Differential mechanisms underlying responses of soil bacterial and fungal communities to nitrogen and phosphorus inputs in a subtropical forest

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Atmospheric nitrogen (N) deposition and phosphorus (P) addition both can change soil bacterial and fungal community structure with a consequent impact on ecosystem functions. However, which factor plays an important role in regulating responses of bacterial and fungal community to N and P enrichments remains unclear. We conducted a manipulative experiment to simulate N and P inputs (10 g $N \cdot m^{-2} \cdot yr^{-1}$ NH_4NO_3 or 10 g $P \cdot m^{-2} \cdot yr^{-1}$ NaH_2PO_4) and compared their effects on soil bacterial and fungal species richness and community composition. The results showed that the addition of N significantly increased NH_4^+ and Al^{3+} by 99.6% and 57.4%, respectively, and consequently led to a decline in soil pH from 4.18 to 3.75 after a five-year treatment. P addition increased Al^{3+} and available P by 27.0% and 10-fold, respectively, but had no effect on soil pH. N addition significantly decreased bacterial species richness and Shannon index and resulted in a substantial shift of bacterial community composition, whereas P addition did not. Neither N nor P addition changed fungal species richness, Shannon index, and fungal community composition. A structural equation model showed that the shift in bacterial community composition was related to an increase in soil acid cations. The principal component scores of soil nutrients showed a significantly positive relationship with fungal community composition. Our results suggest that N and P additions affect soil bacterial and fungal communities in different ways in subtropical forest. These findings highlight how the diversity of microbial communities of subtropical forest soil will depend on future scenarios of anthropogenic N deposition and P enrichment, with a particular sensitivity of bacterial community to N addition.

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Abstract

Atmospheric nitrogen (N) deposition and phosphorus (P) addition both can change soil bacterial and fungal community structure with a consequent impact on ecosystem functions. However, which factor plays an important role in regulating responses of bacterial and fungal community to N and P enrichments remains unclear. We conducted a manipulative experiment to simulate N and P inputs (10 g $N \cdot m^{-2} \cdot yr^{-1}$ NH_4NO_3 or 10 g $P \cdot m^{-2} \cdot yr^{-1}$ NaH_2PO_4) and compared their effects on soil bacterial and fungal species richness and community composition. The results showed that the addition of N significantly increased NH_4^+ and Al^{3+} by 99.6% and 57.4%, respectively, and consequently led to a decline in soil pH from 4.18 to 3.75 after a five-year treatment. P addition increased Al^{3+} and available P by 27.0% and 10-fold, respectively, but had no effect on soil pH. N addition significantly decreased bacterial species richness and Shannon index and resulted in a substantial shift of bacterial community composition, whereas P addition did not. Neither N nor P addition changed fungal species richness, Shannon index, and fungal community composition. A structural equation model showed that the shift in bacterial community composition was related to an increase in soil acid cations. The principal component scores of soil nutrients showed a significantly positive relationship with fungal community composition. Our results suggest that N and P additions affect soil bacterial and fungal communities in different ways in subtropical forest. These findings highlight how the diversity of microbial communities of subtropical forest soil will depend on future scenarios of anthropogenic N deposition and P enrichment, with a particular sensitivity of bacterial community to N addition.

40

41 **Keywords:** Nitrogen deposition, phosphorus addition, microbial diversity, community

42 composition, amplicon sequencing, subtropical forest, soil bacteria and fungi

Introduction

Nitrogen (N) and phosphorus (P) inputs to the ecosystem have greatly increased due to anthropogenic activities, mainly originating from fossil fuel combustion, agricultural fertilization, and dust or ash production (Galloway et al. 2008; Stevens 2019; Wang et al. 2014). Excessive N and P inputs could result in many adverse impacts, including soil acidification (Guo et al. 2010; Mao et al. 2017; Tian & Niu 2015) and nutrient imbalance (Peñuelas et al. 2013), although N and P have been considered as limiting factors for plant growth (Harpole et al. 2011). Moreover, elevated N availability may aggravate P limitation of ecosystem productivity (Li et al. 2016). A large body of research focusing on aboveground plant community responses have demonstrated multiple effects with nutrient additions, including biodiversity loss (Hooper et al. 2012), species composition, and associated ecosystem services, such as terrestrial carbon dynamics (Isbell et al. 2013; LeBauer & Treseder 2008). However, the responses of belowground microorganisms to nutrient additions, including general taxonomic traits and related function shifts, remain unclear (Leff et al. 2015; Ma et al. 2019; Ma et al. 2016; Wei et al. 2018). In particular, integrated field experimental investigations of both bacterial and fungal responses to N and P additions are needed to improve our understanding of how the soil microbial community structure shifts in response to nutrient addition and whether the bacterial and fungal responses are consistent.

Soil microbial communities are sensitive to N enrichment (Dai et al. 2018; Ramirez et al. 2012; Treseder 2008). On average and based on a global meta-analysis, across all of the studies

in agro-ecosystems, the abundances of *Proteobacteria* and *Actinobacteria* significantly increased by 2.2% and 1.1%, respectively, and the abundance of *Acidobactria* decreased by 2.3% under N addition, which suggest differential sensitivity of bacterial phyla to N inputs (Dai et al. 2018). N addition could lead to elevated N availability (Chen et al. 2019), ammonium toxicity (Van Den Berg et al. 2005), acid cation toxicity (Chen et al. 2016; Tian & Niu 2015), and loss of base mineral cations (Bowman et al. 2008). Meanwhile, several potential driving factors were proposed to account for shifts in sensitive microbial communities in some ecosystems (Nie et al. 2018). First, soil pH has been reported as a strong predictor of soil bacterial community composition at the continental scale (Lauber et al. 2009; Rousk et al. 2010). A significantly positive correlation between diversity of bacteria and soil pH might be attributable to narrow pH ranges for optimal growth of bacteria (Rousk et al. 2010). However, fungal community composition is less affected by soil pH because fungi generally exhibit wider pH ranges for optimal growth (Rousk et al. 2010). Second, N addition could directly affect soil bacterial community composition through modified ammonium N concentration (Nie et al. 2018; Zeng et al. 2016; Zhou et al. 2017). Soil bacterial community composition is closely related to soil NH_4^+-N content in tropical forest soil under N enrichment due to a narrow pH decrease in severely acidic soil ($pH < 4.5$) (Nie et al. 2018). Finally, a positive relationship between plant and fungal beta diversity has been reported under N enrichment, soil properties including soil inorganic N, pH, and associated extractable cations being correlated with compositional changes in plant and fungal communities (Chen et al. 2018b). Although those mechanisms have previously been

85 investigated, comprehensive studies on different regulatory pathways of N addition on bacterial
86 and fungal community structure in forest ecosystems are limited.
87 P availability plays an important role in affecting microbial growth; however, our
88 understanding of soil microbial community responses to elevated P inputs remains limited (Leff
89 et al. 2015; Ma et al. 2019; Ma et al. 2016). Moreover, the effects of P input on bacterial and
90 fungal community composition can differ (Cassman et al. 2016; Jorquera et al. 2014; Liu et al.
91 2013; Liu et al. 2012b). Previous studies have demonstrated that P enrichment induces shifts in
92 microbial community composition by increasing P availability and altering soil and plant
93 chemistry (DeForest & Scott 2010; Lagos et al. 2016; Leff et al. 2015; Liu et al. 2012a; Liu et al.
94 2013; Teng et al. 2018). Other studies have reported that only N enrichment influences bacterial
95 abundance and community composition, whereas P input did not because soil pH decreases only
96 with N addition (Jorquera et al. 2014; Wang et al. 2018) or because bacterial community was
97 limited by other resources (Ma et al. 2019). The abovementioned pathways have largely been
98 studied separately, and the relative contributions of these pathways to N- and/P-induced changes
99 in bacterial and fungal communities, however, have not been investigated using field
100 experiments.
101 Subtropical forests in southern China undergo extensive N deposition with signs of N
102 saturation, such as soil acidification (Zhu et al. 2015). Previous studies have demonstrated that
103 forest productivity and soil respiration are sensitive to N and P enrichment, but little is known
104 about how P addition affects microbial communities (Li et al. 2018a; Li et al. 2018b; Yu et al.
105 2017). To better understand the responses of bacterial and fungal communities to N and P

addition and the underlying mechanisms, we conducted a N and P addition experiment in this region. We specifically addressed the following questions: (1) How do bacterial / fungal taxa and community structures respond to N and P enrichment? (2) How do soil biotic and abiotic factors regulate the responses of bacterial / fungal taxa and community structures to N and P enrichment? Finally, (3) What are the mechanisms underlying shifts in bacterial / fungal taxa and community structures to N and P enrichment?

