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Nitrogen addition reduces soil bacterial richness, while phosphorus addition alters community composition in an old-growth N-rich tropical forest in southern China

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ABSTRACT

Increased nitrogen (N) deposition endangers the biodiversity and stability of forest ecosystems, and much of the original phosphorus (P) parent material continues to decrease in most lowland tropical forests. It remains poorly understood as to how soil microbial diversity at a molecular level responds to the addition of excess N and mitigation of soil P limitation, as well as their influencing factors, in the N-rich tropical forest ecosystems. To reach a better understanding, we conducted a six-year N– and P-addition experiment consisting of three treatments: N-addition (150 kg N ha^{-1} yr^{-1}), P-addition (150 kg P ha^{-1} yr^{-1}), and NP-addition (150 kg N ha^{-1} yr^{-1} plus 150 kg P ha^{-1} yr^{-1}), besides a control treatment in an old-growth tropical forest in southern China. We examined the snapshot responses of soil bacterial richness and community composition to the elevated N and P levels after six years using a 16S rRNA gene MiSeq sequencing method. The soil bacterial α-diversity, which is represented by Chao1 index in terms of bacterial richness, was 783 ± 87 (mean ± SD) across all samples in this study. The N addition caused a decline in soil bacterial richness, most likely through its negative effect on soil pH. The decrease in soil pH resulted from the direct N input and indirect NO_3^- increase. However, the P treatment had no effect on soil bacterial richness. The NP treatment also reduced the soil bacterial richness as the N addition. These results suggested that the P input could not alleviate the loss of soil bacterial richness induced by excess N deposition in the old-growth N-rich tropical forest. The Acidobacteria, which comprised 31.1% of the soil bacterial community, were the most dominant bacteria across all samples. The addition of P shifted the soil bacterial community composition. The elevated P availability with P-addition and the decreased understory plant coverage in the N-input treatment altered the soil bacterial β-diversity. Our results highlight the different roles of N and P depositions in shaping the soil bacterial richness and community composition, thereby causing concomitant changes in understory plant and underground microbial communities in this ecosystem.

1. Introduction

Many tropical forests are species rich, functionally complex, and highly dynamic ecosystems, containing an estimated 30% of terrestrial carbon (C) stocks and regulating hydrological and climatic cycles (Siddique et al., 2010; Camenzind et al., 2014). Nevertheless, they are endangered by increasing human activities (Brummitt and Lughadha, 2003). The increased nitrogen (N) deposition or fertilization in tropical areas may alter these diverse N-limited or N-saturated ecosystems (Galloway et al., 2004; Wright et al., 2011; Fisher and Huasco, 2013). Humid tropical forests with acidic soils are considered to be more sensitive to N deposition than the temperate forests (Lu et al., 2014).

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Most lowland tropical forests occur on highly weathered soils, where much of the original phosphorus (P)-rich parent material has been continuously decreasing through leaching and immobilization during the process of soil formation, and N is, therefore, expected to be relatively more available than P in old soils (Vitousek et al., 2010; Alvarez-Clare et al., 2013). The combined N and P addition has a considerable synergistic influence on ecosystems, possibly because the addition of a single nutrient leads to the limitation of an alternative nutrient, suggesting that the N and P supplies are closely balanced in most natural ecosystems (Elser et al., 2007).

Microbes comprise the most diverse and abundant group of organisms on Earth and are critical to the maintenance of plant diversity and ecosystem function (Jackson et al., 2008; Bardgett and van der Putten, 2014; Bennett et al., 2017). The shifts in the soil microbial community composition, as well as the richness of the functional genes specific to recalcitrant C decomposition with N addition, affect the soil C and N cycles (Liu and Greaver, 2010; Freedman et al., 2013). Incorporating soil microbial communities into ecosystem models is necessary for accurately predicting ecosystem processes under N addition conditions (Miki et al., 2010). Several studies have reported negative effects on soil bacterial and fungal species diversity as a result of N fertilization (cf. Yao et al., 2014; Freedman et al., 2015; Kaspari et al., 2017). Apart from the changes in microbial α-diversity, N addition also gives rise to the changes in soil microbial community composition (Eisenlord et al., 2013; Freedman and Zak, 2014). The N addition alters the community composition of soil bacteria in the northern hardwood forests (Freedman et al., 2013), but has no effect on the bacterial phylotype diversity in grasslands (Fierer et al., 2012), suggesting that the effects of N deposition on soil microbes are likely ecosystem-specific. The changes in N availability, soil pH, vegetation diversity, and plant-derived C resulting directly or indirectly from N addition could affect soil microbial diversity and community composition (Ramirez et al., 2010; Rousk et al., 2010; Weand et al., 2010; Klironomos et al., 2011; Kaspari et al., 2017). Continuous N addition promotes soil acidification, increases soil C storage, and changes soil C fluxes and microbial enzymatic activities in tropical forests (Mo et al., 2007; Cusack et al., 2011; Yavitt et al., 2011). These pivotal underground ecological processes are closely related to soil microbial community dynamics. Recent research has revealed that the plant-soil-microbe interactions play a critical role in ecosystem C and nutrient cycles (Lange et al., 2015). The understory plants are of ecological importance in forest regeneration and are usually sensitive to soil nutrients (Dirnböck et al., 2014). Several studies explored the ecological functions of understory vegetations in humid subtropical plantations. For example, understory removal significantly reduced fungal biomass, the fungi to bacteria ratio and litter decomposition rate (Zhao et al., 2011, 2012; Liu et al., 2012b), and impacted soil CO_2 and N_2O fluxes (Li et al., 2010; Wu et al., 2011). High levels of N addition reduced the understory species diversity due to the declines in tree seedling and fern functional groups in an N-rich tropical forest (Lu et al., 2010). Therefore, there is an urgent need to improve the understanding of the mechanisms underlying the responses of soil microbial diversity and community composition to N deposition in tropical forests.

