al., 2015). Changes in the microbial community structure and the shift of functions under changing nutrient conditions are correlated with environmental factors such as pH, total organic C, total N (TN), mineral N content, and soil C: N: P stoichiometry (Abarenkov et al., 2010; Högberg et al., 2007; Zhou et al., 2013). The driving factors depend on the form of N and P addition, its amount and duration, plant species and their nutritional uptake, and soil properties such as texture and pH (Dai et al., 2020; Widdig et al., 2020). For example, a

previous study demonstrated that long-term N addition resulted in the decrease of pH and was the main driver of shifts in microbial community composition for soil with pH values ranging from 4.5 to 8.5, but not when the soil pH fell below 4.5 (Rousk et al., 2010; Lu et al., 2014). P is a common limiting element for the growth of soil microorganisms in highly weathered tropical ecosystems (Mori et al., 2018). P addition alleviates P limitation and changes soil nutrient stoichiometry, such as the C/P and N/P ratios. Whether these changes are driving factors of shifts in microbial community composition and changes in associated functions in tropical forests requires further investigation.

Microbial community and activity were more responsive to P addition than to N addition in an N-rich and P-poor tropical forest (Li et al., 2015; LeBauer and Treseder, 2008). Microorganisms have various adaptations and exhibit different responses to N and P addition. Fungal microorganisms are more sensitive to increases in nutrient availability compared with bacteria (Widdig et al., 2020). Specifically, fungi are more sensitive to P availability because they play a critical role in P mineralization and assimilation (Smith and Read, 2008). Within a soil microbial community, different taxonomic or functional microbial groups may have divergent responses to N and P addition. For example, some groups have higher P mobilization ability; their abundance may decrease with P addition. Microbial responses to N and P addition will in turn affect soil organic matter (SOM) turnover, nutrient cycles, and plant growth (Bardgett and van der Putten, 2014; Castrillo et al., 2017; Duhamel et al., 2021). Investigating the responses of soil microorganisms to N and P addition will improve the prediction of the structure and function of tropical forests under global environmental changes.

Another consequence of N and P addition to microbial communities is their effects on the contribution of microbial necromass to SOM (Bellemain et al., 2010; Hu et al., 2022). Amino sugars have been widely used to indicate the contribution of microbial residues to SOM accumulation in various habitats (Bellemain et al., 2010; Huang et al., 2019). Three amino sugars, namely, glucosamine (GluN), galactosamine (GalN) and muramic acid (MurN), have often been quantified in order to assess bacterial and fungal residues (Joergensen, 2018; Dippold et al., 2019). MurN is mainly found in the peptidoglycans of bacterial cell walls, whereas GluN primarily originates from fungal chitin (Huang et al., 2018; Joergensen, 2018). In addition to contributing to SOM formation, microorganisms mediate SOM decomposition through the synthesis and release of extracellular enzymes that catalyze the depolymerization of macromolecules into oligomers and monomers (Shah et al., 2016; Carreiro et al., 2000). Microorganisms produce enzymes to acquire nutrients, energy, and carbon (Asmar et al., 1994), but their synthesis and release are energy-consuming processes (Gunina and Kuzyakov, 2022). The activity of specific enzymes is thus increased or decreased by strict cellular regulation, which is dependent on the nutrient requirements of the microbial cells related to the soil nutrient stoichiometry. For instance, if N deposition aggravates P limitation, phosphatase activity will be increased to acquire more P. In contrast, P addition decreases phosphatase activity once the P demand is satisfied (Marklein and Houlton, 2012). The cycling of P and C are tightly coupled; for example, P addition can increase the soil CO_2 flux (Soong et al., 2018). Microorganisms in tropical forest soils are often C-limited (Soong et al., 2018). A previous study showed that P and NP addition could alleviate P limitation in microbes and trees (Liu et al., 2012; Jiang et al., 2018b). How the removal of nutrient

limitations will affect the C cycling of tropical forests remains to be revealed (Soong et al., 2020).

Extracellular enzymes are widely considered to be proxies of microbial-mediated SOM decomposition (Allison et al., 2010; Treseder et al., 2012). However, the relationships between changes in microbial community composition, functional gene abundance, and enzyme activities under a changing environment are seldom studied and remain disputed (Chen and Sinsabaugh, 2021). Long-term P input decreased the relative abundance of the P-starvation response gene (*PhoR*) and increased the low-affinity inorganic phosphate transporter gene (*pit*) (Dai et al., 2020). Microbial functional gene abundance has been found to be closely related to specific C-degrading enzyme activities (Trivedi et al., 2016; Moore et al., 2021), while other studies have found no relationship between C mineralization and the abundance of related genes (Wood et al., 2015). Such contrasting outcomes result from the fact that the microbial functional gene abundance merely reveals the potential of microbial communities, rather than reflecting the levels of gene expression underlying the enzymatic activity (Bergkemper et al., 2016; Trivedi et al., 2016). Elucidating the systematic links between microbial community composition, functional gene abundance, and enzyme activities related to N and P addition is a critical prerequisite to predict the future nutrient cycling and functions of tropical forest ecosystems.

This study was based on a 10-year N and P addition field experiment in a monsoonal evergreen broadleaf primary tropical montane forest in southern China. Previously, this research group reported that microbial community structure, enzyme activity, and microbial C cycling were not modified by short-term (4–5 years) N and P fertilization (Jing et al., 2020;

Ma et al., 2020). Accurate information on the changes in microbial community structure and function under long-term N and P addition obtained through amplicon sequencing and the mechanism of this process have not yet been investigated (Ma et al., 2020, 2021). This study explored the effects of 10 years of N and P addition on soil biochemical properties, microbial community composition, enzyme activities, functional gene abundances and microbial necromass accumulation, and examined the drivers behind the observed responses. In addition, this study revealed the relationship between enzyme activities and their related functional gene abundance. We hypothesize that (1) P addition will decrease soil C/P and N/P ratios, which are the main drivers of microbial community and function shifts, because P is the most limited element in highly weathered tropical forest soils; (2) fungi have a strong response and are more sensitive to N and P addition than bacteria, especially in relation to P addition, because fungi play a key role in organic P hydrolyzation and assimilation; and (3) P and C cycling are tightly coupled, and the removal of P limitation will facilitate C cycling.

2 Material and Methods

2.1 Site description

This study was conducted in a tropical montane primary rainforest in Jianfengling (JFL) (18°23–18°52′ N, 108°46–109°02′ E, elevation 870 m), southwestern Hainan Island, China. This experimental site is part of the Network of Nutrient Enrichment Experiments in China's Forests (Du et al., 2013). The study site has an obvious seasonal change between wet (May–October) and dry (November–April) seasons, with an average annual precipitation of 2200 mm. The area has a typical humid climate, with a mean relative humidity of 88% (Jiang et al., 2018b). The average temperatures in the coldest and warmest months are 10.8°C and 27.5°C,

respectively. The soil is predominantly typical lateritic yellow soil with a pH of 4.8, and the soil C, N, and P contents are 22 g kg^{-1}, 1.6 g kg^{-1}, and 140 mg kg^{-1}, respectively, in the topmost 10 cm (Table 1). The total ambient N and P deposition amounts are approximately 9 kg N ha^{-1} yr^{-1} and 1.0 kg P ha^{-1} yr^{-1}, respectively (Du et al., 2016; Wang et al., 2018). The ambient N deposition is lower than the mean deposition rate on Hainan Island (~25 kg N ha^{-1} yr^{-1}) (Jiang et al., 2018b). Thus, unlike most lowland forests in the tropics and subtropics, which are N-saturated, the primary forest of JFL may still be N-limited. The forest is dominated by *Cryptocarya chinensis*, *Cyclobalanopsis patelliformis*, *Mallotus hookerianus*, *Gironniera subaequali*, and *Nephel iumtopengii* (Zhou et al., 2013). The topography is relatively homogeneous, with slopes ranging from 0° to 5° (Du et al., 2013).

2.2. Experimental design

The N- and P-addition experiment in the primary tropical forest was set up in a randomized block design with three replicates, where the distance between any two blocks was > 1 km. Each randomized block consisted of four 20 × 20 m nutrient addition plots with 10-m buffers between plots to prevent runoff effects. Four treatments were implemented (kg ha^{-1} yr^{-1}): Control (0), N (50), P (50), and N + P (50+50). N and P were added in the form of NH_4NO_3 and $Ca(H_2PO_4)_2$, respectively. The experiment began in October 2010. The fertilizers were divided into 12 dosages, then dissolved in 100 L of water to obtain concentrations of 119 mmol L^{-1} N, 53.8 mmol L^{-1} P, and 119 mmol L^{-1} N +53.8 mmol L^{-1} P, and sprayed onto the soil surface of the corresponding plots on a monthly basis. The same amount of water was sprayed onto each control plot.