Materials & Methods

Site description

This nitrogen (N) and phosphorus (P) addition experiment was setup at the Jigongshan Nature Reserve, China (31°51'58"N, 114°5'12"E). The site is experiencing northern subtropical to warm temperate climates because it is located within a climate-transitional region. The mean annual surface air temperature and mean annual rainfall at the reserve is 15.2° C and 1,118 mm, respectively. The soil type is yellow-brown and soil thickness is about 0.3–0.6 m (Yan et al. 2014). The forest type is deciduous oak mixed forest. The predominant tree species in the canopy layer include *Quercus acutissima* and *Q. variabilis* and *Liquidambar formosana*, *Lindera glauca*, and *Viburnum dilatatum* dominates the understory arborous layer. The age of the stand is about 45–50 years and it is a secondary forest due to harvest in the late 1950s (Xu & Liang 1965). The total N deposition in this region is about 30 kg N ha^{-1} yr^{-1} (Zhu et al. 2015). The soil properties at the beginning of the experiment is listed in Table S1.

127

128 **Experimental design**

129 The detailed information of N and P addition experiment could be found in previous studies
130 (Li et al. 2018a; Li et al. 2018b). Briefly, this N and P addition experiment was setup in July
131 2013 using a complete randomized block design. Each block had four treatments that were
132 randomly assigned to 10 m × 10 m plots and four replicate blocks were established. Control (CK,
133 without N and P addition), N addition (N, 10 g m^{-2} yr^{-1} NH_4NO_3), P addition (P, 10 g m^{-2} yr^{-1}
134 NaH_2PO_4), and NP addition (NP, 10 g·m^{-2}·yr^{-1} NH_4NO_3 + 10 g·m^{-2}·yr^{-1} NaH_2PO_4) were
135 included. Backpack sprayer were used to spray 50 L of water dissolved additions for each plot
136 onto the forest floor monthly from May to October each year. The control plot received 50 L of
137 water (equivalent to 0.5 mm precipitation) each time.

138 **Soil properties**

139 Six soil cores (0–10 cm depth) were randomly collected from each plot and mixed to obtain one
140 composite sample in October 2017. The samples were passed through a 2-mm sieve and three
141 parts were divided. One part of the soil was used for the analysis of ammonium (NH_4^+), nitrate
142 (NO_3^-), microbial biomass carbon and nitrogen (MBC and MBN), and dissolved organic carbon
143 (DOC). The second part of fresh soil was collected in a 50-mL centrifuge tube, which was stored
144 at -80° C for soil DNA extraction. The remaining soil was air-dried for the determination of soil
145 pH, total soil organic carbon (SOC), total nitrogen (TN), available phosphorus (AP), and
146 extractable cations including Al^{3+}, Ca^{2+}, Mg^{2+}, and Na^+. The plant fine roots collected from 6 soil
147 cores using the 2 mm-sieving were washed, dried, and then weighted. Soil ammonia and nitrate

concentrations were determined by colorimetric analysis on a FIAstar 5000 Analyzer (FIAstar 5000 Analyzer, Foss Tecator, Hillerød, Denmark). Soil DOC was analyzed using a TOC analyzer (multi N/C 3100, Analytik Jena, Germany). Soil MBC and MBN were determined by a chloroform fumigation extraction method (Brookes et al. 1985). Soil pH was analyzed in a soil water solution (1:2.5 w/ v). SOC and TN were analyzed by a C/N analyzer (vario EL III, CHNOS Elemental Analyzer, Elementar, Germany). Available P was determined following by molybdenum blue colorimetry (Jin et al. 2019). A modified extraction procedure was used to measure extractable cations including Al^{3+}, Ca^{2+}, Mg^{2+}, and Na^{+} (Rauret et al. 2000).

Soil DNA extraction, PCR amplification, and sequencing

Soil DNA was extracted from each sample using a Fast DNA Stool Mini Kit (Tiangen Biotech Beijing Co., Ltd., Beijing, China) according to the manufacturer's instructions. The quality of the purified DNA was assessed based on the 260/280 nm and 260/230 nm absorbance ratios obtained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Microbial community diversity and composition were assessed by amplification of the *16S rRNA* gene for the bacteria and the internal transcribed spacer (ITS) region of the fungi, as described in Prober et al. (2015). Marker genes in the isolated DNA were PCR-amplified and barcoded in triplicate reactions for both the *16S rRNA* gene (using the 515f/907r primer pair) and the ITS1 region (using the ITS1F/ITS2R primer pair). Sequencing was conducted on an Illumina Miseq platform at Majorbio Biopharm Technology Co., Ltd., Shanghai, China.

Sequencing data accession numbers

Raw sequences were trimmed of reads containing ambiguous bases and long homopolymers and merged using QIIME v 1.7.0 (Caporaso et al. 2012). All filtered sequences from 16S rRNA and ITS gene amplicons were clusted into OTUs at 97% similarity cutoff using UPARSE (version 7.1, http:// drive5.com/uparse), followed by chimera filtering usingn the Ribosomal Database Project (RDP) (Tian et al. 2018) and UCHIME (Edgar et al. 2011). The bacterial and fungal OTU sequences were classified using the RDP classifier against Greengenes and UNITE reference database, repectively. All of the raw sequencing data (.fastq files) were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under accession number PRJNA531787 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA531787).

Statistical analysis

The bacterial and fungal richness was determined by the number of OTUs, and alpha diversity was estimated using the Shannon index. Linear regressions were applied to assess the relationships between bacterial and fungal Shannon index and richness and environmental factors. Stepwise multiple regressions were used to identify the most influential environmental variables on bacterial and fungal Shannon index and richness due to collinearity among environmental factors. Differences in microbial species richness, Shannon index, soil properties and fine root biomass among different treatments were determined by one-way ANOVA with Duncan test. To determine the effect size of N, P and combined NP treatment on the relative abundances of the dominant bacterial and fungal taxa, the response ratio was calculated as ln

(X_t/X_c), where X_t is the mean value of experimental treatment and X_c is the mean value of the control treatment. One-sample t-test was used to estimate whether each response ratio was significantly different from zero (She et al. 2018).

Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) were used to determine changes in the bacterial and fungal communities. Analyses, including Anosim, Adonis, and MRPP, were further performed to assess the significant differences in community among different treatments. The partial Mantel test was used to evaluate the linkages between the microbial community structure and the environmental variables. Pairwise taxonomic distance between microbial communities (Bray-Curtis) and Euclidean distance of environmental variables were chosen for partial Mantel. PCA, NMDS, and partial Mantel were conducted using the *vegan* package with R v3.5.0 (R Core Team 2018).

Structural equation modelling (SEM) was performed to analyze the hypothetical pathways of N and P addition effects on bacterial and fungal diversity and community composition. Before the SEM analysis, soil N availability including NH_4^+ and NO_3^-, soil acid cations including H^+ and Al^{3+}, soil nutrients including SOC, TN, DOC, MBC and MBN, and microbial community composition (OTUs) were subjected to principal component analysis (PCA) (Chen et al. 2013). Soil N availability, soil acidity, soil nutrients, and microbial community composition were presented by the first principal components (PC1) in the following SEM analysis. Maximum likelihood estimation method was applied in the process of SEM. The goodness of the models was determined by χ^2 tests, Akaike information criteria (AIC), and root square mean errors of approximation (RMSEA) (Li et al. 2018a).

211

212 Results

213 Fine root biomass and soil properties

214 Nitrogen (N), phosphorus (P), and combined NP additions significantly reduced fine root
215 biomass (Table S2). Soil pH decreased with N addition, whereas P and NP additions did not have
216 any effect compared to the control. Soil NH_4^+ was increased by 99.6% under N addition, whereas
217 it did not change after P and NP additions (Table S2). P and NP additions resulted in a
218 significant increase in soil available phosphorus (AP), but N addition did not (Table S2). Soil
219 Al^{3+} increased by 57.4%, 27.0% and 59.1%, respectively, under N, P and NP additions. Soil Ca^{2+}
220 decreased by 33.6% and 25.4%, respectively, under N and NP additions, but did not significantly
221 change under P addition. N, P, and NP additions did not significantly change soil organic carbon
222 (SOC), total nitrogen (TN), ratio of SOC to TN (C/N ratio), dissolved organic carbon (DOC),
223 microbial biomass carbon, and nitrogen (MBC and MBN), NO_3^-, Mg^{2+}, and Na^+.