Several studies have been conducted to elucidate the effects of P addition on microorganisms in tropical soils (cf. Cleveland et al., 2002; Cleveland and Townsend, 2006; Turner and Wright, 2014), where P limitation for tree productivity and plant biomass is often expected (Wardle et al., 2004). Moreover, P availability can constrain the activity of soil microorganisms in lowland tropical forests, and a threshold for strong belowground P limitation is 2 mg P kg^{-1} resin phosphate (Turner et al., 2018). For example, the P addition caused an increase in the soil microbial C, N, and P, and decreased the soil phosphatase activity, indicating that P limits microbial biomass in the strongly-weathered lowland tropical forest soil (Turner and Wright, 2014). Meanwhile, relieving soil P limitation through exogenous P addition could mitigate N-derived soil acidification by (i) reducing excess N through promoting uptake of nitrates by plants before it leaches out, and (ii) by increasing the release of hydroxyl ions during the uptake of nitrate and phosphate anions (Fujii, 2014; Mao et al., 2017). Modest increases in soil pH were observed in the short-term or modest P-addition studies in tropical forests (Zhang et al., 2011; Turner et al., 2013). P addition increased soil pH in the first few years, while in the sixth year, the stimulated effect of P addition on soil pH disappeared in an old-growth tropical forest (Mao et al., 2017). P addition increased soil microbial biomass and altered the microbial community composition quantified by phospholipid fatty acids (PLFAs) analysis in the first two years in an old-growth tropical forest (Liu et al., 2012a). However, it remains unclear how soil microbial community change after three or more years of P addition in this ecosystem.

Previous studies have investigated the responses of arbuscular mycorrhizal fungi (AMF) abundance, richness and diversity in a tropical montane forest (Camenzind et al., 2014) and the short-term (< 4 year) responses of soil microbial biomass and the fungi to bacteria ratio in old-growth and reforested tropical forests to N addition, P addition, and NP interaction (Liu et al., 2013a; Li et al., 2015). AMF richness was reduced by N− and P-addition with a significant effect in the NP-addition treatment, though the interaction effect of N and P was not significant in the tropical montane forest (Camenzind et al., 2014). In old-growth and reforested tropical forests, no interactions between N− and P-addition on soil bacterial and fungal biomass and microbial community composition analyzed by PLFAs were observed (Liu et al., 2013a, 2015). To the best of our knowledge, the interaction effects of long-term (> 6 year) N− and P-addition on soil bacterial diversity and community composition at a molecular level in forest ecosystems, particularly in N-rich tropical forests, are not well documented.

Therefore, we conducted a full-factorial N− and P-addition experiment in a typical N-rich old-growth tropical forest in southern China, where high N deposition rates of over 30 kg N ha^{-1} yr^{-1} occur downwind from the centers of human activity, in February 2007 (Cui et al., 2013; Liu et al., 2013b). The present study aimed to investigate the effects of a six-year N addition, P addition, and NP interaction on the richness and community composition of soil bacteria, as well as the underlying mechanisms, in an N-rich tropical forest. We first hypothesized that the N addition would negatively affect soil bacterial richness and community composition because of the high N status in the forest ecosystem, and the P addition would cause relatively less decline in soil bacterial richness and lead to a change in soil bacterial community composition by mitigating soil P limitation in the N-rich tropical forest studied. We further hypothesized that the responses of soil bacterial diversity to N and P addition would be correlated with the changes in understory plants in the studied forest after a continuous six-year nutrient addition.

2. Materials and methods

2.1. Study site

This study was conducted in the Dinghushan Biosphere Reserve (DHSBR), which is a UNESCO's Man and the Biosphere (MAB) site (112° 10′ E, 23° 10′ N), on the outskirts of Zhaoqing City, in the middle of Guangdong Province, southern China. The DHSBR is characterized by a monsoon climate, with a mean annual precipitation of 1927 mm, nearly 75% of which falls between March and August (Huang and Fan, 1982). The mean annual temperature is 21.0 °C with average temperatures of 12.6 and 28.0 °C in the coolest (January) and the hottest (July) months, respectively. The total N input from precipitation was 34.4 kg N ha^{-1} yr^{-1} from 2009 to 2010 (Lu et al., 2013). The deposition flux of total P was less than 1 kg P ha^{-1} yr^{-1} (Zhang et al., 2010). We established our research site in an old-growth monsoon evergreen broadleaf forest located at an elevation of 300 m. This forest represents the climax community in this area, which has been well protected from direct human disturbance for over 400 years (Mo et al., 2003). The soil was formed

from sandstone and classified as a lateritic red earth in the Chinese soil classification system (Mo et al., 2003), equivalent to the Oxisol based on the United States Department of Agriculture (USDA) Soil Taxonomy (USDA, 1996). The major plant species included *Castanopsis chinensis* Hance, *Schima superba* Gardn. et Champ., *Cryptocarya chinensis* (Hance) Hemsl., *Cryptocarya concinna* Hance, *Machilus chinensis* (Champ. ex Benth.) Hemsl., and *Syzygium rehderianum* Merr. et Perry in the tree layer and *Calamus rhabdicladus* Burret, *Ardisia quinquegona* Blume and *Hemigramma decurrens* (Hook.) Copel in the understory layer (Lu et al., 2010). The stem densities of *Castonopsis chinensis*, *M. chinensis*, *Schima superba*, *Cryptocarya chinensis*, *Syzygium rehderianum*, and other plants of the old-growth tropical forest were 268, 131, 185, 270, 185 and 1587 tree ha^{-1}, respectively, before the start of N and P fertilization in February 2007 (Liu et al., 2013a).

