Science of the Total Environment 607–608 (2017) 806–815

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Nitrogen deposition has minor effect on soil extracellular enzyme activities in six Chinese forests

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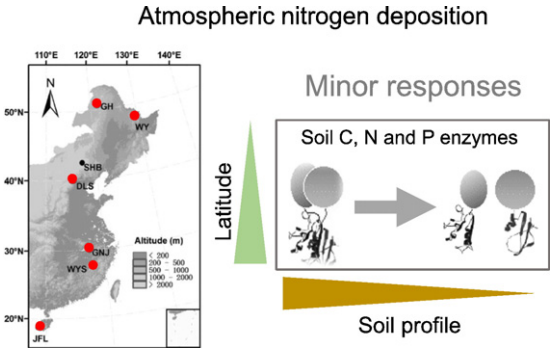
HIGHLIGHTS

- The effect of N deposition on soil enzymes was studied across six Chinese forests.
- N addition had no effect on the activities and ratios of soil enzymes in most cases.
- N addition effect did not vary with site and soil depth except acid phosphatase.
- Soil enzyme activities do not track N deposition in the six Chinese forests.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 March 2017
Received in revised form 7 July 2017
Accepted 7 July 2017
Available online 27 July 2017

Keywords:
Nitrogen deposition
Soil enzyme
Stoichiometry
Decomposition
Nutrient cycling
Forest ecosystem

ABSTRACT

Soil extracellular enzymes play a key role in mediating a range of forest ecosystem functions (i.e., carbon and nutrients cycling and biological productivity), particularly in the face of atmospheric N deposition that has been increasing at an unprecedented rate globally. However, most studies have focused only on surface soils in a single ecosystem. In this study, we aimed to determine whether the effect of simulated N deposition on the activities and ratios of soil enzymes changes with soil depth across six forest ecosystems in eastern China. We collected soil samples from three blocks × four soil depths (0–10 cm, 10–20 cm, 20–40 cm and 40–60 cm) × three N treatment levels (control, 50 and 100 kg N ha^{-1} $year^{-1}$) at each of the six forest ecosystems. We measured the activities of seven soil enzymes involved in C-, N- and P-cycling. We found that 4–5 years of N addition had no significant effect on the activities and ratios of these enzymes in most cases. The interactions among N addition, site and soil depth on soil enzyme activities were not significant, except that acid phosphatase activity showed site-specific responses to N addition. Our findings suggest that the activities of soil enzymes involved in C- and N-cycling generally do not track simulated N deposition in the six forest ecosystems. Further work on plant, soil and microbial characteristics is needed to better understand the mechanisms of soil enzyme activities in response to N deposition in forest ecosystems.

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1. Introduction

Anthropogenic emissions of reactive N have substantially increased atmospheric N deposition globally (Galloway et al., 2008). The

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http://dx.doi.org/10.1016/j.scitotenv.2017.07.060
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additional atmospheric N deposition can affect climate, soil, biodiversity and functions of natural and semi-natural ecosystems (Vitousek et al., 1997; Clark and Tilman, 2008; Liu et al., 2013). These changes caused by N deposition can further influence forest C storage (Pan et al., 2011). In forest ecosystems, soil enzymes play a key role in catalyzing several important reactions in the process of soil C and nutrient cycling (Sinsabaugh et al., 2005; Xu et al., 2017). This is because the most widely assayed soil enzymes are involved in the degradation of cellulose, lignin, chitin, proteins, nucleic acids, phospholipids and the other ester phosphates (Sinsabaugh et al., 2008, 2009). Furthermore, all soils contain suits of enzymes that determine the process of ecologically-connected soil organic matter decomposition, in return, the activities of soil enzymes depend on the physicochemical and biological properties of their habitats (Burns et al., 2013). A more detailed characterization of soil enzymes in forest soils and their responses to atmospheric N deposition is needed to understand soil microbial feedbacks to contemporary environmental changes locally and globally.