2.3. Soil sampling

Soil was sampled in January 2021 (the middle of the dry season). The litter layer was carefully removed before sampling. Five soil cores (5 cm inner diameter) were taken at a depth of 0–10 cm from each plot and mixed to form a pooled sample. The soil samples were stored in airtight polypropylene bags and placed in a cool box at 4°C during transportation to the laboratory. After the removal of visible litter, roots, worms, and other debris, the soils were sieved through a 2-mm mesh. Each sample was separated into four subsamples. Subsamples used to measure the TN, total phosphorus (TP), pH, SOC, and exchangeable cations were air dried. Subsamples for the analysis of dissolved organic carbon (DOC), ammonium N (NH_4^+), nitrate N (NO_3^-), and available P concentration were stored at 4°C and analyzed within one week. Subsamples for the measurement of enzyme activity were stored at −20°C. Subsamples for determining microbial community composition and functional gene (GeoChip) analysis were stored at −80°C.

2.4 Soil physical and chemical properties

Soil water content was determined by drying 10 g soil at 105°C. Soil pH was determined with a pH meter after shaking the soil in deionized water (1:2.5 w/v) for 30 min. The SOC and TN were determined by a CHN analyzer using Dumas combustion (Elementar vario EL III, Elementar, Hanau, Germany). TP was determined using digestion with H_2SO_4-H_2O_2-HF (Bellemain et al., 2010). The available P was extracted with 0.03 M NH_4F-0.025M HCl and determined by the ammonium molybdate method. DOC was extracted with 0.05 M K_2SO_4 and quantified by a multi 3100N/C TOC analyzer. Soil NH_4^+ and NO_3^- were extracted with 2 M KCl and measured by a continuous flow analyzer. Exchangeable cations (Al^{3+}, Fe^{3+}, Ca^{2+}, and

K^+) were extracted with 0.1 mol/L $BaCl_2$ (50:1, solution: soil), and analyzed using an inductively coupled plasma-optical emission spectrometer (Perkin-Elmer).

2.5 Microbial communities

For the extraction of total DNA, 0.5 g of soil was processed using a Power Soil kit (MoBio Laboratories) according to the manufacturer's instructions. After purification, the DNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The V3V4 regions of the bacterial 16S rRNA gene and fungal ITS1 region were amplified using the primers 338F/806R (Tian et al., 2015) and ITS2F/ITS5R (Bellemain et al., 2010) with the attached barcode, respectively. The polymerase chain reaction (PCR) was conducted on a BioRad S1000 thermal cycler (Bio-Rad Laboratory, CA, USA) in triplicate. The PCR products were then purified with a DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and mixed in equimolar ratios before sequencing. High-throughput sequencing of 16S rRNA and ITS gene amplicons was performed on an Illumina HiSeq platform (PE250) by the MAGIGEN Company (Guangzhou, China). All of the raw Hiseq sequencing data were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under accession number PRJNA822009.

Raw sequences were quality-checked and assigned to samples based on corresponding barcodes using QIIME (version 1.17, http://qiime.org/index.html). Sequences with an average quality score ≤ 20, or those comprising vague base calls, were discarded (Caporaso et al., 2012). The remaining high-quality sequences were clustered based on a 97% similarity cutoff using UPARSE (Edgar et al., 2013) (version 7.1 http://drive5.com/uparse/) to generate the

operational taxonomic units (OTUs). The taxonomic information of bacterial OTUs was assigned by the Ribosomal Database Project (RDP) classifier (http://rdp.cme.msu.edu/), and the fungal OTUs were assigned to taxonomic information using the UNITE 7.1 database (Abarenkov et al., 2010). Sequences were rarefied to 10,000 for all of the samples, and then used for further analysis. We focused on the dominant species. Rarefaction curves evaluating the OTU richness per sample at a sequence depth of 10,000 nearly approached saturation (Fig. S1), containing most of the dominant species. The relative abundances (%) of individual taxa for each community were calculated. The Shannon diversity index was calculated with the 'vegan' package in R. To reveal the differences in OTU composition between treatments, the matrix of β diversity distance was calculated based on the unweighted UniFrac distance using the QIIME software. The effects of fertilization on the bacterial and fungal communities were assessed using permutational multivariate analysis of variance (PerMANOVA) via the 'adonis' function in the 'vegan' package of R (v3.4.0).

2.6 GeoChip analysis

DNA hybridization was conducted using GeoChip 5.0 according to the procedure described by Zhou et al. (2013). Briefly, DNA samples were labeled with Cy-5 fluorescent dye via a random priming method and purified using the QIA quick purification kit (Qiagen). The DNA was dried in a SpeedVac (ThermoSavant) at 45°C for 45 min. GeoChip hybridization was performed at 42°C for 16 h on a MAUI® hybridization station (BioMicro). After purification, GeoChips were scanned by a NimbleGen MS200 scanner (Roche) at 633 nm, with a laser power and photomultiplier tube gain of 100% and 75%, respectively.

Raw GeoChip data were analyzed with a data analysis pipeline as described previously (Zhou et al., 2013). Briefly, the following steps were performed: (i) spots flagged, or those with a signal-to-noise ratio (SNR) of less than 2.0 were regarded as poor-quality spots and removed; (ii) a minimum of two valid values for three biological replicates was required for each gene; (iii) normalized intensities of each spot were calculated by dividing the signal intensity of each spot by the total intensity of the microarray and then multiplying the average signal intensity of all of the GeoChip data; and (iv) natural logarithmic transformation was applied.

2. 7 Soil enzyme assay

The potential activity of six hydrolytic enzymes, β-glucosidase (β-Glu), xylosidase (XYL), N-acetylglucosaminidase (NAG), leucine-aminopeptidase (LAP), acid phosphatase (ACP) and alkaline phosphatase (ALP), was measured using fluorescent substrates. The β-Glu was detected using 4-methylumbelliferyl-β-D-glucoside, the XYL using 4-methylumbelliferyl-β-D-xylopyranoside, the NAG using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, the LAP using 7-amino-4-methylcoumarin hydrochloride, and the ACP and ALP using 4-methylumbelliferyl-phosphate. Suspensions of 1 g soil with 100 mL universal buffers (50 mM acetate buffer, with pH = 5.0 for β-Glu, XYL, NAG, LAP, and ACP; and 50 mM borate buffer, with pH = 10 for ALP) were prepared using low-energy sonication (40 J s^{-1} output energy) for 2 min. The fluorescent substrates were dissolved in deionized water to obtain concentrations of 150 μM for the enzymes of β-Glu, XYL, NAG, and LAP; 1500 μM for ACP; and 600μM for ALP. Fifty microliters of soil suspension, 100 μL of substrate solution, and 50 μL of buffer were added to 96-well microplates. Plates were incubated in the dark at

30°C for 4 h. Fluorescence was measured using a microplate fluorometer with 365 nm excitation and 450 nm emission filter. A calibration line was prepared with increasing concentrations of 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) (0, 120, 200, 500, 800, and 1200 picomoles/well). Enzyme activities were expressed as MUF or AMC release in nmol per g dry soil per hour based on the calibration line (nmol g^{-1} dry soil h^{-1}).

2.8 Soil microbial biomass

The fumigation extraction method was applied to determine the microbial biomass C, N, and P. Two subsamples of 5 g fresh soil each were weighed into glass bottles. One subsample was fumigated with chloroform in a vacuum for 24 h and the other was untreated. The total DOC and dissolved nitrogen concentrations from both the fumigated and un-fumigated samples were extracted with 0.05 M K_2SO_4 and analyzed using a multi-3100N/C TOC analyzer (Analytik Jena, Germany). The microbial biomass carbon (MBC) and nitrogen (MBN) were calculated as the differences in DOC or dissolved nitrogen concentrations between the fumigated and the unfumigated samples, corrected by the extraction factors of 0.45 and 0.54, respectively (Brookes et al., 1985; Wu et al., 1990). Inorganic P was extracted with 0.5 M $NaHCO_3$, and the P concentration was determined using the ammonium molybdate stannous chloride method (Brookes et al., 1982). Microbial biomass phosphorus (MBP) was determined as the difference in P concentration between the fumigated and unfumigated samples, corrected with the extraction factor of 0.4 (Kouno et al., 1995). The stoichiometries of MBC, MBN and MBP were expressed as MBC: MBP, MBC: MBN, and MBN: MBP, respectively.