2.2. Experimental design

The soil N and P addition experiment included three treatments: N-addition (150 kg N ha^{-1} yr^{-1}), P-addition (150 kg P ha^{-1} yr^{-1}), and NP-addition (150 kg N ha^{-1} yr^{-1} plus 150 kg P ha^{-1} yr^{-1}), and a control treatment. There were twenty 5-m × 5-m plots on the same slope, with five replicates of each treatment, separated by > 5-m wide buffer strips to avoid interference from different plots. The plot size and fertilizer levels were similar to those in the experiment conducted by Cleveland and Townsend in Costa Rica (Cleveland and Townsend, 2006). The solutions of NH_4NO_3 and NaH_2PO_4 were applied bimonthly. Fertilizer was dissolved in 5 L of water and applied in each plot using a backpack sprayer. We added N and P at high rates to eliminate all possible nutrient constraints by N and P, as well as to enhance the response of ecological processes (Cleveland and Townsend, 2006; Wright et al., 2011). Each control plot received equal volumes (5 L) of water corresponding to an annual increase of 1.2 mm precipitation.

2.3. Sampling and measurements

Soil samples were collected in July 2013 to determine the effects of N and P additions on soil microbial diversity and community composition, soil pH, the contents of soil organic C (SOC), soil total N (STN), NH_4^+, and NO_3^-, available P (AP), and total P (TP). Five randomly placed soil cores, 2.5 cm in diameter and 10 cm deep were drilled per 5-m × 5-m plot. These five cores from each plot were pooled and sieved through a 2-mm mesh (to carefully remove plants, roots, and gravel) in order to minimize the influence of plant residues on chemical and microbial analyses. To trace the long-term and general dynamics of NH_4^+ and NO_3^- concentrations in the soil, we additionally sampled soils in July 2012, March 2013, and January 2014. The sum of NH_4^+ and NO_3^- concentrations represents the soil available N. Soil pH was measured in deionized water using a glass electrode. The SOC and STN contents were determined using the dichromate oxidation and Kjeldahl methods, respectively (Sparks et al., 1996). The NH_4^+ concentration was measured spectrophotometrically using the indophenol blue method, and the NO_3^- concentration was measured using the cadmium reduction method followed by a sulfanilamide-NAD reaction (Liu et al., 1996). The TP content was measured using the alkaline fusion molybdenum-antimony colorimetric method, and the AP content was measured spectrophotometrically after extraction with a solution of hydrochloric and sulfuric acids (Bao, 2000).

2.4. Understory vegetation status

All understory plants in this study were identified as vascular plants < 1.5 m in height. During soil sampling for microbial diversity analysis in July 2013, a 1-m × 1-m subplot was randomly selected and installed for monitoring understory changes in each 25-m^2 plot. The understory vegetation status was based on the measurements of species richness (mean number of species m^{-2}), individual abundance (stems m^{-2}), and coverage (percentage). We defined species richness as the total number of species in a specific area, considering that species richness is the most significant measure of diversity. The individual coverage was estimated visually by using a square grid method.

2.5. DNA extraction and MiSeq sequencing of 16S rRNA gene amplicons

DNA was extracted using the MO BIO Power Soil DNA Extraction kit (MO BIO Laboratories, Carlsbad, USA) and quantified using a NanoDrop Spectrophotometer. The polymerase chain reaction (PCR) amplification was conducted using the primers 515F (5′-GTGCCAGCM GCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR was performed in a 25-μL reaction volume containing 1 μL each of 10 μM primer, 10 ng of template DNA, and 0.5 units of Accuprime high-fidelity Taq polymerase (Invitrogen, USA). Each sample was amplified in triplicate under the following conditions: 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s, and a final extension at 67 °C for 10 min. The PCR products from the same sample were mixed in one tube and were then purified using a gel extraction kit (Zhang et al., 2016). An equimolar amount of PCR product from each sample was pooled for library construction. Libraries were prepared using the Illumina Truseq DNA kit. The libraries were mixed with PhiX in an amount equal to 30% of the total DNA and then sequenced on an Illumina Miseq system with the MiSeq Reagent Kit v2 for 2 × 250 bp at the Environmental Genome Platform of Chengdu Institute of Biology, Chinese Academy of Sciences, Sichuan, China.

2.6. Sequence analyses

The sequence data were extracted based on the unique sample barcodes, trimmed for sequence quality, and then denoised using the QIIME Pipeline (http://qiime.org/tutorials/tutorial.html). The Operational taxonomic units (OTUs) were identified using USEARCH v7.0 (http://www.drive5.com/usearch/download.html) with a threshold of 97%. Subsequently, the chimeric sequences and singletons were removed from clustered sequences using the USEARCH algorithm. Resampling to the same sequence depth (8000 sequences per sample) was performed using daisychopper.pl (http://www.festinalente.me/bioinf/downloads/daisychopper.pl) for downstream analysis.