Numerous studies have shown that N addition has positive, neutral and negative effects on the activities of soil enzymes (Keeler et al., 2008; Jian et al., 2016; Chen et al., 2017). For instance, Chen et al. (2017) found that N addition significantly increases glycosidase activity (a group of hydrolytic enzymes, including α-1,4-glucosidase, β-1,4-glucosidase, β-1,4-xylosidase and cellobiosidase), and the effect is stronger for long-term, high-rate and mixed fertilizer of the N addition treatment. Their findings are consistent with the resource allocation theory that soil microorganisms will produce enzymes to obtain resources most in demand, and will decrease enzyme production when simple resources are available (Allison and Vitousek, 2005; Allison et al., 2011; Weintraub et al., 2013). According to the predictions of resource allocation theory, N addition would inhibit the activities of protein- and chitin-degrading enzymes (Olander and Vitousek, 2000; Allison et al., 2008), and stimulate phosphatase activity (Olander and Vitousek, 2000; Marklein and Houlton, 2012), thus enhances plant growth and production in P-limited ecosystems (Marklein and Houlton, 2012). It is also generally believed that N addition increases soil organic matter content through suppressing phenol oxidase and peroxidase activity in both forest floor and mineral soil (DeForest et al., 2004; Waldrop et al., 2004; Zak et al., 2008). However, the effect of added N on soil enzymes is not always consistent with the theoretical predictions. For example, Jing et al. (2016) found a neutral effect of N addition on C-, N- and P-cycling enzymes in an alpine grassland ecosystem. Saiya-Cork et al. (2002) found that N addition increases leucine aminopeptidase activity in the litter layer of a temperate forest. These results highlight that uncertainties remain in our understanding of the responses of soil enzymes to N addition. The type (organic, inorganic and mixed), duration (short, medium and long), amount, rate and frequency (low, medium and high) of N addition and the site-specific variation in soil properties, vegetation types and climate have been proposed to explain the N addition effect on soil enzymes (Keeler et al., 2008; Sinsabaugh et al., 2008; Du et al., 2014; Jian et al., 2016; Chen et al., 2017). Studies across multiple sites and soil depths with coordinated distributed N-addition field experiments are warranted to understand the mechanisms of nutrient addition effect on soil enzyme activities.

Soil microbial community composition and diversity exhibit distinct biogeographical patterns (Fierer and Jackson, 2006; Tedersoo et al., 2014), yet most of studies on the N deposition effect on soil enzyme activities are limited to a single ecosystem (but see Keeler et al., 2008; Cusack et al., 2010, 2011). As a result, little is known about how these groups of soil C-, N- and P-cycling enzymes respond to N addition ranging from tropical to temperate forest ecosystems. There are a series of biotic and abiotic factors that influence the activities of soil enzymes, such as soil pH, nutrient availability, microbial biomass and community composition, temperature and precipitation (Sinsabaugh et al., 2008; Sinsabaugh and Shah, 2011; Sinsabaugh and Follstad Shah, 2012; Burns et al., 2013; Xu et al., 2017). All of these factors can change with latitude. For instance, a global-scale synthesis found that the activities of seven soil C, N and P cycling enzymes are all correlated with soil pH, mean annual temperature and precipitation (Sinsabaugh et al., 2008). Along a north-south transect in eastern China, Xu et al. (2017) observed that soil phosphatase activity is higher in tropical forests than in temperate forests, and is negatively correlated with soil pH. In contrast, soil β-1,4-glucosidase and β-1,4-*N*-acetyl-glucosaminnidase activities are negatively correlated with mean annual temperature and precipitation and soil C:P and N:P ratio. In addition, Keeler et al. (2008) found that N addition enhances hydrolytic enzyme activities, but has no effect on the activity of oxidative enzyme in N-limited grassland and forest ecosystems. Cusack et al. (2010, 2011) reported that N addition increases soil C stocks by decreasing oxidative enzyme activity in two P-limited tropical forests. These findings collectively showed that the activities of soil enzymes can be indicators of energetic and nutrient limitation of microbial metabolism.