2.9 Soil amino sugars

Soil amino sugars were extracted according to the methods described by Bellemain et al. (2010) and Indorf et al. (2011). Briefly, 0.5 g freeze-dried soil was hydrolyzed using 10 ml of 6 M HCl for 6 h at 105°C. After hydrolysis, samples were cooled to room temperature and mixed uniformly, and then filtered. An aliquot of 0.5 ml filtrate was evaporated to dryness using N_2 at 40–45°C to eliminate HCl. The dried residues were dissolved in 0.5 ml deionized water, dried using N_2, redissolved in 2 ml deionized water and stored at −20°C before analysis. The concentrations of three amino sugars (MurN, GluN, and GalN) were measured with a high-performance liquid chromatograph (Dionex Ultimate 3000, Thermo Fisher Scientific) equipped with an octadecylsilylated silica gel column (Acclaim120 C18; 150 mm, 4.6 mm, 3 μm; Thermo Fisher Scientific) following pre-column derivatization with ortho-phthaldialdehyde. The individual amino sugars (MurN, GluN, and GalN) were identified and the quantity was measured according to the chromatograms of standard solutions containing a mixture of the three amino sugars. The concentrations of individual amino sugars were calculated based on the internal standard. The concentrations of individual and total amino sugars were expressed as mg/kg dry soil. Because GluN is present in both fungal and bacterial cell walls, F-GluN can be calculated by subtracting the bacterial-derived GluN from the total GluN, assuming that MurN and GluN have a molar ratio of 1:2 in bacterial cell walls (Engelking et al., 2007) (Eq.1):

$$\text{F-GluN } (\mu g/g) = \text{total GluN } (\mu g/g) - 2 \times \text{MurN } (\mu g/g) \times (179.2/251.2), \quad (1)$$

where 179.2 and 251.2 are the molecular weights of GluN and MurN, respectively (Shao et al. 2017).

2.10 Data analysis

All of the data and residuals were tested for normality before analysis using the Shapiro–Wilk and Levene tests, respectively. The least significant difference (LSD) was used for multiple comparisons ($p < 0.05$) to analyze the effects of nutrient addition on soil properties, microbial biomass, enzyme activity, amino sugar contents, functional gene abundance, and microbial community composition using SPSS 23.0 software. Redundancy discriminate analysis (RDA) was used to determine the main soil properties affecting the changes in the soil microbial community composition, and the main soil chemical properties and microbial community composition affecting enzyme activity and functional gene abundance. The RDA was performed using CANOCO 5.0 software (Wageningen UR, Netherlands). The correlations between enzyme activities and functional gene abundance were tested using linear regression. The correlations between the functional genes responsible for C degradation and those involved in P cycling, and between functional genes and microbial taxa (expressed as the relative abundance at the phylum level), were tested using Pearson correlation.

3 Results

3.1 Effects of N and P addition on soil properties and microbial biomass

Ten years of N, P, or N+P addition did not change pH, SOC, DOC, TN, NH_4^+, NO_3^-, Ca^{2+}, Al^{3+}, Fe^{3+}, or K^+ significantly compared with the unfertilized control soil (Table 1). Soil total P increased by 35%–57% under P and N+P addition but, owing to large variations, this increase was not significant ($p > 0.05$, Table 1). Even if the pool size did not reflect a significant change, the stoichiometry did; P and N+P addition decreased soil C: P and N: P ($p < 0.05$) compared with the control soil (Table 1), and increased the available P pool by 2.5–4.8 times compared with

unfertilized soil ($p < 0.05$) (Table 1). This had implications for the microbial biomass: P and N+P addition increased soil MBP by 48%–80%, while MBC: MBP and MBN: MBP decreased by 47%–50% and 25%–44% compared with unfertilized control soil, respectively ($p < 0.05$) (Table 1).

3.2 Changes of bacterial and fungal community composition

The addition of P decreased the relative abundance of Proteobacteria by 33%, especially for the Alphaproteobacteria class (Figs. 1a and S2). C: P and N: P were the most important factors influencing the relative abundance of Proteobacteria (Fig. 1b). With respect to the fungal communities, the relative abundance of Mortierellomycota under P and NP addition was one-third that in the unfertilized control (Fig. 1c). The changes in Mortierellomycota were mainly affected by P addition (available P and total P) and subsequent soil stoichiometry changes (C/P and N/P) (Fig. 1d). The relative abundance of Mortierellomycota was positively correlated with phosphatase activity and P cycling functional gene abundance (Fig. 1e and f, Table S3).

Fertilization did not change the bacterial community structure, but markedly altered the fungal community structure, as indicated by principal coordinates analysis (PerMANOVA: $p = 0.382$ and PerMANOVA: $p = 0.001$, respectively) (Fig. 1g and h). The first principal coordinate explained 31.5% of the variation in the fungal community dataset, and separated the fertilization with and without P (Fig. 1h). In summary, fungi were more sensitive to fertilization than bacteria, and the fungal community shift was mainly driven by P addition.

3.3 Effects of N and P addition on enzyme activities, amino sugars, and gene abundance

involved in P and C cycling

The addition of N increased ACP and ALP activity by 17% and 90%, respectively (Fig. 2). In contrast, P addition increased β-Glu (24%), and LAP activity (16%), but decreased the activity of NAG (−28%), ACP (−57%), and ALP (−43%). The addition of N+P increased β-Glu, NAG, XYL, and LAP activity by 14%–149%, while it decreased ACP and ALP by 43–54% ($p < 0.05$) (Fig. 2). Generally, GluN and F-GluN decreased by about −20% under P addition compared with N addition ($p < 0.05$) (Fig 3).

The abundances of alkaline phosphatase (*phoA*), *phytase*, C-P lyases (*phn*), and exopolyphosphatase (*ppx*) genes, which are involved in organic P hydrolysis, were 1.55, 2, 1.6, and 1.5 times higher, respectively, under N addition than in the control soil. Nitrogen addition also strongly increased the abundances of a central gene involved in the P-starvation regulation gene (*phoB*) and a high affinity P-uptake and transportation gene (*pst*) by about 80% each compared with the control (Fig. 4). In contrast, P and N+P addition decreased *phoA*, *phytase*, *ppx*, *phn*, *phoB*, and *pst* by 5–40% compared with unfertilized control soil. The addition of N increased the abundances of genes responsible for starch, hemicellulose, cellulose, chitin, and lignin degradation by 66–86% ($p < 0.05$). In contrast, P addition decreased the abundances of these five genes, which were involved in C degradation, by 20–28% ($p < 0.05$), and N+P addition decreased them by about 10% ($p < 0.05$) (Fig. 5).

3.4 Drivers of changes in enzyme activities and functional gene abundance

Soil C: P and N: P were the most important factors explaining changes in phosphatase activity (ACP and ALP), but not of C degradation enzyme activities (XYL and β-Glu) (Fig. 6a). Soil C: P, N: P, and available P were the three most important factors explaining the changes in C

and P functional gene abundance ($p < 0.05$) (Fig. 6b).

A Pearson correlation analysis showed that ACP and ALP activity increased with the abundance of each of the functional genes responsible for P-starvation regulation and for P mining in soils (Fig. 7a and b). In contrast, hydrolytic enzyme activity responsible for cleaving major microbial C sources was independent of the abundances of major genes controlling C metabolism and degradation (Fig. 7c and d). Fertilization-induced changes in gene abundance linked to P cycling and those responsible for organic C decomposition were highly positively correlated (Table S2).

4. Discussion

4.1 Responses of soil properties and microbial biomass to N and P addition

There is considerable evidence that N addition causes soil acidification in tropical forests, and that the degree of acidification is often linked to the amount of N loading. However, in the present study, the soil pH remained stable after 10 years of N and P application (Table 1). Whether fertilization affects soil pH depends on many factors, such as the fertilization intensity (the applied N amounts), the soil buffering capacity, the initial pH, the land use history, the plant species, and the types of added nutrient, especially the speciation of nitrogen (Hu et al., 2022). The applied N speciation in the present study was half ammonium and half nitrate; their combination minimized the effect on acidification. Additionally, the soil in the present study had a relatively low pH even before the experiment began (pH = 4.8). There is a strong soil buffering capacity due to hydroxyl aluminum and base cation exchange in this pH range. This was reflected by the similar content of exchangeable cations (including Ca^{2+}, Al^{3+}, Fe^{3+}, and K^{+}) irrespective of the fertilizer applied (Table 1). Furthermore, substantial amounts

of the added N were likely lost via leaching, considering the heavy rainfall of 2200 mm year^{-1} in the study area (Tang et al., 2018). This would explain the lack of any increase in TN, NH_4^+, and NO_3^- following N fertilization (Table 1). Together, these factors explain the absence of N-induced soil acidification effects in the present study.

Fertilization had no effect on the total C and microbial C pool (Table 1). However, P and N+P addition increased the total P content (35–57%), resulting in a decrease in soil C: P and N: P. Similarly, MBC: MBP and MBN: MBP decreased under P and N+P addition, and in most cases, this was linked to an increase in MBP content. This clearly confirmed the high degree of P depletion of these deeply weathered tropical soils, which made soil nutrient and microbial biomass stoichiometry sensitive to fertilization-induced P pool changes (Bergkemper et al., 2016; Carrara et al., 2018). Thus, P addition and subsequent soil stoichiometry (C/P and N/P) changes may be the main drivers of microbial community composition shifts and function changes (will be discussed following).