2.7. Data analyses

Repeated-measures ANOVA was used to determine the effects of sampling time and nutrient (N, P, and NP) addition on soil NH_4^+ and NO_3^- concentrations. No significant interaction was observed between the sampling time and nutrient addition (Supplement Table 2). The average values of NH_4^+ and NO_3^- concentrations from four-time samples were subsequently used throughout the analyses. A two-way ANOVA was used to examine the main effects of N addition, P addition, and their interaction on soil properties and understory plant richness, individual abundance and coverage, soil bacterial α-diversity, and the abundances of the bacterial groups with relative abundances higher than 1% in total bacterial communities. A one-way ANOVA was used to compare the means of soil and plant properties, and soil bacterial α-diversity among the treatments. The statistical analyses of ANOVA were performed using the SPSS software v19.0 for Windows (SPSS Inc., Chicago, IL, USA). The effects of N addition, P addition, and NP interaction on soil bacterial community composition were assessed by a PerMANOVA (permutations = 999).

A distance-based redundancy analysis (dbRDA) was used to partition the variation in soil microbial community composition into the fractions explained by soil and plant community variables, in which the vectors of soil parameters (pH, SOC, TN, NH_4^+, NO_3^-, TP, and AP), understory plant richness, individual abundance, and coverage were taken into consideration. The model construction, variables and axes significance permutation test, variance inflation factor analysis, and

forward selection were conducted using the functions "dbrda", "anova.cca", "vif.cca", and "ordiR2step", respectively, in R package Vegan.

The structural equation modeling analysis was performed with the specification of a conceptual model of hypothetical relationships, assuming that the N addition and P addition alter edaphic factors and understory plant richness, which, in turn, affect soil microbial diversity. The analysis was performed with the AMOS v17.0 software (SPSS Inc., Chicago, IL, USA), and the data were fitted to the models using the 'robust' maximum likelihood estimation procedures. The adequacy of model fitting was determined by several tests, including the χ^2 test ($p > 0.05$, CMIN/df < 2), goodness of fit (GFI) (values > 0.8 and < 1), the root square mean error of approximation (RMSEA) (values < 0.05), and the comparative fit index (CFI) (values > 0.9) (Jassey et al., 2013; Zhang et al., 2013). To obtain the most parsimonious model, the non-significant indicators and pathways were eliminated in the final path model. For each set of analyses, the R^2 values were obtained for each dependent matrix, representing the proportion of total variance explained by the model (Grace et al., 2010; You et al., 2014). All significant differences were set at a level of $p < 0.05$.

3. Results

3.1. Effects of N and P additions on soil and plant parameters

N addition significantly increased available N, primarily as NO_3^- (Fig. 1a and b), and decreased soil pH (Fig. 2a). P addition significantly increased TP and AP (Fig. 1b and c) and negatively impacted NO_3^- (Fig. 1a). NO_3^- was significantly lower in the NP addition plots than in the N-addition plots, while there was no difference in NO_3^- between the control plots and the NP addition or the P addition plots (Fig. 1a). Soil organic C was neither affected by N addition nor by P addition ($p > 0.05$). Soil pH was significantly higher in the NP addition plots than in the N addition plots, while there was no difference in soil pH between the control plots and the NP addition or the P addition plots (Fig. 2a). N addition significantly decreased understory plant richness, coverage and abundance (Fig. 2b–d). P addition significantly decreased understory plant abundance (Fig. 2d). The understory plant coverage was significantly lower in the N-addition plots than in the control plots, while there was no difference in understory plant coverage between the control plots and the NP addition or the P addition plots (Fig. 2c). The understory plant richness and individual abundance were significantly lower in N−, P−, and NP-addition plots than in the control plots (Fig. 2b, d). Meanwhile, the interaction effect of N and P addition on understory plant individual abundance was observed (Fig. 2d). However, the interaction effect of N and P addition on soil pH, available N, NO_3^-, TP, or understory plant richness and coverage was not observed (Figs. 1 and 2).

3.2. Effects of N and P additions on soil bacterial α-diversity

N addition had a significant negative impact on soil bacterial α-diversity in terms of bacterial richness represented by the Chao1 index (Fig. 3), whereas P addition did not affect bacterial richness (Fig. 3). Soil bacterial richness was significantly lower in the NP addition plots than in the control plots, while no significant difference was observed between the N and NP addition plots (Fig. 3). No significant interaction effect of N and P addition was observed on bacterial richness (Fig. 3). The Shannon-Wiener index was neither affected by N addition nor by P addition ($p > 0.05$).

The regulatory pathway model of plant richness and soil bacterial richness associated with N and P additions was supported by all of the statistical tests ($\chi^2 = 15.714$, $p = 0.265$, CMIN/df = 1.209, GFI = 0.815, CFI = 0.967, RMSEA < 0.05) (Fig. 4). The structural equation modeling analysis accounted for 64, 44, 88, 66, and 20% of the variations in soil pH, NO_3^-, AP, plant richness, and soil bacterial α-diversity, respectively (Fig. 4). A decrease in soil pH resulted from the direct N addition and indirect NO_3^- increase (Fig. 4). N addition caused a decline in soil bacterial richness, most likely through its negative effect on soil pH (Fig. 4). An increase in soil NO_3^- and AP concentrations due to nutrient additions led to a reduction in understory plant richness (Fig. 4). However, the changes in understory plant richness did not result in concomitant shifts in soil bacterial richness (Fig. 4).