224

225 Relative abundance of dominant microbial taxa

226 16,573 to 26,745 (average: 21,019) and 46,414–70,354 (average: 64,655) valid sequences were
227 consequently obtained per sample for bacterial and fungi, respectively. These sequences were
228 grouped into 1,666 and 2,487 OTUs at the 97% similarity level for bacterial and fungi,
229 respectively. All of the samples were compared at an equivalent sequencing depth of 16,573 and
230 46,414 per sample for bacteria and fungi, respectively.

231 The predominant bacterial phyla across all of the samples were *Proteobacteria*,
232 *Acidobacteria*, and *Actinobacteria* (mean relative abundance > 5%), which accounted for more
233 than 82% of the bacterial sequences on average (Fig. 1a). In addition, *Planctomycetes*,
234 *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, and *Gemmatimonadetes* were also present at relatively
235 low abundance. The dominant fungal phyla across all of the samples were *Ascomycota*,
236 *Basidiomycota*, *Zygomycota*, and *Rozellomycota* (Fig. 1b).
237 At the phylum level, *Omnitrophica*, *Chlorobi*, and *Nitrospirae* presented significant
238 decrease and *Saccharibacteria* increased in relative abundance under N treatment (Fig. 2a). P
239 addition significantly increased the relative abundances of *Elusimicrobia*, but N addition had no
240 effect compared to the control plots (Fig. 2a). Only NP treatment increased the relative
241 abundance of *Firmicutes* and decreased that of *Chloroflexi* (Fig. 2a). No significant differences
242 were observed in the fungal relative abundance of *Ascomycota*, *Rozellomycota*, and
243 *Chytridiomycota* at the phylum level among different treatments (Figs. 2b). *Basidiomycota*
244 presented significant decrease in their relative abundance under N and NP additions and only NP
245 addition decreased the relative abundance of *Zygomycota* (Fig. 2b). At the fungal genus level,
246 *Chaetomium* and *Purpureocillium* presented significant decrease in their relative abundance
247 under N and P treatments, and N and NP treatments decreased the relative abundance of
248 *Mycoarthris* (Fig. 2b). The relative abundance of *Mortierella* significantly decreased only under
249 NP addition (Fig. 2b).
250
251 **Bacterial and fungal α diversity**

252 N addition significantly decreased bacterial α diversity, including phylotype richness (OTU
253 numbers) and Shannon diversity index, but did not change fungal α diversity (Fig. 3). P addition
254 had no effect on both bacterial and fungal α diversity. NP addition only decreased fungal
255 phylotype richness (Fig. 3). N addition decreased the number of unique phylotypes compared to
256 the control (Table S3).

257 The bacterial richness exhibited the largest correlation with soil pH among all of the
258 environmental factors (Table 1, $r = 0.80$, $P < 0.01$). In addition to DOC ($r = -0.72$, $P < 0.01$), soil
259 pH was the best predictor of bacterial diversity (Table 1, $r = 0.71$, $P < 0.01$). Bacterial richness
260 and diversity also significantly correlated with fine root biomass, SOC, MBC, Al^{3+}, and Ca^{2+}
261 (Table 1). Soil Al^{3+} ($r = -0.62$, $P < 0.05$) presented the largest correlation with fungal richness,
262 followed by soil pH ($r = 0.57$, $P < 0.05$). No significant correlations were noted between fungal
263 diversity and any of the environmental variables (Table 1).

264 Stepwise regression showed that bacterial richness significantly correlated with pH (64.3%)
265 and DOC (7.1%) (Table S4). Soil pH explained the largest part of the variation in bacterial
266 diversity (50.1%), followed by TN (14.9%), DOC (13.4%), and MBC (9.7%). Soil pH explained
267 33.0% of the variation in fungal richness, whereas pH (17.0%), SOC (19.1%), and MBC (41.4%)
268 collectively contributed to 65.2% of the variation in fungal diversity (Table S4).

269

270 **Bacterial and fungal community structure and relationships with environmental factors**

271 Significant differences in bacterial community structure between N and NP additions and the
272 control were observed in NMDS and PCA plots (Figs. 4 and S1). However, only NP addition

exhibited significant differences in fungal community structure compared to the control (Fig. 4). The results of ANOSIM, ADONIS, and MRPP analyses further confirmed the significant differences among treatments (Table S5). This finding was confirmed by a cluster analysis based on a Bray-Curtis distance matrix of soil samples under different treatments (Fig. S2).

A partial Mantel test was performed to reveal the major environmental variables shaping microbial community structure. pH ($r_M = 0.57$, $P = 0.001$), Al^{3+} ($r_M = 0.40$, $P = 0.002$) and fine root biomass ($r_M = 0.36$, $P = 0.005$) were the most important factors that independently contributed to variations in soil bacterial community structure (Table 2). TN, C:N ratio, DOC, and MBC showed weak but significant correlations ($r_M = 0.21$, $r_M = 0.22$, $r_M = 0.27$, $r_M = 0.24$, respectively, $P < 0.05$) (Table 2). pH ($r_M = 0.57$, $P = 0.001$) and MBC ($r_M = 0.40$, $P = 0.004$) were the most important factors that independently contributed to variations in soil fungal community structure (Table 2). C:N ratio, DOC, Al^{3+}, and Mg^{2+} showed weak but significant correlations ($r_M = 0.25$, $r_M = 0.26$, $r_M = 0.21$, $r_M = 0.27$, respectively, $P < 0.05$) (Table 2).

Pathways determining the responses of bacterial and fungal diversity and community composition

The SEM explained 12% of the variations in soil N availability, 83% of the variation in soil acid cations (H^+ and Al^{3+}), 5% of the variation in soil nutrients and 80% of the variation in soil available P (Fig. 5). The total variation in bacterial Shannon index was mainly explained (64%) by soil acid cations and soil available P (Fig. 5a), but the total variation in fungal Shannon index was mainly explained (39%) by soil available P (marginally significant, $P = 0.08$, Fig. 5b). The

294 total variation in bacterial community composition was mainly explained (79%) by soil acid
295 cations (Fig. 5a), but the total variation in fungal community composition was mainly explained
296 (45%) by soil nutrients (Fig. 5b). Linear regression demonstrated that principal component (PC1)
297 scores of fungal community composition was significantly correlated with PC1 scores of soil
298 nutrients ($R^2 = 0.41$, $P = 0.008$, Fig. S3).
299 Standardized total effects from SEM demonstrated that N enrichment had stronger effects
300 on bacterial diversity and community composition compared to P addition (Fig. 6). Specifically,
301 acid cations showed the most powerful negative effect on bacterial diversity (Fig. 6a) and had a
302 stronger positive effect on bacterial community composition than soil N availability (Fig. 6b).
303 Both N and P enrichment had negative effects on fungal diversity, and soil nutrients exerted the
304 most powerful positive effect on fungal diversity (Fig. 6c). N enrichment had stronger positive
305 effects on fungal community composition, and soil nutrients exerted strongest negative effect on
306 fungal community composition (Fig. 6d).
307

308 ## Discussion

309 **N but not P addition significantly influences bacterial communities**

310 Our results showed that N addition significantly decreased bacterial OTU richness and Shannon
311 index and altered the bacterial community composition, with *Saccharibacteria* increasing, but
312 *Chloroflex* decreasing in terms of their relative abundances (Figs. 2–4, Table S5). These results
313 indicate that N addition tends to favor the copiotrophic phylum (*Saccharibacteria*) and
314 counterselects the oligotrophic phylum (*Chloroflex*) in bacterial communities, which is in line

315 with the copiotrophic hypothesis in previous reports (Fierer et al. 2007; Fierer et al. 2012; Leff et
316 al. 2015). However, N addition did not change the relative abundance of dominant phylum of
317 *Proteobacteria*, *Acidobacteria,* and *Actinobacteria* (Fig. 2). The results are in contrast with the
318 findings of previous studies, which showed that added N stimulates and decreases the relative
319 abundances of *Proteobacteria* (copiotrophic) and *Acidobacteria* (oligotrophic), respectively
320 (Chen et al. 2019; Fierer et al. 2012; Leff et al. 2015). This discrepancy could be attributable to
321 neutral effects of N enrichment on soil available C and N (including DOC and NO_3^-, Table S2)
322 (Turlapati et al. 2013). It has been well documented that *Proteobacteria* contains N-fixing and N
323 transforming genera (e.g., *Bradyrhizobium*, *Burkholderia*, *Magnetospirillum*, and
324 *Mesorhizobium*) and thus are closely related to N cycling. However, in our study, added N only
325 increased NH_4^+ and did not affect NO_3^-, indicating the N transforming-related bacterial phylum
326 (*Proteobacteria*) may not be affected by the addition of N. Additionally, *Acidobacteria* has been
327 reported to be negatively correlated with soil C availability (Fierer et al. 2007). The neutral effect
328 of elevated N on DOC in our study may have contributed to the unaltered relative abundance of
329 *Acidobacteria*, which is favored by low C availability (Fierer et al. 2007; Turlapati et al. 2013).
330 Finally, the absence of a decrease in the abundance of *Actinobacteria* may be attributed to its
331 relatively high tolerance to environmental stress such as low pH (Dai et al. 2018).
332 In contrast to most previous findings (Ling et al. 2017; Tan et al. 2013), neither the bacterial
333 diversity nor the community composition was substantially influenced by P addition in our study
334 (Figs. 3-4, Table S5), but this finding is consistent with a previous study reporting that most
335 bacterial taxa were not limited by P in Tibetan meadow (Ma et al. 2019). Among all of the