4.2 Response of microbial community composition to N and P addition

For bacteria, the relative abundance of the Proteobacteria phylum, especially the Alphaproteobacteria class, decreased under P addition (Figs. 1a and S2). Similarly, within the fungal kingdom, the relative abundance of Mortierellomycota decreased under P and NP addition (Fig. 1b). P addition and subsequent changes in soil stoichiometry (C/P and N/P) were the main factors influencing the relative abundances of Proteobacteria and Mortierellomycota, which was consistent with our first hypothesis. The changes of soil C/P and N/P were mainly due to P addition, because total C and N were stable among treatments (Table 1). Increased P availability can reduce the abundance of microorganisms with P

mobilization abilities. Alphaproteobacteria are known to have high alkaline phosphatase production and P_i transport abilities, and contain genes involved in P solubilization and mineralization (Morrissey et al., 2016; Dai et al., 2020). As a result, the abundance of Alphaproteobacteria decreased under P addition. Because the relative abundance of Mortierellomycota was negatively correlated with soil total P and available P (Fig. 1d), it was therefore predicted that Mortierellomycota also plays a key role in P mineralization and assimilation. Moreover, the changes in phosphatase activity and P cycling functional genes were to a large extent related to the relative abundance of Mortierellomycota (Fig. 1e and f). Thus, P-related soil stoichiometric changes control the relative abundance of Mortierellomycota, which has direct implications for organic P hydrolyzation and P assimilation in P-depleted tropical forest soils. However, underlying ecophysiological reasons for this pronounced response of Mortierellomycota to P addition remain to be elucidated.

Fertilization markedly changed soil fungal community structure, but it did not change bacterial community structure (Fig. 1g and h). This indicated that fungi were more sensitive to nutrient addition than bacteria. The change in fungal community structure was mainly driven by the P addition-induced increase in microbial P availability (Fig. 1h), which verified the second hypothesis. Fungi play a critical role in organic P hydrolysis and inorganic P uptake, especially in severely P-limited tropical soils (Smith and Read, 2008). For instance, saprotrophic fungi and ectomycorrhiza can hydrolyze organic P (Boot et al., 2016), and arbuscular mycorrhiza and ectomycorrhiza can efficiently assimilate P_i (Phillips et al., 2013; van der Heijden et al., 2015). P addition may alleviate tree and microbial P limitation, with implications for fungal community structure; in particular, P addition may reduce the need for

trees to intensively interact with mycorrhizal partners. In the present study, less interaction ultimately led to the reduction of fungal biomass (Yuan et al., 2021; Ma et al., 2019), which was supported by the reduction of fungal residues (F-GluN) under P addition (Fig. 3c).

4.3 Response of phosphatase and P cycling functional genes to N and P addition

The addition of N for 10 years increased acid and alkaline phosphatase potential activities and the underlying microbial functional genes involved in organic P release, such as *phoA*, *phytase*, *phn*, and *ppx* (Figs. 2 and 4). Phosphatase activity increased with the abundance of functional genes related to cellular P starvation and P mining (Fig. 7a and b). Phosphorus deficiency in extremely P-poor soils is boosted once N deficiency is eliminated following N fertilization. Nitrogen addition boosts plant and microbial P demand and aggravates P limitation, and thus increases the abundance of organic P hydrolysis genes and related phosphatase production (Dai et al., 2020). Microorganisms not only increase the abundances of genes responsible for organic P hydrolysis, but they also boost the abundances of genes enabling efficient P_i uptake and transport (Dai et al., 2020). For example, the high-affinity P_i-specific transporters (*pstSCAB*) and their related regulation gene (*phoB*) were increased by about 80% under N deposition in the present study (Fig. 4b and c). The increase of phosphatase activity, organic P hydrolysis, and the larger number of genes for high-affinity P_i transporters were jointly demonstrated to be successful in alleviating further P deficiency under N fertilization, as the total and available P as well as microbial P could be kept at an identical level under the unfertilized control and under N addition (Table 1). The addition of P and NP, in contrast, increased soil-available P ($p < 0.05$) (Table 1), and therefore microorganisms reduced phosphatase production. The addition of P also resulted in the

decrease of abundances of genes responsible for organic P hydrolysis and high-affinity P_i-specific transporters (*pstSCAB*) (Figs. 2 and 4). Thus, the soil microbial PHO operon was greatly affected by the removal of or increase in the P-starvation status of the P-limited tropical forest. The C/P ratio was the main factor affecting changes in acid and alkaline phosphatase activity, and C/P, N/P, and available P were the three main factors affecting P functional gene abundance (Figs. 6a and b). This indicated that P-related soil stoichiometry was the key driver shaping changes in P cycling functional gene abundance and phosphatase activity. Phosphatase activity was positive correlated with P cycling functional gene abundance (Fig. 7a and b), indicating that the microbial community composition was correlated with P cycling functions.

4.4 Response of enzyme-degrading complex organic compounds and their functional gene abundances to N and P addition

Generally, P and N+P addition increased the activity of enzymes with major roles in soil C cycling (Fig. 1), which was consistent with the third hypothesis. This was another clear confirmation of a severe P limitation in this tropical soil. P fertilization removed this growth-limiting nutrient deficiency, directly stimulating microbial growth and thus revealing which element was further limited (Bergkemper et al., 2016). The enhanced C and energy requirement of the growing microbial population increased the activities of all of the enzymes, releasing easily accessible C sources, such as oligosaccharides (GLU), hemicelluloses (XYL) and proteins (LAP) (Fig. 1). Globally, microbes are generally C-limited (Soong et al., 2020), but the current global dataset lacks data from the tropics and subtropics, especially from highly P-depleted tropical soils where P limitation might also be dominant. This experiment

was valuable in supplementing global data on tropical fertilization experiments, clearly demonstrating that P deficiency may exceed C deficiency in these ecosystems and that P fertilization is required to shift the microbial metabolism towards C limitation (Soong et al., 2020). The DNA-based GeoChip approaches revealed the genetic potential of microbial communities (Bergkemper et al., 2016; Trivedi et al., 2016), showing that C and P functional genes had the same trends and were closely correlated in response to fertilization (Figs. 3 and 4, Table S2). This suggested C and P co-limitation (or at least one limitation appearing immediately after the other was eliminated by fertilization). Previous studies have shown that organic P hydrolysis is intrinsically coupled with C cycling (Boot et al., 2016; Chen et al., 2018). For the first time, the results of the present study further demonstrated that this coupling was at least partly related to the simultaneous changes in C and P functional gene abundance. However, the C hydrolases were independent of C cycling functional gene abundance. This indicated that the increase in SOM hydrolyzing enzyme activities was not linked to a shift in the genetic potential, but rather appeared to be attributable to enhanced gene expression. Microbial functional gene abundance merely reveals the potential of microbial communities rather than reflecting the actual levels of gene expression (Bergkemper et al., 2016; Trivedi et al., 2016). Further analyses, such as RNA-based high-throughput sequencing, are suggested in order to test the response of gene expression and its correlation with enzyme activity under long-term N and P inputs.

Microbial community composition shifted, functional gene abundance changed, and enzyme activities were altered with 10 years of nutrient addition. Previous studies in the field found that N and P fertilization did not influence microbial community structure, enzyme activity, or

microbial C cycling (Jing et al., 2020; Ma et al., 2020). Such contrasting phenomena may be due to multiple factors. First, the duration of nutrient addition, such as in short-term (4–5 years) versus long-term (10 years) studies, may yield contrasting results. Soil properties may be stabilized under short-term nutrient addition due to the strong buffering capacity of the soil. Soil microbial communities adapt to site-specific soil and environmental conditions. Therefore, long-term investigations of the microbial response to environmental changes should be conducted. Second, different methods were used. Previous studies used phospholipid fatty acids to quantify microbial community structure (Ma et al., 2020, 2021). High-throughput amplicon sequencing, which can provide more accurate information, was used in the present study. However, the current study was based on the one-time sampling of surface soils (January 2021, 0–10 cm). Soil microbial community structure and function may vary between different seasons and soil depths. Thus, sampling at multiple time points and various soil depths is required to comprehensively reveal the microbial responses to environmental changes. The Network of Nutrient Enrichment Experiments in China's Forests was built with a small number of replicates (three). Considering the large variation in the field, more replicates are needed in the future.

Conclusions

Ten years of nutrient addition shifted the soil microbial community composition. Fungi were more sensitive to nutrient addition than bacteria, and the fungal community shift was mainly driven by P availability (Fig. 8). N addition aggravated P limitation, to which microbes responded by an increase in the abundance of P cycling functional genes and phosphatase

activity. In contrast, P addition alleviated P deficiency and thus decreased the P cycling functional gene abundance and phosphatase activity (Fig. 8). The shift of microbial community composition, changes in the functional genes involved in P cycling, and phosphatase activity were mainly driven by P addition and the subsequent alteration of soil stoichiometry (C/P and N/P). Alleviating P deficiency through fertilization accelerated C cycling by increasing the activity of C degradation enzymes. The abundance of C and P functional genes was positively correlated, indicating the intensive coupling of C and P cycling in P-limited forest soil (Fig. 8). In summary, the long term fertilization experiment demonstrates that soil microorganisms can adapt to induced environmental changes in soil nutrient stoichiometry, not only through shifts in the microbial community composition and functional gene abundances, but also through the regulation of enzyme production. Altogether, these adaptations ensure the maintenance of microbial nutrient requirements and have large-scale implications for ecosystem nutrient cycling.