3.3. Effects of N and P additions on soil bacterial community composition

The soil bacterial community was dominated by Acidobacteria

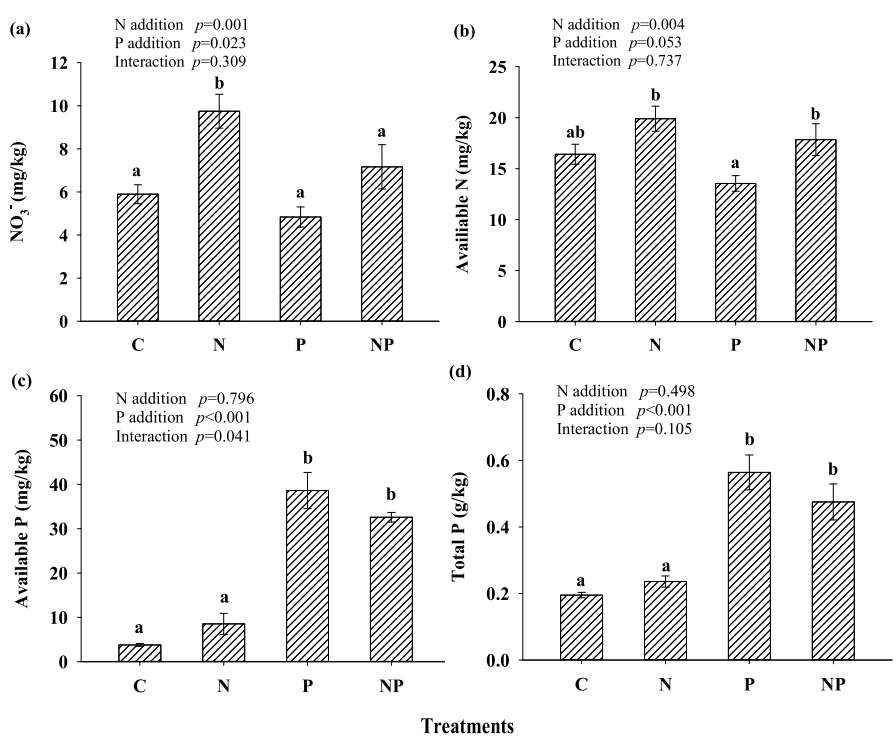

Fig. 1. Effects of nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) additions compared to the control treatment (C) on soil NO_3^--N (a), soil available N (b), soil available P (c), and soil total P (d) in an old-growth N-rich tropical forest in southern China. The results of the two-way ANOVA for the primary factors (N and P) and NP interaction on these soil properties are shown in the sub-figures. The soil samples were analyzed in July 2013 (after 6 years of manipulation). Values are means with SE (n = 5). Different lowercase letters represent multiple comparisons of means among treatments determined by SNK-test (α = 0.05).