bacterial phyla identified, only *Elusimicrobia* were negatively correlated with soil available P (Table S6). There are several potential explanations for the insensitive response to added P. First, it is well established that nutrient addition-induced pH decline is an important mechanism in shifting the bacterial community composition (Chen et al. 2015; Ling et al. 2017; Zeng et al. 2016). However, P addition did not change the soil pH in our study (Table S2). Additionally, P input could increase the P availability, and thus affect the bacterial richness and community structure, especially in P-limited ecosystems (Li et al. 2015; Liu et al. 2013). Our results showed that increased soil P availability did not change microbial biomass, suggesting that microbial growth is not limited to P in this subtropical forest. Finally, Liu et al. (2013) pointed out that a decrease in labile SOC is associated with alterations in soil microbial community structure under P addition. In our study, however, neither SOC or DOC was not significantly influenced by P addition (Table S2). Overall, our results suggest that only increased P availability may not be able to change the bacterial community composition in subtropical forest.

Neither N nor P addition significantly influenced fungal community

Our results demonstrated that fungal OTU richness is positively correlated to fine root biomass and soil pH and negatively related to MBC and soil Al^{3+}. However, fungal Shannon index was not significantly associated with soil parameters (Table 1). Fungal community structure was closely related to soil pH and MBC, followed by soil C:N ratio, DOC, Al^{3+}, and Mg^{2+} (Table 2). In contrast to the bacterial community, both N and P additions did not significantly change fungal richness and diversity (Fig. 3), which suggests that the tolerances of fungi to environmental changes are generally stronger than those of bacteria (Fierer et al. 2012; Rousk et

357 al. 2010). The responses of fungal diversity to N enrichment is not always consistent due to
358 ecosystem type and nutrient dose (Chen et al. 2019; Mueller et al. 2014). Some studies have
359 demonstrated that experimental N deposition results in increased fungal richness and diversity in
360 a low fertility loblolly pine forest (Mueller et al. 2014; Weber et al. 2013), but other studies
361 showed the opposite response partly due to variations in nutrient availability (Chen et al. 2019;
362 Chen et al. 2018a). Previous studies have demonstrated that N enrichment increases the relative
363 abundance of copiotrophic phylum *Ascomycota* and decreased that of oligotrophic phylum
364 *Basidiomycota* according to the copiotroph-oligotroph concept (Chen et al. 2018b; Fierer et al.
365 2007; She et al. 2018; Yao et al. 2017). Although no significant effect of N enrichment on fungal
366 community composition was observed (Fig. 4, Table S5), regression analysis also showed that
367 the relative abundance of *Basidiomycota* was negatively correlated with soil N availability (NH_4^+
368 and NO_3^-, Table S6), indicating that N enrichment likely directly affects *Basidiomycota* via
369 increasing N availability. Overall, the limited responses of fungal richness, diversity, and
370 community composition under N enrichment in our study may be due to increased N loss from
371 soil through denitrification and leaching (Niu et al. 2016).

372 Compared to many studies analyzing the response of soil microorganisms to N addition,
373 relatively little research has been done on the effects of P enrichment on soil fungi in grasslands
374 and forests (Li et al. 2015; Liu et al. 2013), and much fewer studies using high-resolution
375 technology, such as *16S rRNA* sequencing (Cassman et al. 2016; He et al. 2016). The
376 understanding of fungal community responses to elevated P inputs remains limited (Leff et al.
377 2015). The effect of P addition on soil fungal community composition depends on ecosystem

type, soil properties, and nutrient type and dose (Bao et al. 2013; Beauregard et al. 2010; Li et al. 2015; Liu et al. 2013). Previous studies have demonstrated that experimental P fertilization reduces the species richness of arbuscular mycorrhizal fungi, which could increase soil nutrient capture of their hosts in return for plant C resource (Camenzind et al. 2014; Cheng et al. 2013; Liu et al. 2013). However, elevated P input could alleviate P deficiency of soil microbes and increase the fungal biomass, suggesting P availability is one of the limiting factors for fungal growth in an old tropical forest (Liu et al. 2012a). Another explanation for the not significant change of fungal community composition in response to P enrichment might be the relatively short-term experimental duration of P addition (Cheng et al. 2013), and these detailed parameters on fungal community composition warrant further investigation.

Mechanisms underlying responses of soil bacterial and fungal communities to N and P inputs

Several mechanisms behind the observed shift of bacterial and fungal community composition under N and P additions have been proposed, including increased N and P availability (Liu et al. 2012a; Liu et al. 2013; Nie et al. 2018; Zhou et al. 2017), a decline in soil pH (Chen et al. 2015; Zeng et al. 2016), and increased soil acid cations availability (Chen et al. 2015). Contrary to the N and P availability theory (Liu et al. 2012a; Nie et al. 2018), our results showed that N and P availability (including NH_4^+, NO_3^-, and AP) had no significant relationship with bacterial and fungal diversity and community structure (Table 1 and 2). Meanwhile, both partial Mantel test and SEM results have demonstrated that N and P availability had no significant relationship with bacterial and fungal community composition (Table 2, Fig. 5), suggesting that N and P

399 availability could not explain the shifts in bacterial and fungal community composition. The
400 remaining possible reasons for the decline of bacterial diversity and shift of bacterial community
401 composition include increased concentration of H^+ and Al^{3+} (Table S2). Furthermore, SEM
402 results showed that soil acid cations (H^+ and Al^{3+}) induced a significant decrease in bacterial
403 diversity and substantial change of bacterial community composition (Figs 5, 6). However, SEM
404 results have demonstrated that soil nutrients are significantly correlated to fungal community
405 composition (Fig. 5). Taken together, in this study, increase of soil acid cations and soil nutrients
406 significantly contributed to the shift in bacterial and fungal community composition,
407 respectively.
408

409 ## Conclusions

410 After a five-year N and P addition in subtropical forest, our results showed that N addition
411 significantly decreased bacterial species richness and diversity, and resulted in a substantial shift
412 of bacterial community composition, whereas P addition did not. Neither N nor P addition
413 changed fungal species richness, diversity, and fungal community composition. A structural
414 equation model showed that the shift in bacterial community composition is attributable to an
415 increase in soil acid cations. The principal component scores of soil nutrients showed a
416 significantly positive relationship with fungal community composition. Our results show how the
417 diversity of microbial communities of subtropical forest soil will depend on future scenarios of
418 anthropogenic N deposition and P enrichment, with a particular sensitivity of bacterial
419 community to N addition

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Table 1(on next page)

Pearson correlations between bacterial and fungal richness and diversity and plant and soil characteristics.

Table 1 Pearson correlations between bacterial and fungal richness and diversity and plant and soil characteristics. * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$

r	Bacteria		Fungi	
	Richness	Diversity	Richness	Diversity
Fine root biomass	0.59*	0.56*	0.51*	0.32
pH	0.80**	0.71**	0.57*	0.41
SOC	-0.52*	-0.40	-0.29	-0.47
TN	-0.47	-0.35	-0.16	-0.33
C:N ratio	-0.40	-0.35	-0.42	-0.47
DOC	-0.75**	-0.72**	-0.32	-0.20
NH_4^+	-0.48	-0.34	-0.23	-0.17
NO_3^-	-0.40	-0.31	-0.15	-0.02
AP	0.07	0.24	-0.34	-0.42
MBC	-0.64**	-0.49	-0.54*	-0.48
MBN	-0.41	-0.27	-0.40	-0.42
Al^{3+}	-0.54*	-0.58*	-0.62*	-0.33
Ca^{2+}	0.58*	0.54	0.40	0.33
Mg^{2+}	0.24	0.09	0.22	0.13
Na^+	-0.06	-0.07	-0.33	-0.17

SOC: soil organic carbon; TN: total nitrogen content; DOC: dissolved organic carbon; AP: available phosphorus; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen.

Table 2(on next page)

Partial mantel test of soil bacterial and fungal community structure with environmental characteristics.