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Declaration of competing interest

The authors declare that there are no conflicts of interest in the present experiment.

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Figures captions

Fig. 1 Relative abundances of the dominant bacterial and fungal groups at the phylum level under the control, nitrogen (N), phosphorus (P), and combined nitrogen and phosphorus (N+P) fertilization treatments in tropical primary forest soils (a and c). Lowercase letters indicate significant differences ($p < 0.05$, least significant difference (LSD) test between treatments. The Shannon diversity (considering phyla as individuals) is given above each bar. Letters beside the diversity values represent significant differences ($p < 0.05$). Redundancy discriminate analysis (RDA) plots illustrate the relationships between soil physico-chemical properties and the dominant bacteria and fungal phyla (b and d), microbial community composition and enzyme activities, and functional gene abundance (e and f). Principal coordinates analysis (PCoA) between treatments was conducted based on the operational taxonomic units for bacteria and fungi (g and h).

Fig. 2 Activities of β-glucosidase (β-Glu), xylosidase (XYL), N-acetylglucosaminidase (NAG), leucine-aminopeptidase (LAP), acid phosphatase (ACP), and alkaline phosphatase (ALP) in the soil of a tropical primary forest under the control, nitrogen (N), phosphorus (P), and combined N and P (N+P) addition treatments. Lowercase letters indicate significant differences ($p < 0.05$, least significant difference (LSD) test between treatments. Values are means ± SE (n =3).

Fig. 3 Content of individual and total amino sugars (reflecting microbial necromass content) in the soil of a tropical primary forest under the control, nitrogen (N), phosphorus (P), and combined N and P (N+P) addition treatments. Lowercase letters indicate significant

differences ($p < 0.05$, least significant difference (LSD) test between treatments. Values are means ± SE (n = 3). GalN, galactosamine; GluN, glucosamine; F-GluN, fungal glucosamine; MurN, muramic acid; Total ASs, total amino sugars.

Fig. 4 Abundances of genes involved in microbial cellular P response and the mineralization of soil organic P: alkaline phosphatase (*phoA*) (a), phytate hydrolysis (*phytases*) (b), C-P lyases (*phn*) (c) and exopolyphosphatase (*ppx*) (d), high affinity P_i-specific transporters (*pst*) (e); P-starvation response regulation (*phoB*) (f) under the control, nitrogen (N), phosphorus (P), and combined nitrogen and phosphorus (NP) addition treatments in tropical primary forest. Lowercase letters indicate significant differences ($p < 0.05$, least significant difference (LSD) test) between treatments. Values are means ± SE (n = 3).

Fig. 5 Abundances of genes indicating the decomposition of organic compounds (starch, cellulose, hemicellulose, chitin, and lignin) in tropical primary soils under the control (C), nitrogen (N), phosphorus (P), and combined N and P (N+P) addition treatments. Signal intensities were summed and normalized by the probe number for each substance. Lowercase letters indicate significant differences ($p < 0.05$, least significant difference (LSD) test between treatments. Values are means ± SE (n = 3).

Fig. 6 Redundancy analysis (RDA) ordination biplot of soil properties and enzyme activities (a), and soil properties and functional genes (b).

Fig. 7 Relationship between acid phosphatase activity and alkaline phosphatase activity with P cycling gene abundance (a and b), and β-glucosidase and xylosidase (XYL) activity with

carbon degradation gene abundance (c and d).

Fig. 8 A conceptual figure illustrating the microbial community, enzyme activity, and functional gene abundance in response to long-term N and P inputs in tropical forests.

Table 1 Effects of nitrogen (N), phosphorus (P), and combined nitrogen and phosphorus (NP) fertilizer addition to the soil of a tropical primary forest on carbon C, N, and P pools, and soil microbial biomass C, N, and P content.

	Treatment (kg ha^{-1} $year^{-1}$)			
	Control	N	P	NP
		50	50	50-50
Soil chemistry				
pH	4.8±0.1 a	4.7±0.1 a	4.8±0.1a	4.7±0.1 a
Ca^{2+} (cmol1/3Al3^{+}kg-1)	0.4±0.10 a	1.1±0.3 b	0.9±0.1 ab	0.4±0.0 a
Al^{3+} (cmol1/3$Al^{3+}kg^{-1}$)	3.6±0.4 a	5.9±0.5 a	4.0±0.3 a	5.9±1.1 a
Fe^{3+} (cmol1/3$Fe^{3+}kg^{-1}$)	76.7±4.2 a	73.7 ±4.9 a	82.8±1.7 a	79.5±3.1 a
K^{+} (cmol $K^{+}kg^{-1}$)	96.3±3.8 a	79.4±21.3 a	79.7±12.6 a	84.8±15.4 a
Soil organic carbon (SOC) (g C^{-1} kg^{-1})	21.9±4.1 a	21.2±5.6 a	20.4±2.7 a	23.1±3.4 a
Dissolved organic carbon (DOC) (mg C^{-1} g^{-1})	255±39.2 a	263±49.1 a	281±104.3 a	284±94.9 a
Total nitrogen (TN) (g N^{-1} kg^{-1})	1.6±0.2 a	1. 7±0.4a	1.5±0.3 a	1.7 ±0.2 a
Nitrate N (mg N^{-1} kg^{-1})	13.2±4.0 a	11.5±2.9 a	9.1±3.1 a	8.5±1.5 a
Ammonium N (mg N^{-1} kg^{-1})	32. 5±3.4 a	28.7 ± 3.0 a	42.1±11.0 a	40.7±11. 9 a
Total phosphorus (TP)(mg/kg)	140±20 a	140±30 a	220±20 a	190±20 a
Available phosphorus (AP) (mg /kg)	2.5±0.7 a	2.5±0.9 a	12.0±1.5 c	6.2±0.4 b
C: N	13.8±1.2 a	14.6±1.1 a	13.7±0.5 a	13.4±0.4 a
C:P	156±10.0 c	167±7.5 c	93±7.3 a	123±3.8 b
N:P	11.3±0.3 c	11.5±0.4 c	6.9±0.8 a	9.2±0.3 b
Soil microbial biomass				
MBN (mg kg^{-1})	68.5±16.4 a	77.6±12.4 a	57.1±12.8 a	93.1±22.8 a
MBC (mg kg^{-1})	554±18.2 a	679±24.3 a	465±142.3 a	573 ±82.9 a
MBP (mg kg^{-1})	22.2±5.1 a	26.5±4.0 ab	32.7±6.3 ab	40.1±3.3 b
MBC: MBP	27.4±5.4 b	26.7±3.6 b	13.7±1.5 a	14.5±2.3 a
MBN: MBP	3.1±0.4 b	3.0±0.4 b	1.7±0.1 a	2.3±0.5 ab

Lowercase letters indicate significant differences ($p < 0.05$, LSD test) between treatments. Values are means ± SE (n = 3).