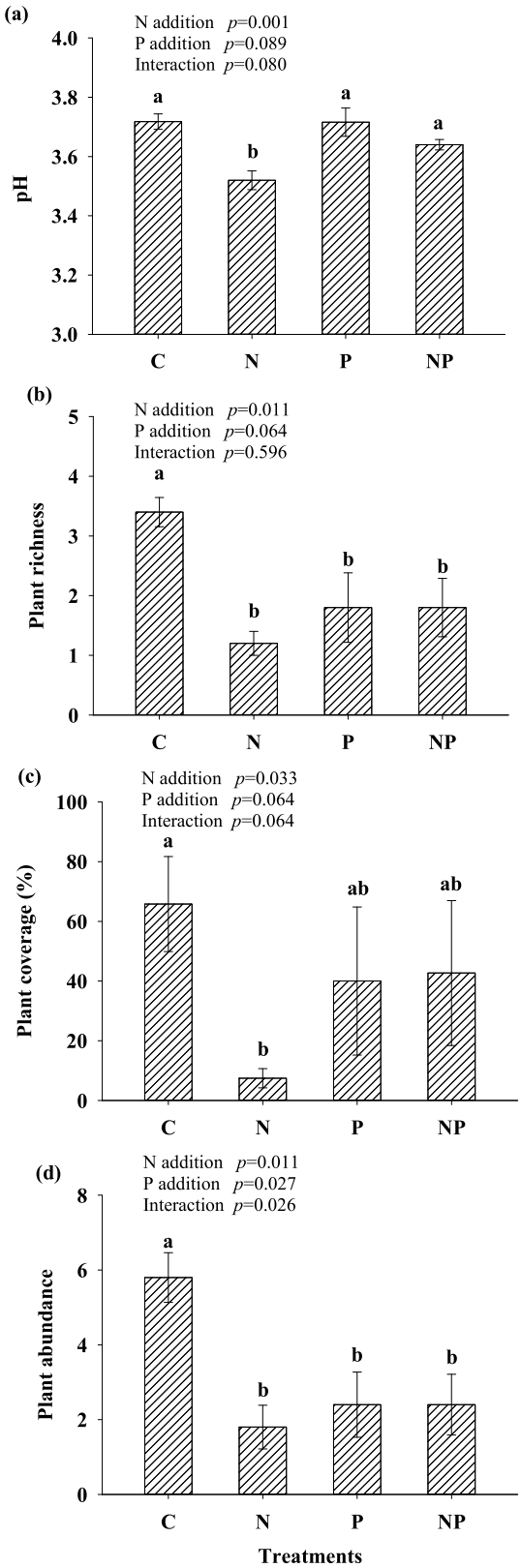

Fig. 2. Effects of nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) additions compared to the control treatment (C) on soil pH (a), understory plant richness (mean number of species m^{-2}) (b), understory plant coverage (%) (c) and understory plant individual abundance (stems m^{-2}) (d) in an old-growth N-rich tropical forest in southern China. The results of the two-way ANOVA for the primary factors (N and P) and their interaction on soil pH (a), plant richness (b), plant coverage (c), and plant abundance (d) are shown in the sub-figures. Values are means with SE (n = 5). Different lowercase letters represent multiple comparisons of means among treatments determined by SNK-test (α = 0.05).

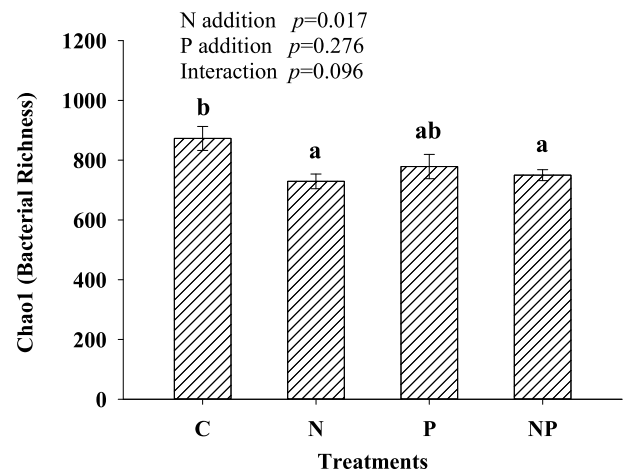

Fig. 3. Effects of nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) additions compared to the control treatment (C) on soil bacterial richness (Chao1) in an old-growth N-rich tropical forest in southern China. The results of the two-way ANOVA for the primary factors (N and P) and their interaction on soil bacterial richness are shown in the sub-figures. Values are means with SE (n = 5). Different lowercase letters represent multiple comparisons of means among treatments determined by SNK-test (α = 0.05).

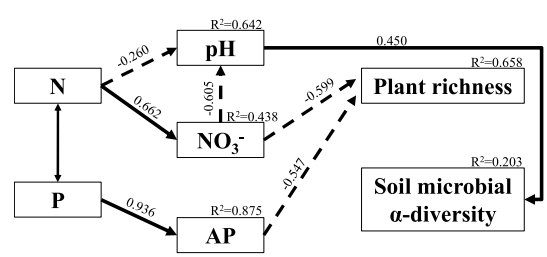

Fig. 4. Structural equation modeling (SEM) analysis depicting the regulatory pathway of the controls of plant richness and soil microbial α-diversity under the conditions of N addition, P addition, pH, NO_3^- concentration, and available phosphorus (AP) factors in an old-growth N-rich tropical forest in southern China. The numbers on the arrow lines are the standardized direct path coefficients. The solid double arrow line between N and P indicates that the interaction effects of N and P addition are considered in the SEM. Other solid arrow lines indicate positive effects, while the dashed arrow lines indicate negative effects.

(31.1%), Actinobacteria (14.0%), Alphaproteobacteria (13.7%), and Gammaproteobacteria (9.0%) across all 20 samples, and other taxa were present in low abundances (Fig. 5). P addition significantly decreased the abundances of Alphaproteobacteria (p = 0.038) and Thermoleophilia (p = 0.029), while neither the N addition nor the NP interaction affected the abundances of dominant bacterial taxa (> 1%).

Differences in the overall soil bacterial community composition were evaluated using dbRDA. A two-way perMANOVA indicated that the P addition (p = 0.006) had a significant effect on soil bacterial community composition, while no significant effects of the N addition (p = 0.131) and NP interaction (p = 0.374) were observed. Furthermore, we observed shifts in the bacterial community composition in a redundancy analysis (Fig. 6). The AP content (p = 0.002) and understory plant coverage (p = 0.004) were selected as the best vectors to explain the variation in the bacterial community data (Fig. 6).

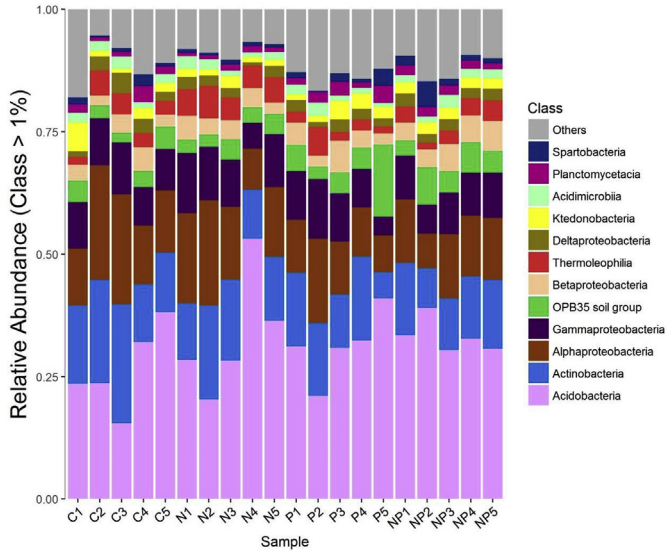

Fig. 5. Relative abundances of the soil bacterial groups under N addition and P addition treatments in each plot in an old-growth N-rich tropical forest in southern China. The groups with relative abundances higher than 1% are shown, while those with less than 1% relative abundance are integrated into "other".