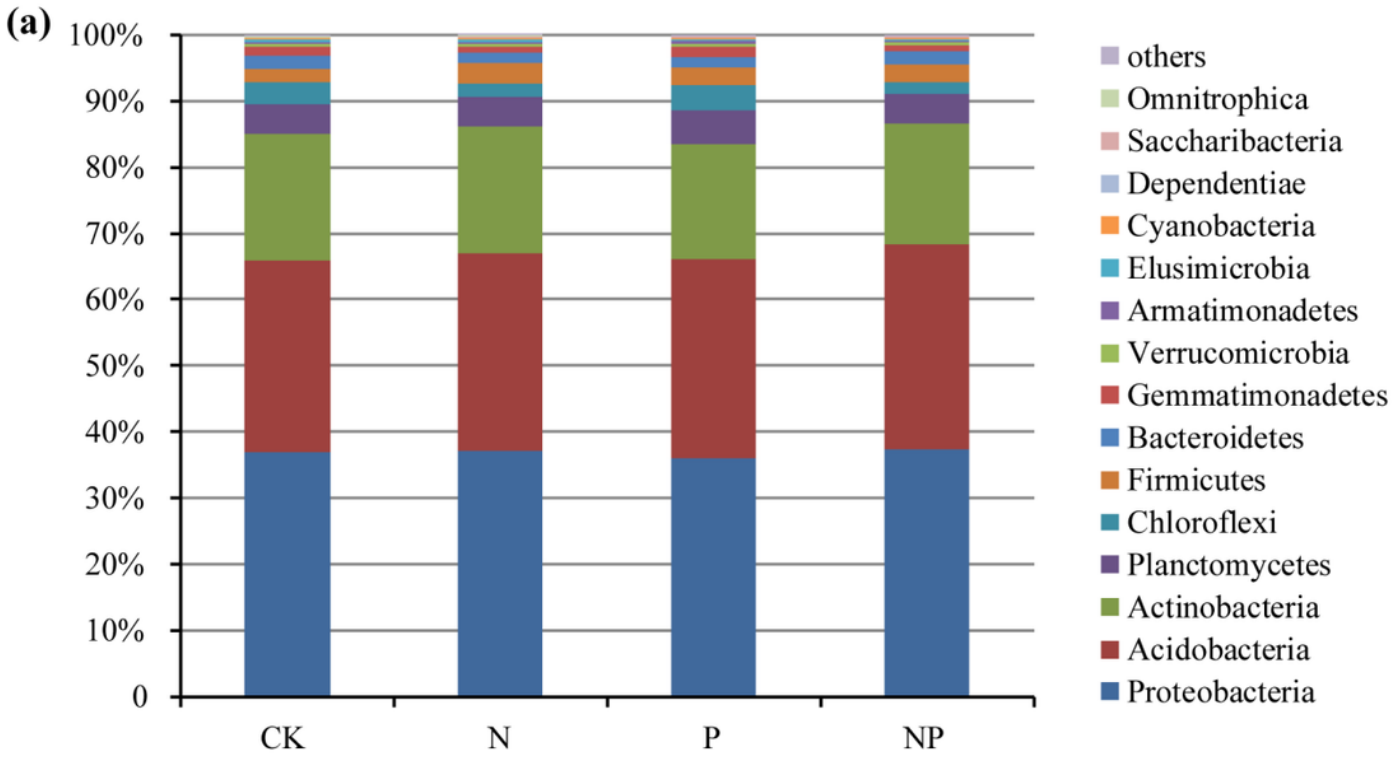
Table 2 Partial mantel test of soil bacterial and fungal community structure with environmental characteristics. The correlation and significance were determined between bacterial and fungal community structure (Bray-Curtis distance) and environmental variables (Euclidean distance) based on 999 permutations.

	Bacteria		Fungi	
	r	*P*	*r*	*P*
Fine root biomass	**0.36**	**0.005**	0.10	0.18
pH	**0.57**	**0.001**	**0.40**	**0.001**
SOC	0.16	0.10	0.19	0.07
TN	**0.21**	**0.04**	0.12	0.14
C:N ratio	**0.22**	**0.04**	**0.25**	**0.02**
DOC	**0.27**	**0.02**	**0.26**	**0.01**
NH_4^+	0.08	0.21	-0.009	0.49
NO_3^-	0.07	0.26	0.02	0.45
AP	-0.14	0.95	-0.13	0.92
MBC	**0.24**	**0.03**	**0.36**	**0.004**
MBN	0.10	0.15	0.17	0.06
Al^{3+}	**0.40**	**0.002**	**0.21**	**0.03**
Ca^{2+}	0.12	0.18	-0.06	0.66
Mg^{2+}	0.06	0.28	**0.27**	**0.02**
Na^+	-0.15	0.91	-0.03	0.62

SOC: soil organic carbon; TN: total nitrogen content; DOC: dissolved organic carbon; AP: available phosphorus; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen.

Figure 1

Relative abundance of the dominant bacterial (a) and fungal (b) groups at phylum level under different treatments.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Cyanobacteria
Elusimicrobia
Armatimonadetes
Verrucomicrobia
Gemmatimonadetes
Bacteroidetes
Firmicutes
Chloroflexi
Planctomycetes
Actinobacteria
Acidobacteria
Proteobacteria

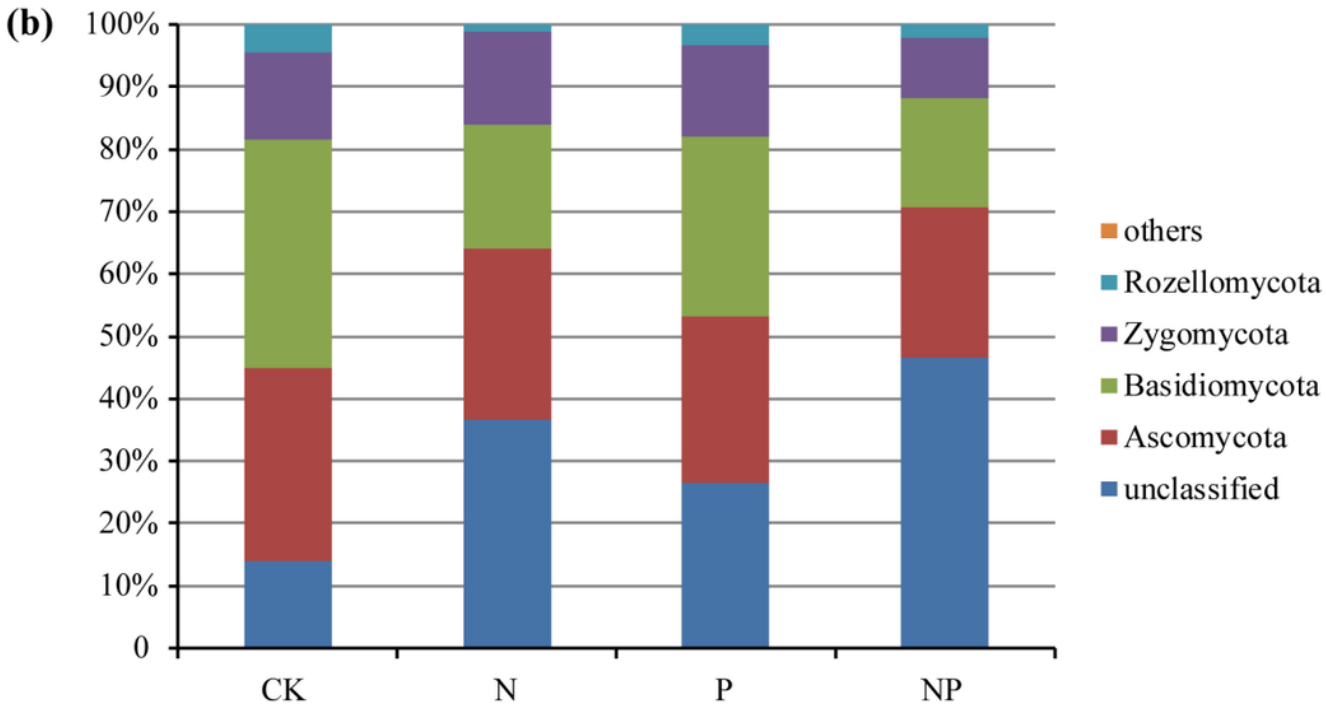

(b)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
CK
N
P
NP
others
Rozellomycota
Zygomycota
Basidiomycota
Ascomycota
unclassified

Figure 2

Responses ratio analysis of changes in the relative abundance of dominant bacterial phyla (a) and fungal phyla/genera (b) in response to N, P and NP treatment compared to the control treatment, at the 95% confidence interval.