Figure 1

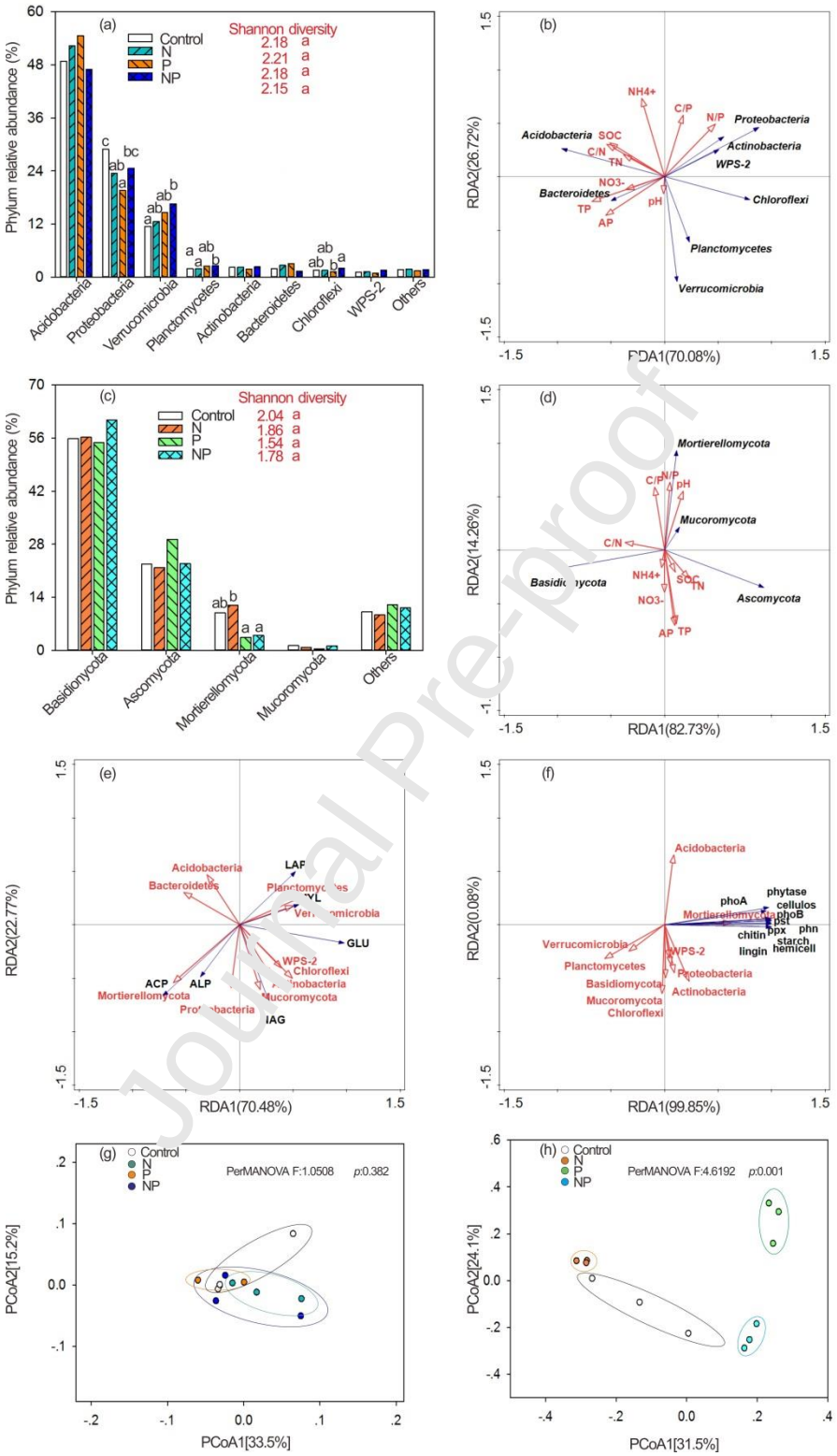
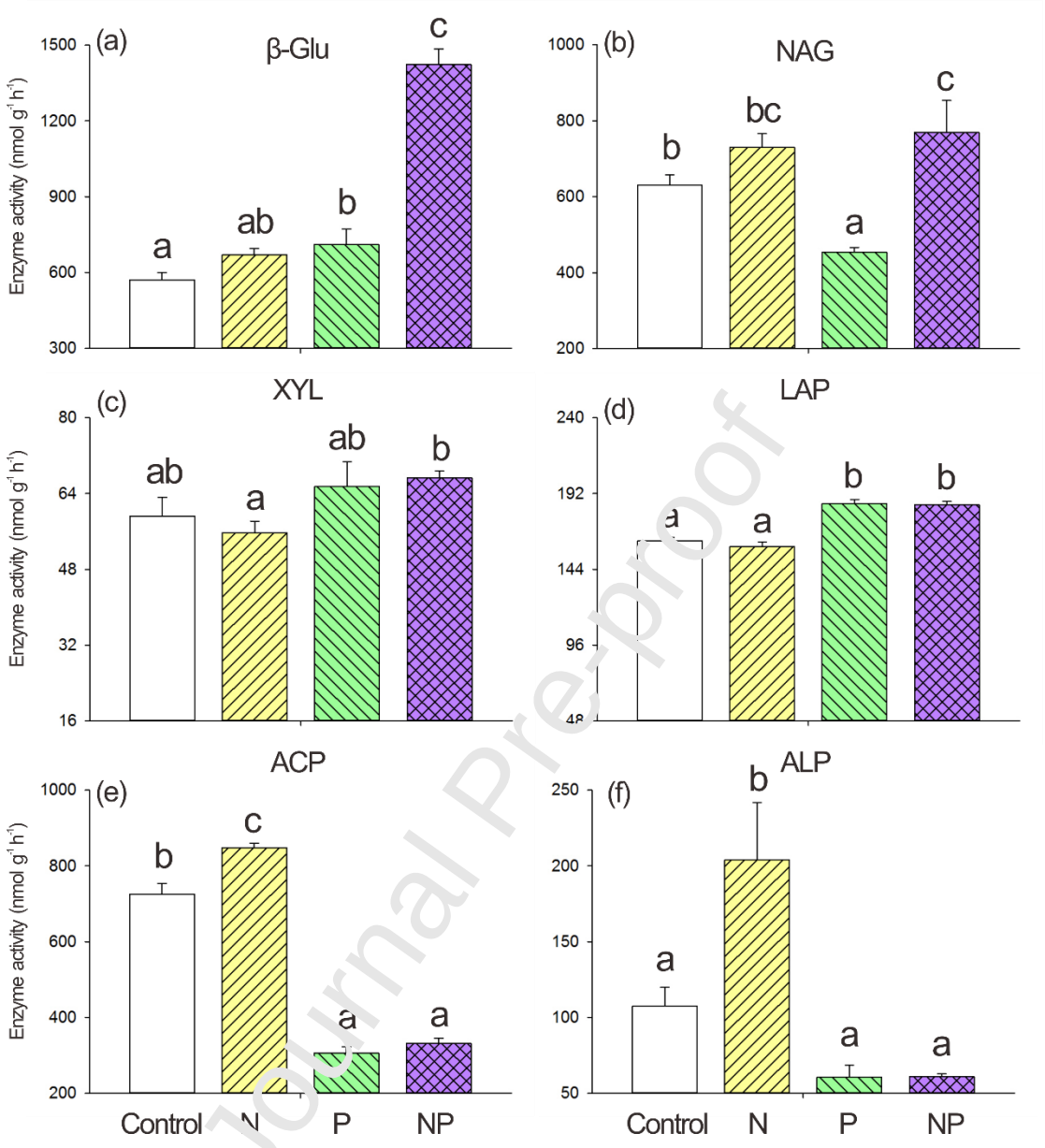