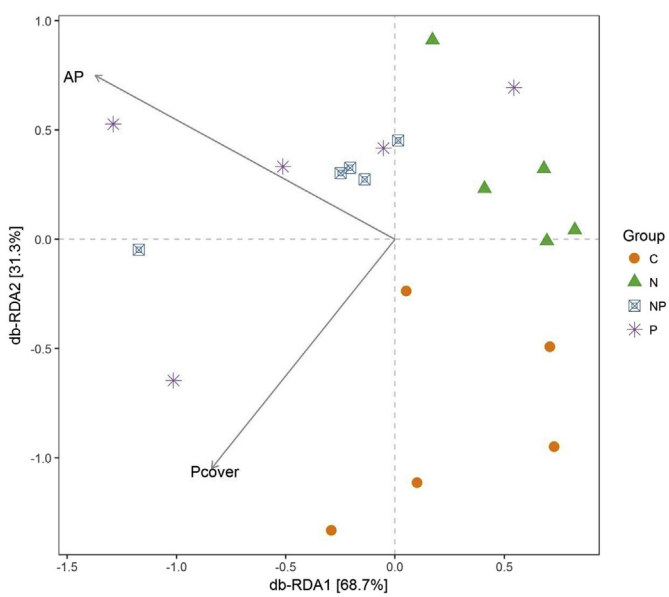

Fig. 6. Soil bacterial community composition and its variation partition ordination plot of a distance-based redundancy analysis (dbRDA) under N and P addition treatments in an old-growth N-rich tropical forest in southern China. The functions "dbrda", "anova.cca", "vif.cca", and "ordiR2step" in R package Vegan were used to perform the model construction, variables and axes significance permutation test, variance inflation factor analysis, and forward selection, respectively. The vectors available phosphorus (AP) and understory plant coverage (Pcover) demonstrate significant correlations with the data and are presented as arrows ($p < 0.05$).

4. Discussion

4.1. Nitrogen, rather than phosphorus, addition reduces soil bacterial richness

Molecular analyses provide insight into the influence of nutrient additions on the soil bacteria in this forest ecosystem. In the present study, N addition contributed to both higher soil NO_3^- concentration and lower soil pH (Figs. 1a and 2a). The soil bacterial richness was reduced under the N addition treatment (Fig. 3), indicating that the six years of continuous N addition primarily decreased the number of bacterial species in the tropical forest ecosystem. These results support our first hypothesis that the soil bacterial richness would decline with N addition. In some studies in the tropical rainforest, temperate forest, steppe, and arctic tundra ecosystems, the experimental addition of N also led to a decrease in soil bacterial diversity (Campbell et al., 2010; Eisenlord et al., 2013; Freedman and Zak, 2014; Zeng et al., 2016; Kaspari et al., 2017). The bacterial response to the projected high rates of N deposition could be highly dependent on the background nutrient availability or acidification status. The studied forest soil was highly acidified and acid-sensitive, with pH < 4, base saturation < 5%, and exchangeable H^+ and Al^{3+} ions dominating the soil exchangeable cation pool (Mao et al., 2017). The soil bacterial diversity is frequently reported to be related to the soil pH, even at large scales, and a narrow pH range for the optimal growth of bacteria is probably the main factor underlying a decrease in bacterial richness (Fierer and Jackson, 2006; Lauber et al., 2009; Rousk et al., 2010; Peay et al., 2017). The overall soil pH in the present study (mean ± SD = 3.65 ± 0.11) was lower than that in the nutrient-addition experiment of a Panama forest (pH = 4.47–5.36) (Turner et al., 2013). In the Panama experiment, the N addition led to a reduction in soil pH by 0.8 units compared to the controls and suppressed the richness of soil prokaryotes (Kaspari et al., 2017). In the present study, the N addition-induced decline of 0.2 pH units was associated with a decrease in soil bacterial richness, suggesting that a small decline of soil pH could have an obvious effect on bacterial α-diversity in the highly acidified N-rich old-growth tropical forest soils.

Moreover, soil acidification can result in changes in the availabilities of calcium and magnesium (Lucas et al., 2011), as well as in the mobilization of the toxic mineral aluminum (Aber et al., 1998), which might indirectly affect the soil microbial community. The increased aluminum/calcium ratio and decreased low base saturation further suggested that soil acidification was accelerated by an N input in the present forest ecosystem (Mao et al., 2017). The cation exchange capacity (CEC) in soil strongly contributes to changes in the soil bacterial community, as soil CEC influences the sensitivity of soil to acidification and its capacity for nutrient cation retention (Clark et al., 2007). In another nutrient addition experiment of this studied tropical forest, a six-year N addition resulted in the depletion of base cations, which further decreased the acid neutralizing capacity and increased the CEC of soil (Lu et al., 2014). In addition, the shifts in the quality and quantity of soil organic matter following N addition are linked to the variations in soil microbial characteristics and enzyme activities (Cusack et al., 2011). Although the content of SOC did not differ among the different treatments in the present study, a seven-year N addition decreased dissolved organic C leaching, which likely increased the soil C sequestration in an adjacent and similar tropical forest (Lu et al., 2013).