Red points indicate significant changes compared with the control treatment.

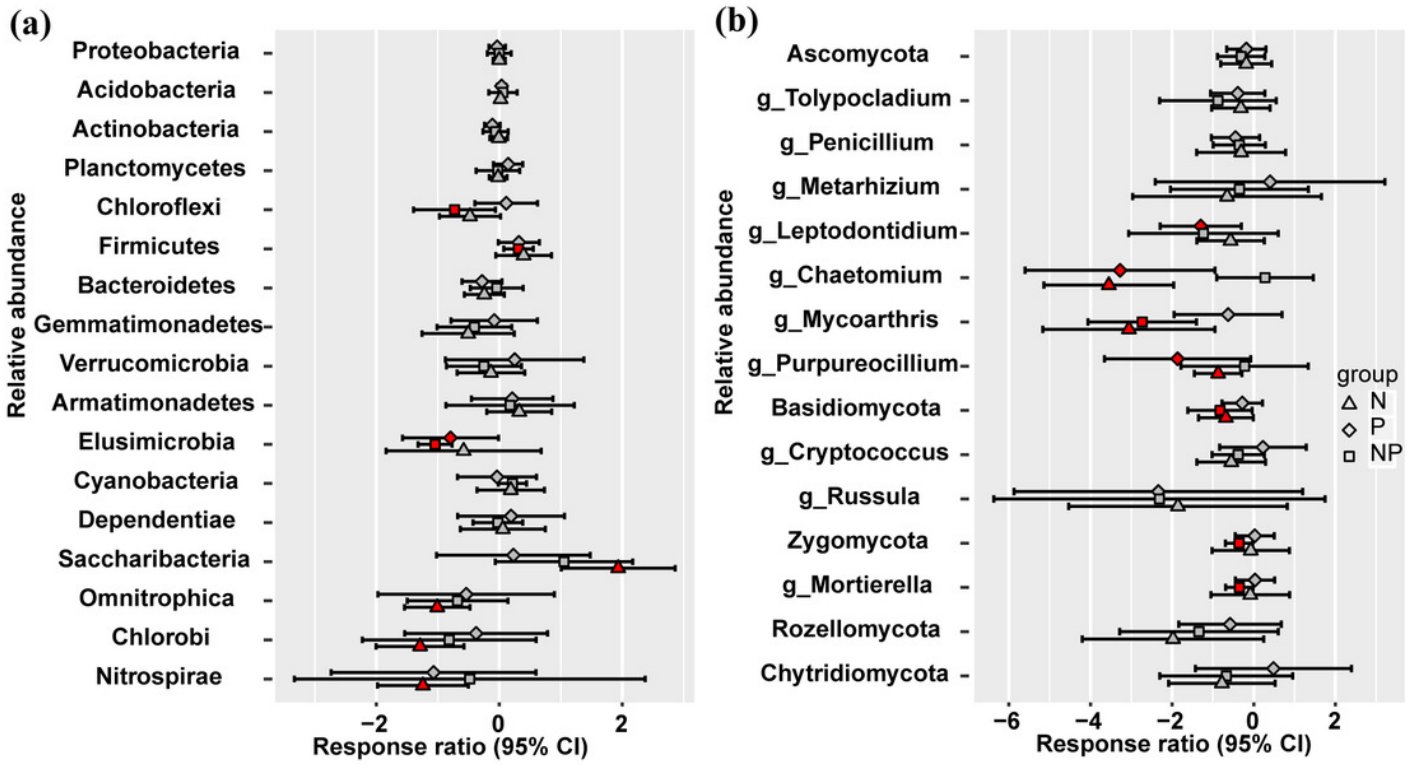


Figure 3

Bacterial and fungal phylotype richness (a - b) and Shannon diversity index (c – d) under different treatments.

The error bars represent the SE of the mean (n = 4).

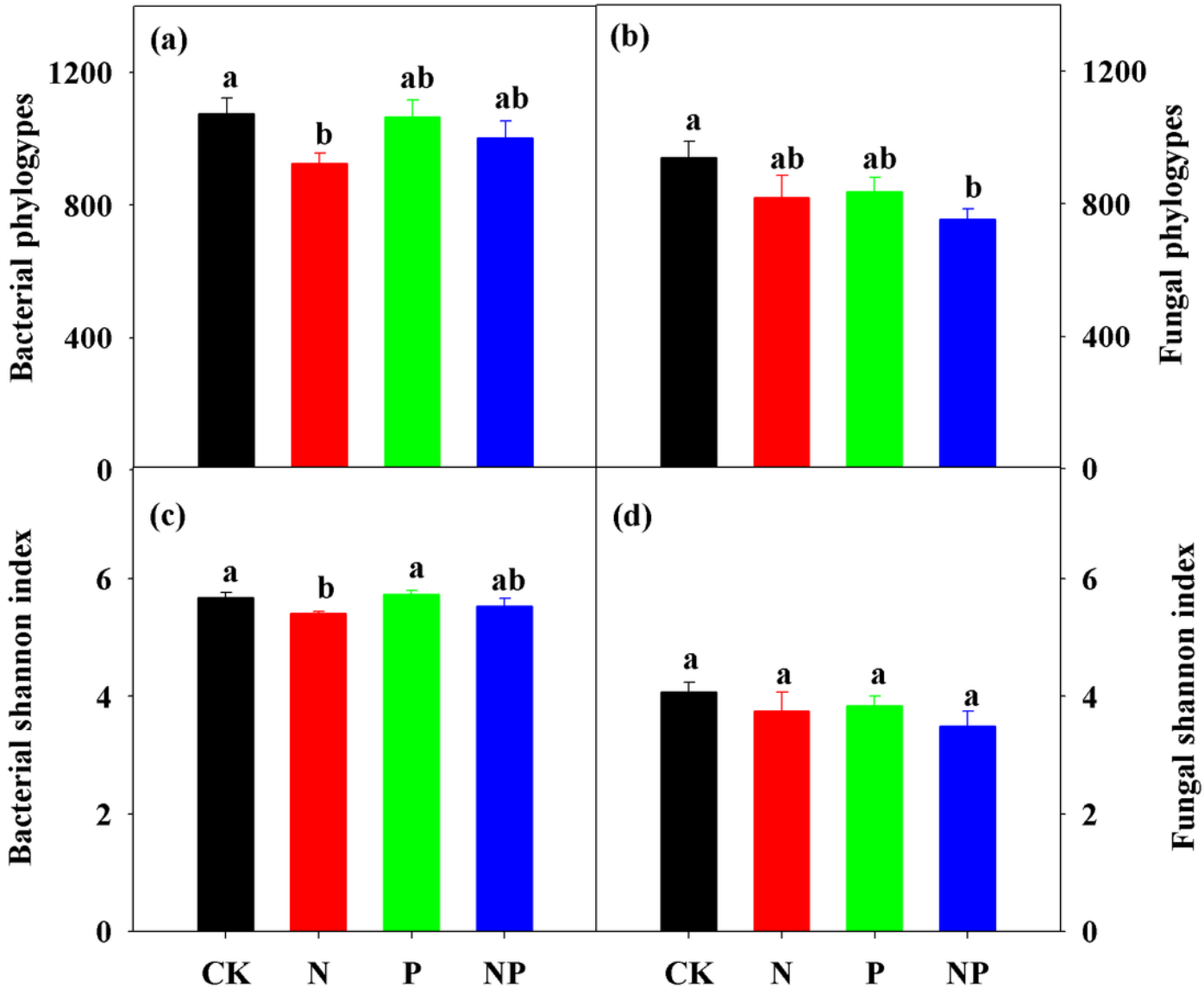
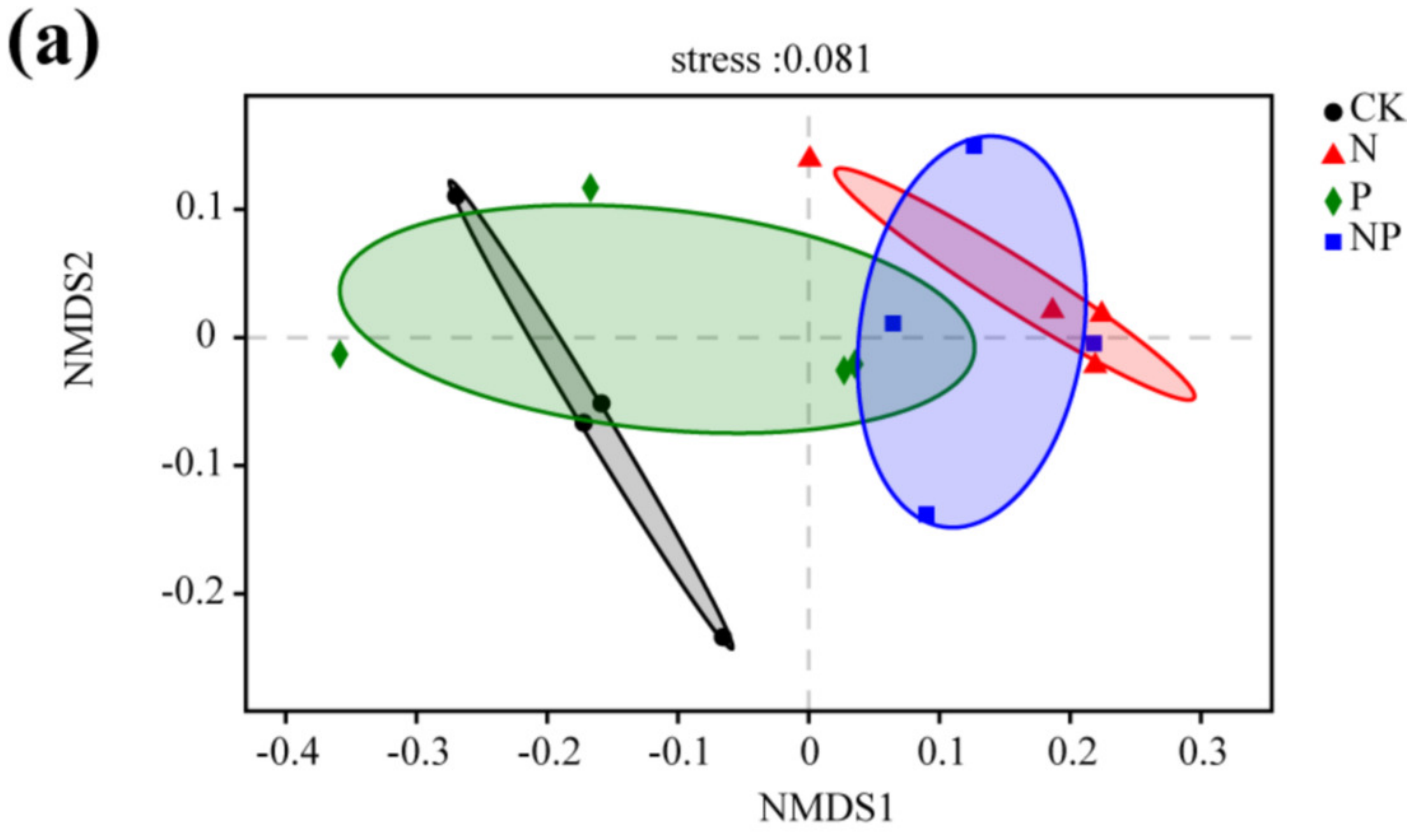