Figure 2



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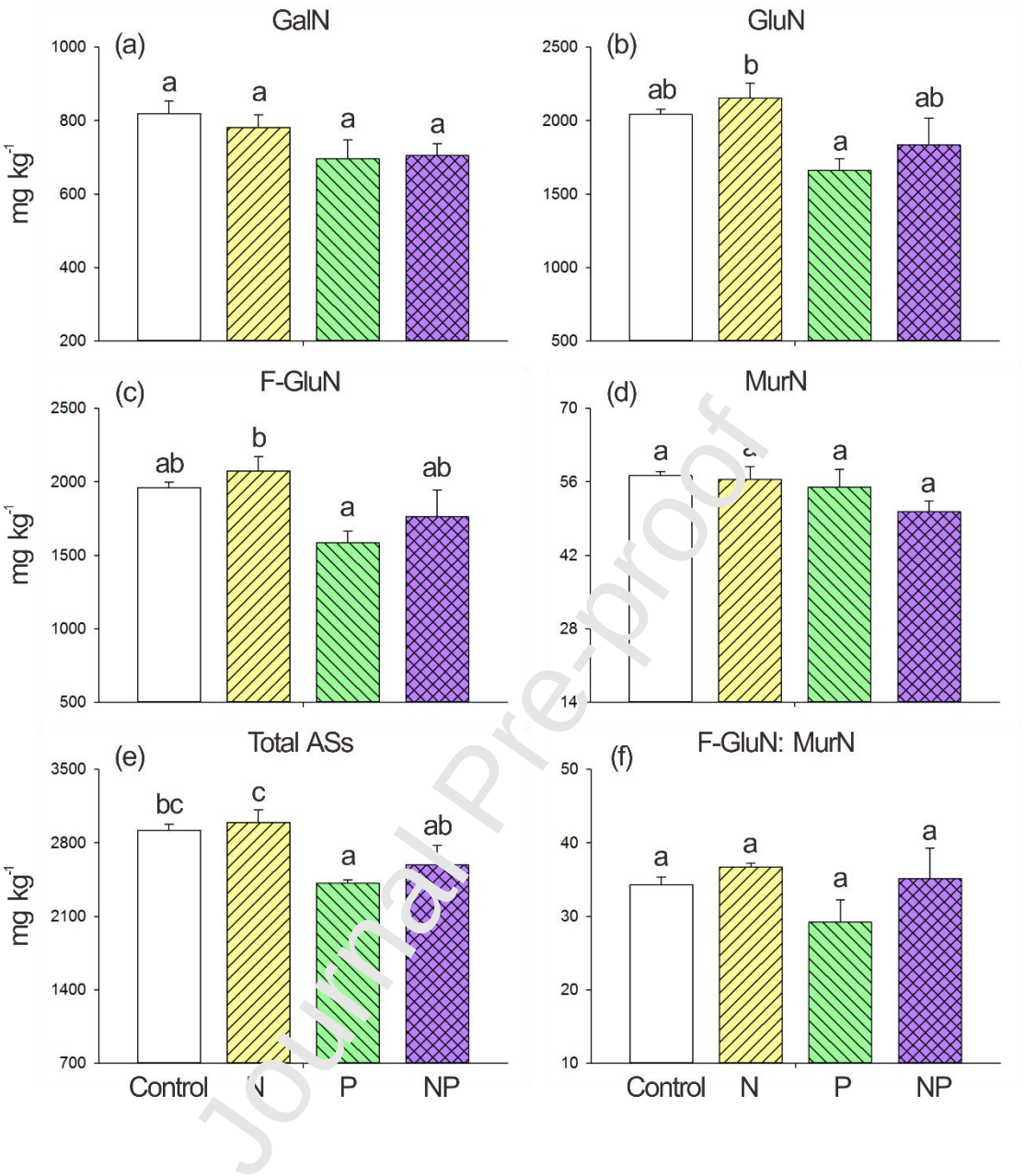
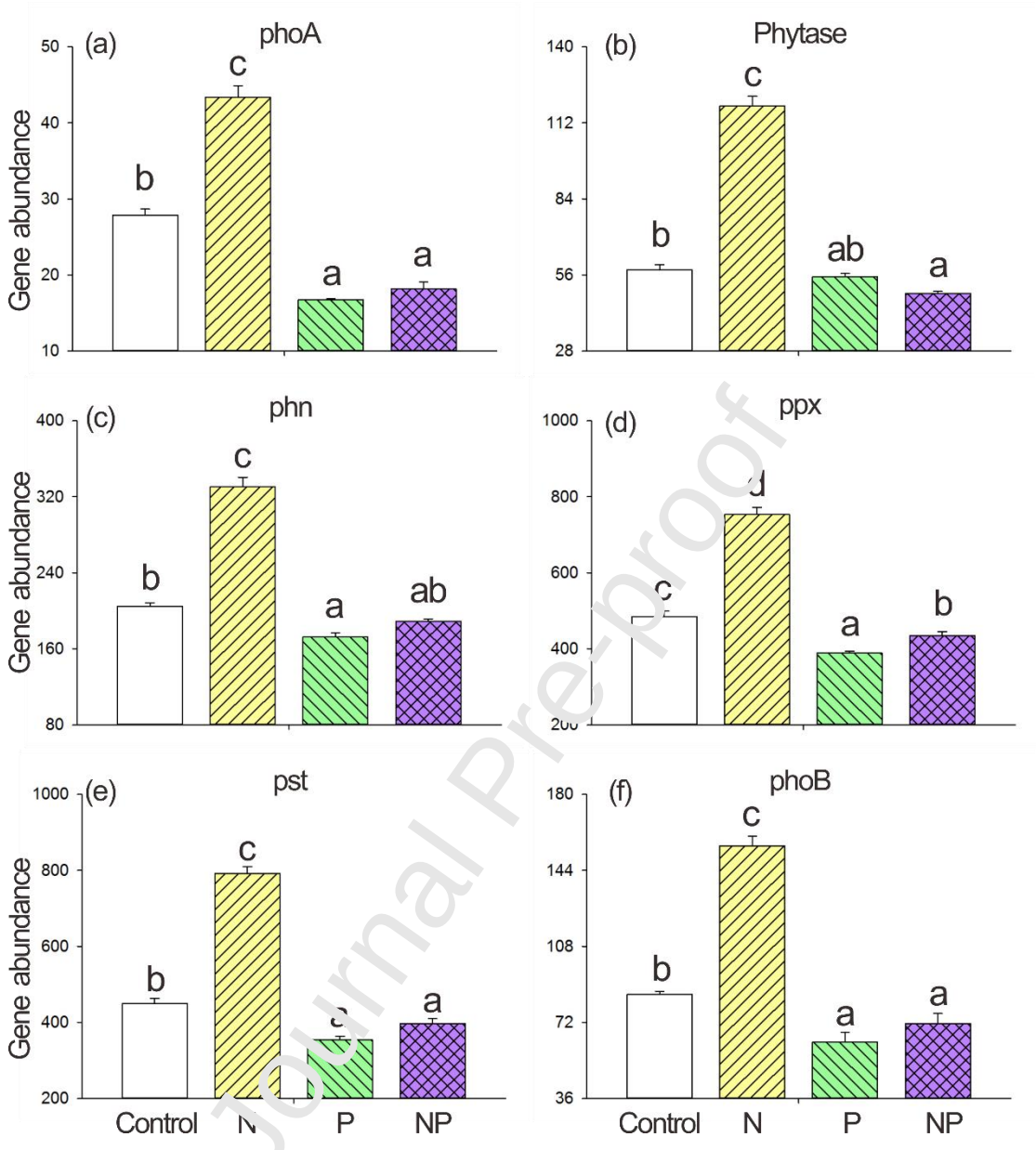


Figure 4



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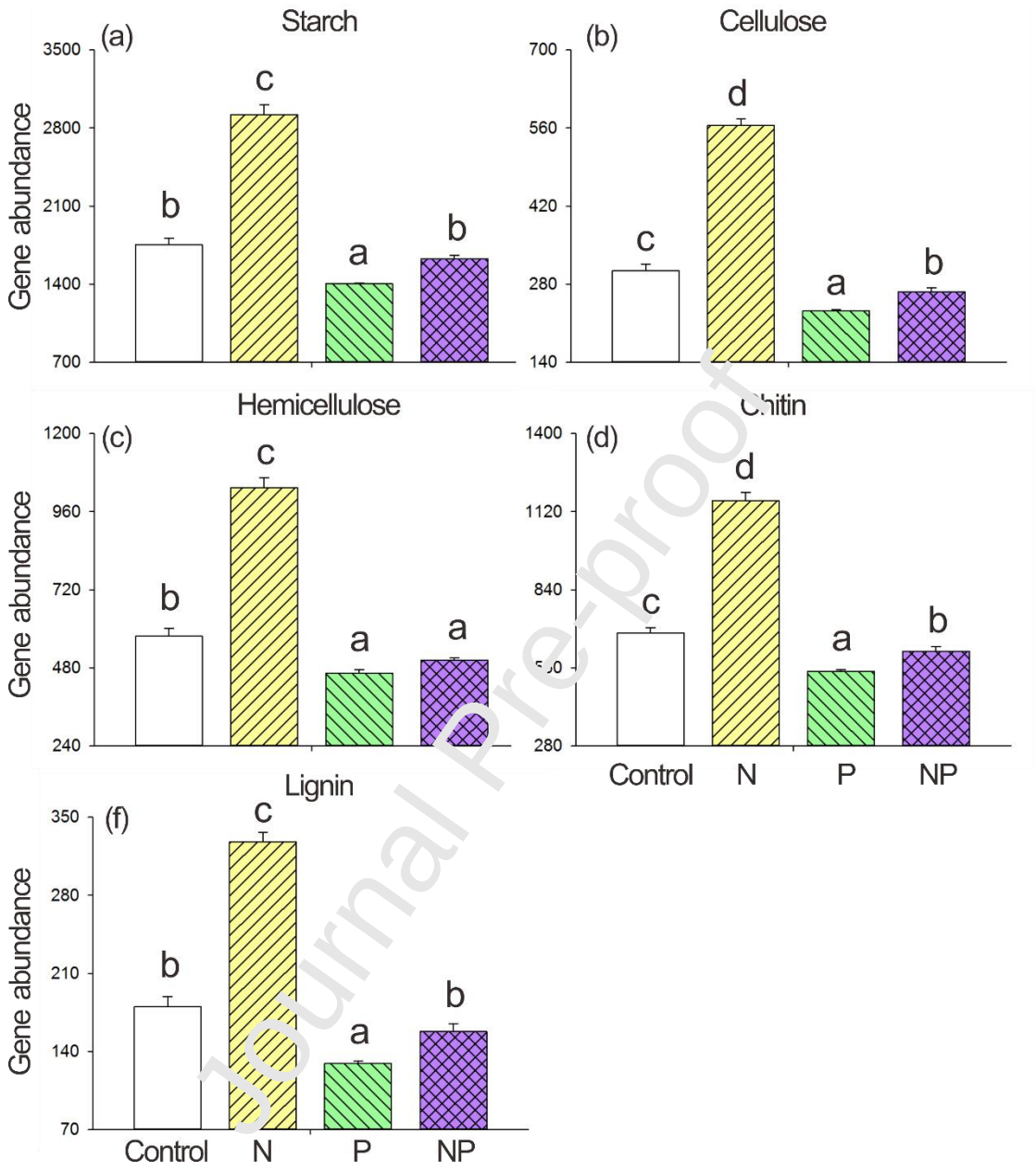