The Gram-positive bacteria have much thicker cell walls than the gram-negative bacteria (Silhavy et al., 2010); the anionic teichoic acids abounding in the gram-positive bacterial cell wall can bind cations and play a role in cation homeostasis (Silhavy et al., 2010); the gram-positive bacteria are usually oligotrophic k-strategists and the gram-negative bacteria are copiotrophic r-strategists (Andrews and Harris, 1986). Elevated N addition reduced the abundance of gram-negative bacteria in the studied tropical forest (Wang et al., 2018), probably leading to the decline of soil bacterial richness under the intensified N addition stress in the present study.

P addition had no significant effect on soil bacterial α-diversity, suggesting that it remains steady with increasing P availability in the present tropical forest. In this study, P addition increased the AP, TP, and NO_3^- contents, but did not affect the soil pH (Figs. 1 and 2). Therefore, the non-significant response of bacterial richness to P addition could be explained by that the P inputs do not alleviate excess N-

derived soil acidification. However, in the previous studies, the soil bacterial and fungal biomass are increased with P addition in old-growth and secondary tropical forests in southern China (Liu et al., 2012a; Li et al., 2015). The soil microbial response to P addition might vary with soil C availability, as several studies have shown that microbial biomass and activity are more likely to be limited by P when C availability is high (Cleveland et al., 2002; Esberg et al., 2010). The increases in plant growth rate and subsequent increases in plant C inputs into soils by P addition possibly enhance the soil microbial activity (Li et al., 2015).

4.2. Phosphorus addition alters soil bacterial community composition

The soil bacterial community composition has been shown to be correlated with soil and plant properties, and plant-microbe interaction (Strickland et al., 2009; Weand et al., 2010). Therefore, we predicted a shift in the soil bacterial community composition following nutrient addition. Indeed, the multivariate analyses revealed a significant shift in the β-diversity of soil bacteria by P addition, and the P availability and understory plant coverage were most correlated to the overall community composition of soil bacteria (Fig. 6). The P availability vector pointed to the main soil bacterial communities in the P and NP addition treatments (Fig. 6). Increasing the P availability and improving the balance between soil nutrients through exogenous P addition in the N-rich tropical forest could contribute to the observed changes in the soil bacterial community composition. Accelerated mineralization of the labile soil C in the soil by P addition can additionally satisfy the soil microbial demands for energy and nutrients (Nottingham et al., 2015). The Thermoleophilia affiliate with the phylum Actinobacteria, and both Actinobacteria and Alphaproteobacteria belong to the copiotrophic microbial groups (Zhao et al., 2017). Therefore, we expected these bacteria to exhibit rapid growth. However, excessive P addition had a negative effect on the abundances of Alphaproteobacteria and Thermoleophilia in the present study. These results suggested that the soil bacterial communities might not encounter P limitation following the long-term P addition in the N-rich forest ecosystem. In the previous studies, the addition of P altered soil microbial community composition in terms of the ratio of fungi to bacteria in the old-growth and secondary tropical forests in southern China (Liu et al., 2012a; Li et al., 2015). The present study further discovered that the community composition of soil bacteria changed with P addition at the operational taxonomic unit (OTU) level in the old-growth tropical forest. Moreover, this study examined the potential interactions between soil parameters, understory plant parameters, and soil bacterial community composition.

Increased plant diversity is likely to enhance soil microbial diversity by increasing the range of available food sources (Van der Heijden et al., 2008). In the present study, the direction of the understory plant coverage vector was opposite to the great bacterial communities in the N addition treatment in the N-rich tropical forest (Fig. 6). The understory plant coverage was lower in the N-addition plots than in the control plots, while there was no difference in understory plant coverage between the control plots and the NP addition or the P addition plots (Fig. 2c). However, the understory plant richness and abundance were lower in all the N, P, and NP addition plots than the control plots (Fig. 2b, d). These results suggested that N addition could indirectly affect the soil bacterial composition through changing understory plant community coverage. The N-induced shifts in plant biomass or composition can influence the amount of C available to microbes (Wallander, 1995). Therefore, the understory plant coverage might be another underlying predictor, apart from the P availability, for the dynamics of soil bacterial communities with nutrient addition in this study. The understory plant species concerned in the N and P addition experiment resulted from the limited plot size and the relatively high stem density of the understory plants in the studied tropical forest. The physiological processes and mechanisms of plant and soil microbial responses to nutrient addition at individual or community level need to be explored further using more advanced methods, such as isotope tracing technique, in the future.

5. Conclusions

In the present study, we observed the changes in the molecular diversity of soil bacteria caused by the long-term N and P additions in the old-growth N-rich tropical forest. The added amounts of N had a negative effect on the richness of soil bacteria, most likely because of the continuous soil acidification caused by the N input. NP-addition mitigated the responses of soil NO_3^- and pH to N-addition alone, but the P inputs could not mitigate the decrease in soil bacterial richness caused by excessive N deposition in the old-growth N-rich tropical forest. Additionally, the P addition shifted the composition of the soil bacterial community. Elevated P availability in soil by P addition and the decreased understory plant coverage with N input contributed to the changes in soil bacterial β-diversity. These findings suggest that the 6-year N and P additions have different influences on soil microbial diversity and community composition in this ecosystem.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.08.022.

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