Figure 4

Non-metric multidimensional scaling (NMDS) ordination of the soil bacterial (a) and fungal (b) community structure under different treatments.

The Bray-Curtis distance matrix based on the abundance of OTUs was used to determine the compositional variation.


(a)
stress :0.081
CK
N
P
NP
NMDS2
NMDS1
0.1
0
-0.1
-0.2
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

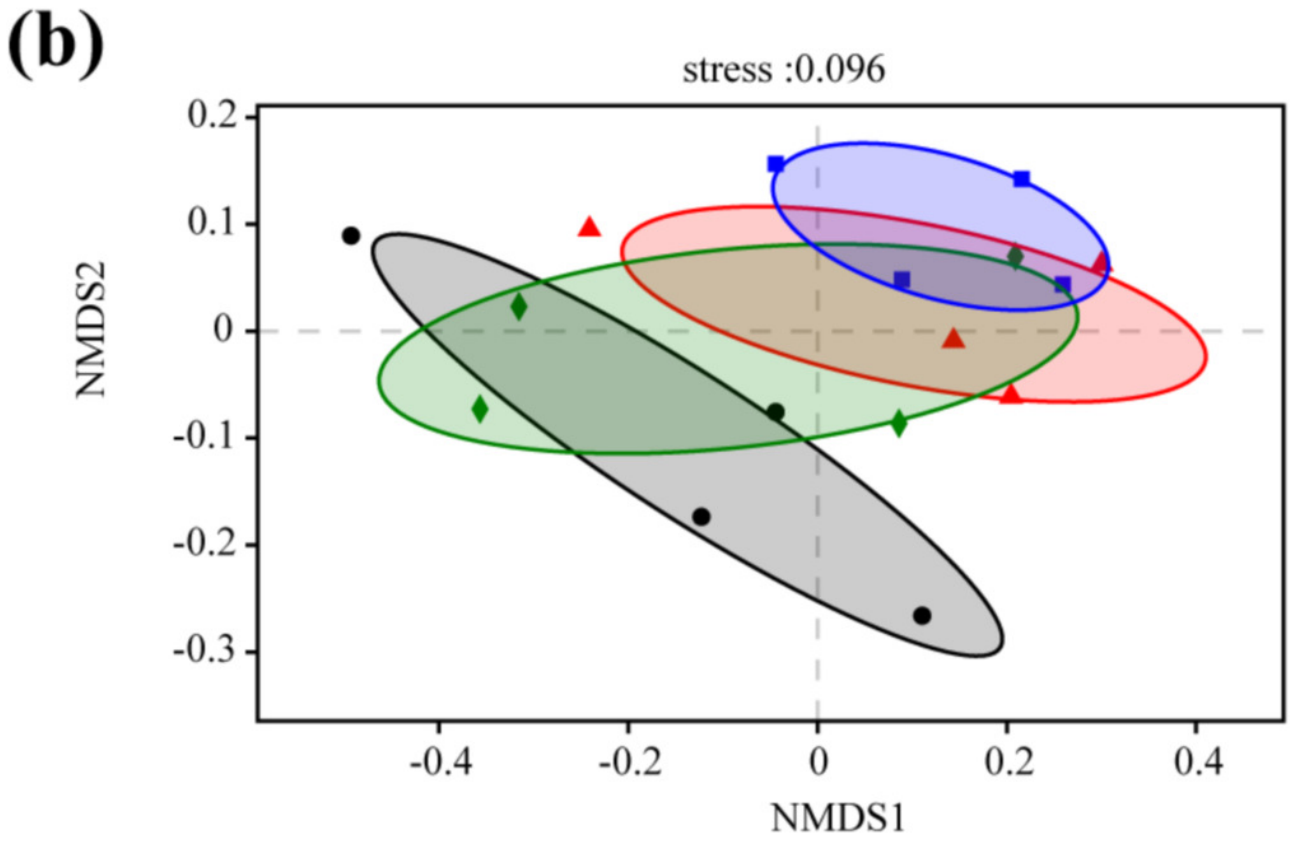
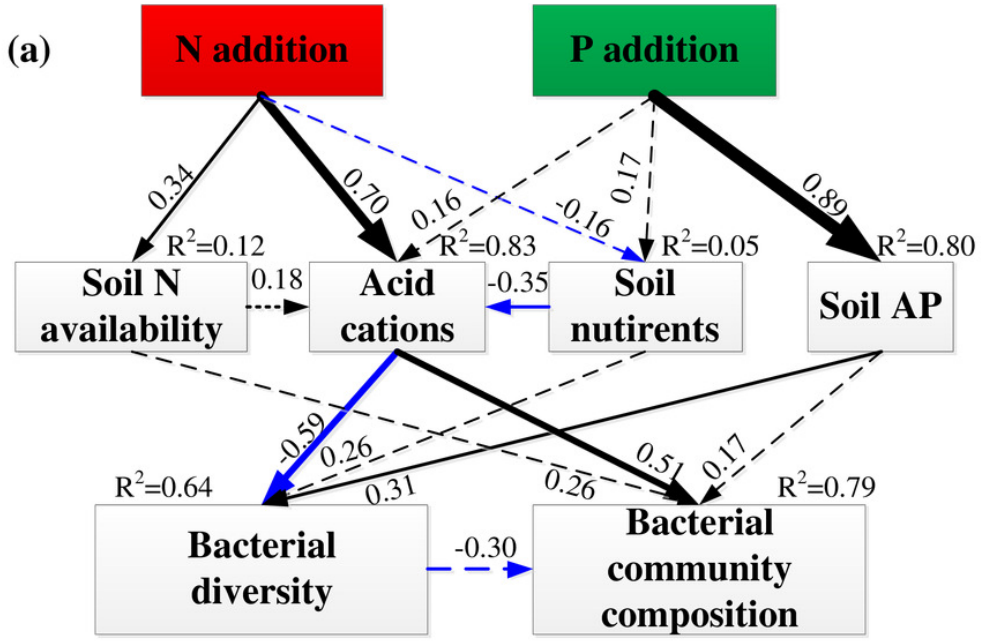

(b)
stress :0.096
NMDS2
NMDS1
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.2
0
0.2
0.4

Figure 5

Structural equation model (SEM) analysis of the effects of N and P enrichment on soil bacterial (a) and fungal (b) community structure.