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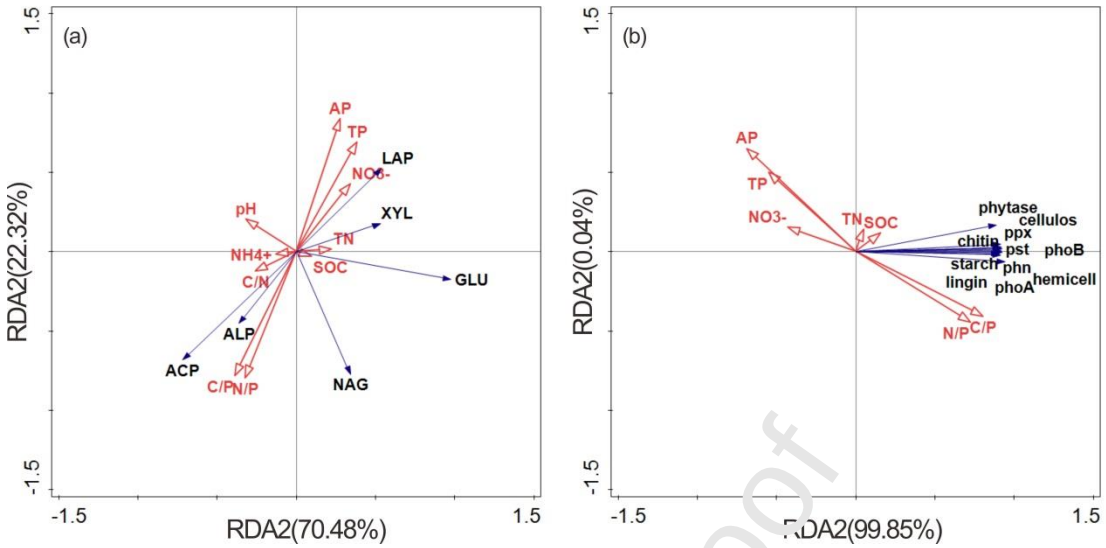


Figure 7

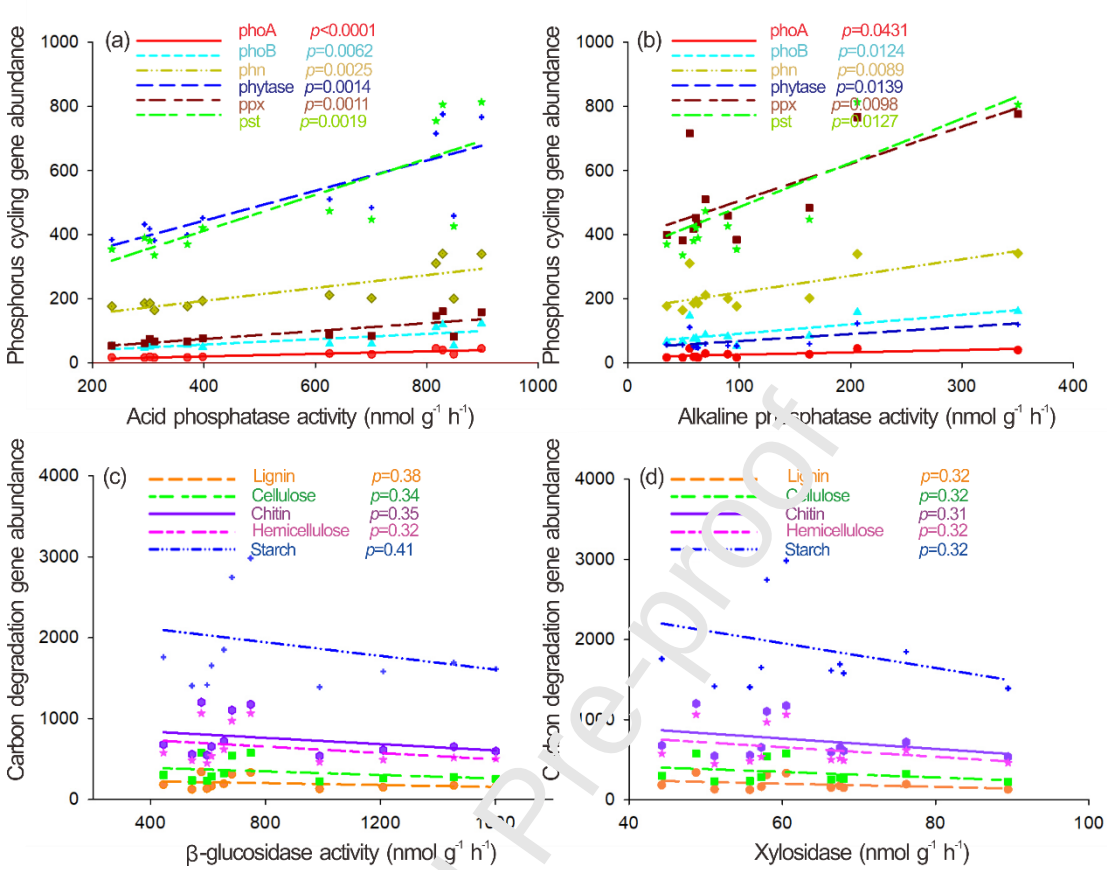
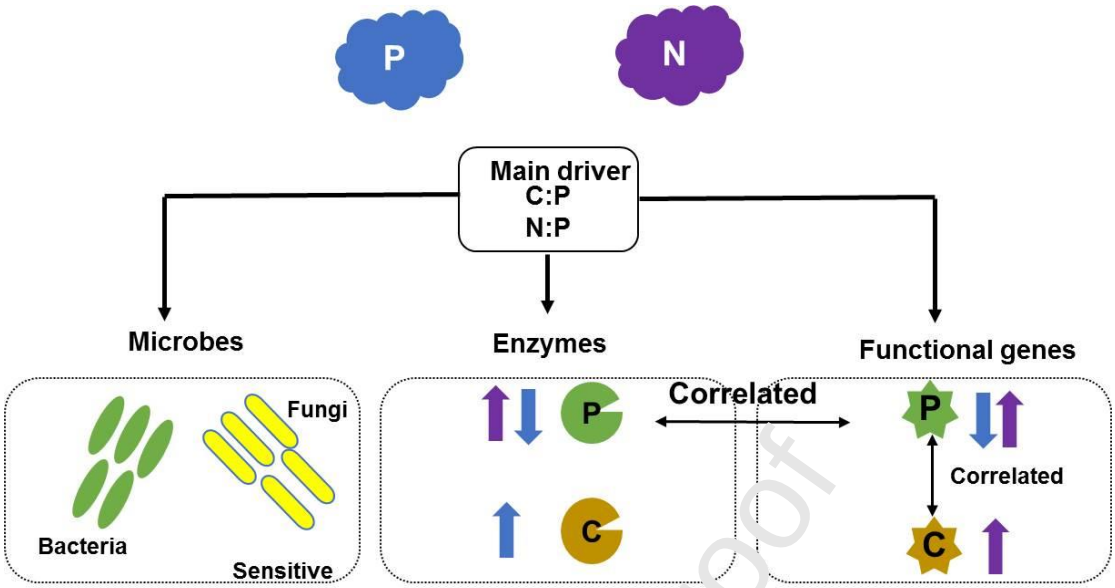


Figure 8



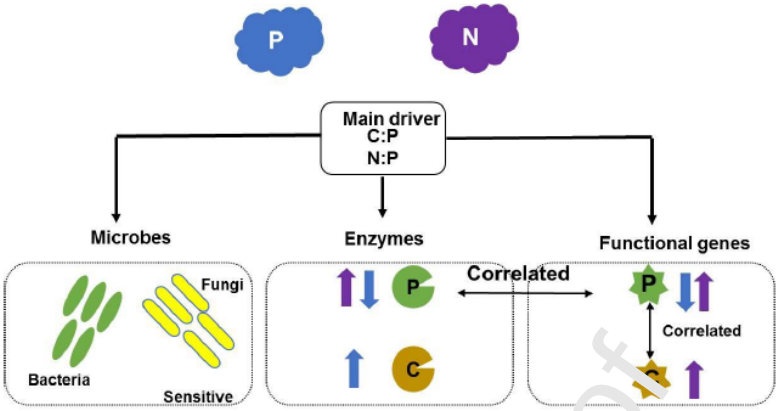
CRediT authorship contribution statement

Xiaomin Ma, Zhang Zhou and Jie Chen Funding acquisition, conducted the experiments, analyzed the data, wrote the manuscript conceived; **Han Xu** conducted the experiments, analyzed the data, **Suhui Ma** built and maintenance of long term experimental platform, **Michaela A. Dippold and Yakov Kuzyakov** read and edited the manuscript

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract

Highlights

- Microbial community composition was more sensitive to P than to N addition.
- Fungi were more sensitive to N and P addition than bacteria.
- Nitrogen addition increased phosphatase activity and P addition decreased it.
- Eliminating P deficiency can accelerate C cycling.
- Carbon and P cycling are tightly coupled.