Results of model fitting: (a) χ^2 = 19.38, df = 13, *P* = 0.11, RMSEA = 0.18, AIC = 65.38; (b) χ^2 = 18.39, df = 13, *P* = 0.14, RMSEA = 0.17, AIC = 64.39. Black and blue arrows represent significant positive and negative pathways, respectively, and dashed arrows indicate nonsignificant pathways. Numbers at the arrows are standardized path coefficients and arrow width is proportional to the strength of the relationship. R^2 values on top of response variables indicate the proportion of variation explained by relationships with other variables.

Prior to SEM analysis, soil N availability (NH_4^+ and NO_3^-), soil acid cations (H^+ and Al^{3+}), soil nutrients (SOC, TN, DOC, MBC and MBN), bacterial and fungal community composition (OTUs) were subject to principal component analysis (PCA) procedure to reduce the number of variables.


(a)
N addition
P addition
0.34
0.70
0.16
-0.16
0.17
0.89
R2=0.12
R2=0.83
R2=0.05
R2=0.80
Soil N availability
0.18
Acid cations
-0.35
Soil nutirents
Soil AP
-0.59
0.26
0.51
0.17
0.31
0.26
R2=0.64
R2=0.79
Bacterial diversity
-0.30
Bacterial community composition

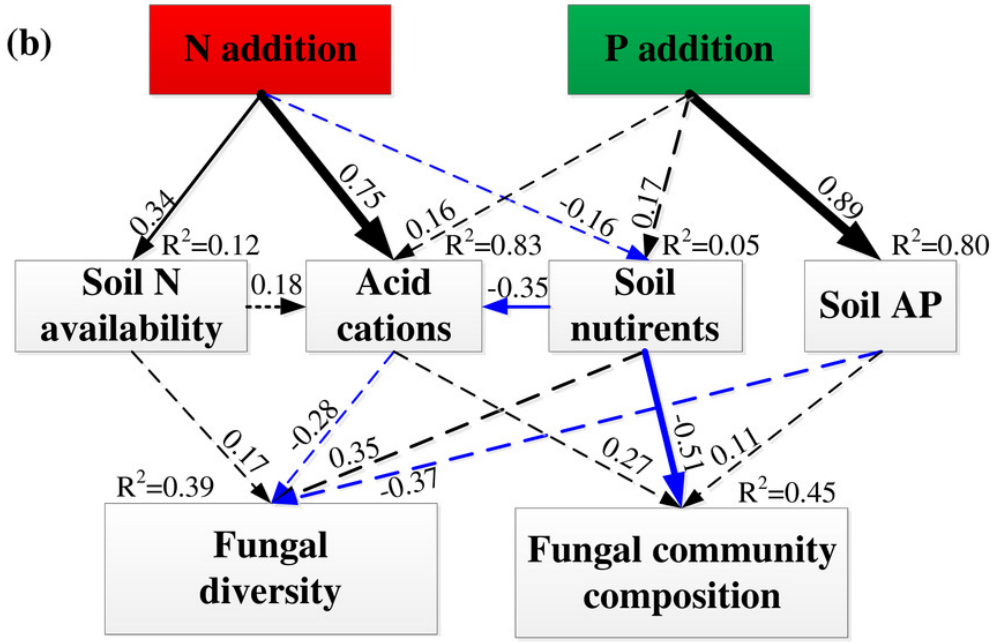
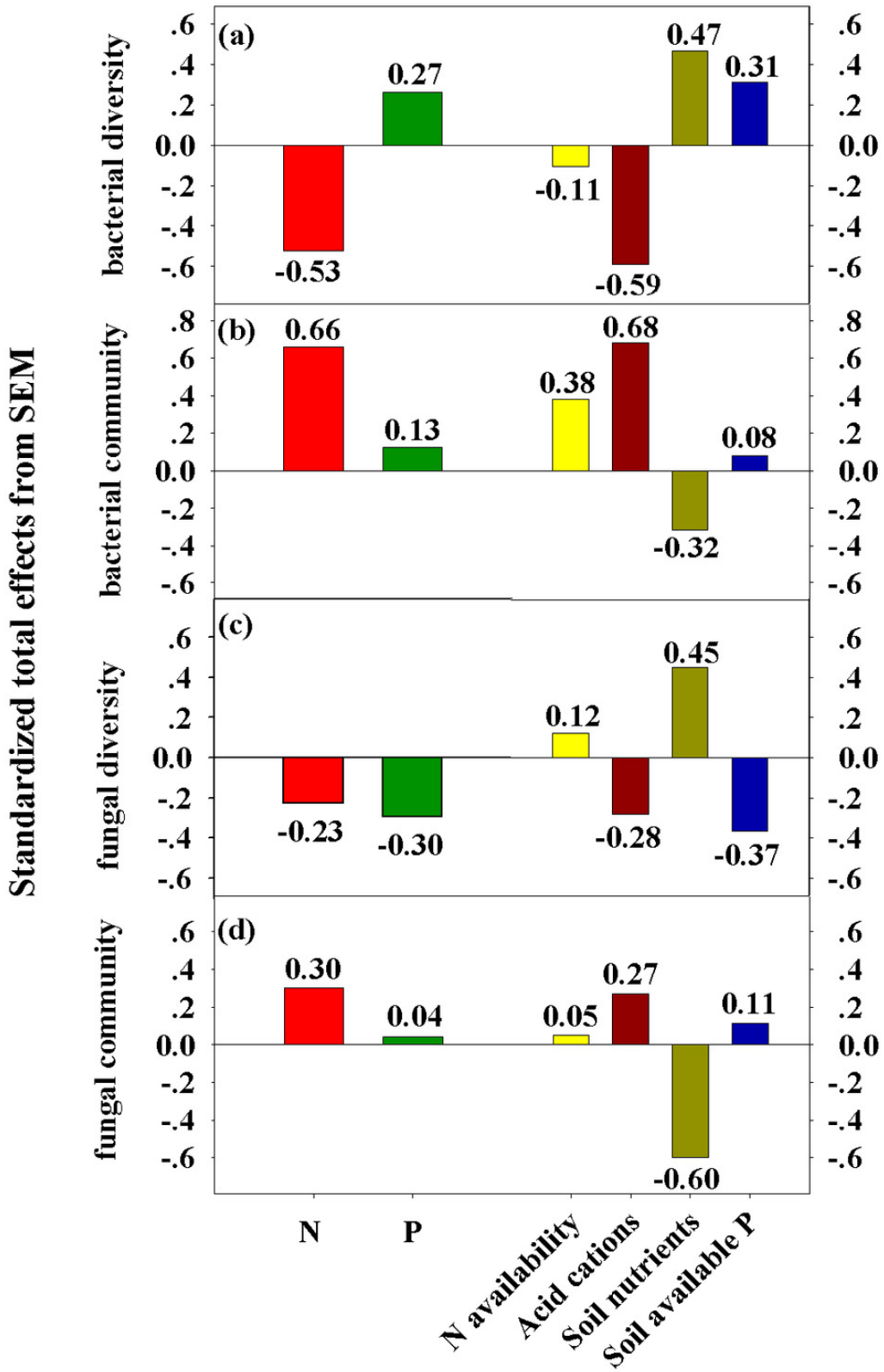

(b)
N addition
P addition
0.34
0.75
0.16
-0.16
0.17
0.89
R2=0.12
R2=0.83
R2=0.05
R2=0.80
Soil N availability
0.18
Acid cations
-0.35
Soil nutirents
Soil AP
-0.17
-0.28
0.35
0.27
-0.57
0.11
-0.37
R2=0.39
R2=0.45
Fungal diversity
Fungal community composition

Figure 6

SEM derived standardized total effects of N and P additions, and variables including N availability, acid cations, soil nutrients, and soil available P on bacterial diversity and community composition (a-b) and fungal diversity (c-d).


Standardized total effects from SEM
(a)
bacterial diversity
0.27
0.47
0.31
-0.53
-0.11
-0.59
(b)
bacterial community
0.66
0.13
0.38
0.68
0.08
-0.32
(c)
fungal diversity
0.12
0.45
-0.23
-0.30
-0.28
-0.37
(d)
fungal community
0.30
0.04
0.05
0.27
0.11
-0.60
N
P
N availability
Acid cations
Soil nutrients
Soil available P