Variation in microbial community composition and diversity throughout soil profile has been well documented (Blume et al., 2002; Fierer et al., 2003; Eilers et al., 2012; Stone et al., 2015). However, we know little on the vertical patterns of soil enzyme activities along soil profile (but see Stone et al., 2014; Stone and Plante, 2014; Peng and Wang, 2016). Soil potential enzyme activities generally decline with soil depth (Taylor et al., 2002; Šnajdr et al., 2008; Stone et al., 2014). However, Stone et al. (2014) observed that the specific activity (normalized to microbial biomass) of β-1,4-glucosidase, β-1,4-xylosidase, cellobiosidase and glucosaminidase enzymes do not change with soil depth, while those of α-1,4-glucosidase and acid phosphatase enzymes increase with soil depth. Additionally, soil biotic and abiotic factors, including microbial biomass and community composition, C and nutrient availability, pH, temperature, moisture, O_2 availability, redox status, texture, can change with soil depth (Fierer et al., 2003; Stone et al., 2014; Stone et al., 2015; Heitkötter et al., 2017). Thus, the responses of microbial community to nutrient addition may also vary with soil depth. For instance, Heitkötter et al. (2017) observed that N addition increases P-cycling enzyme activity in both topsoil and upper subsoil, but has no effect in the lower subsoil. They also found that N addition decrease α-1,4-glucosidase activity in both subsoil depths of a sandy Cambisol. Jing et al. (2016) reported that N addition has no effect on soil C-, N- and P-cycling enzymes in both topsoil and subsoil, while P addition has negative effect on glycosidase enzymes in topsoil but has no effect in subsoil in an alpine grassland ecosystem.

In this study, we used 4–5 years of N addition field experiments in six forest ecosystems in eastern China with distinct climate, vegetation types and soil properties. We investigated the effect of N addition on the activities and ratios of soil enzymes involved in C-, N- and P-cycling, and examined whether the effect of N addition change with sampling site and soil depth along a latitudinal transect of climate, vegetation types and soil properties. Based on previous studies, we hypothesized that N addition would increase the activities of soil enzymes involved in C- and P-cycling and decrease soil enzymes involved in N-cycling according to the resource allocation theory. In addition, N addition would decrease the activities of soil oxidative enzymes. We also hypothesized that the effect of N addition would be greater in the N-limited temperate forest ecosystems than in the P-limited tropical forest ecosystems. Finally, we hypothesized that the effect of N addition would be greater in topsoil than in subsoil.

2. Materials and methods

2.1. Study site

The study was conducted in six forest ecosystems of 'NEECF', a project of Nutrient Enrichment Experiments in China's Forest (Du et al., 2013). Ranging from south to north, the six forest ecosystems can be grouped into two categories of vegetation types—tropical/subtropical and temperate forest ecosystems, including Jianfengling (JFL; tropical), Wuyishan (WYS; subtropical), Guniujiang (GNJ; subtropical),

Donglingshan (DLS; temperate), Wuying (WY; temperate) and Genhe (GH; temperate) (18°43′N–50°56′N, 108°53′E–129°11′E). Specifically, the study covers five vegetation types in eastern China, including tropical montane rain forest, subtropical evergreen broadleaved forest, temperate deciduous broadleaved forest, temperate broadleaved and conifer mixed forest and temperate coniferous forest, and three soil types including Inceptisols, Ultisols, and Alfisols (USDA Soil Taxonomy). The mean annual temperature ranges from −5.4 to 24.7 °C, mean annual precipitation from 481 to 2265 mm, and elevation from 350 to 1400 m. Atmospheric N deposition ranges from 5.5 to 25.0 kg N ha^{-1} $year^{-1}$. Soil pH ranges from 4.08 to 7.04 (average 5.28), soil total C from 18 to 323 mg g^{-1}, soil total N from 1.6 to 12.7 mg g^{-1}, and soil total P from 0.13 to 1.23 mg g^{-1}. More site information can be found in Table 1 and Du et al., 2013.

2.2. Experimental design

The NEECF project was designed to assess the effect of N deposition on the functioning of typical forest ecosystems in eastern China (Du et al., 2013). Specifically, the nutrient addition experiments in JFL, WY and GH were established in 2010, and those in WYS, GNJ and DLS were established in 2011. At three randomly distributed blocks within the six forests, the experimental plots (20 × 20 m^2; >10 m buffer area) were set up. Three or four N treatment levels were applied in each block at each forest, including control (CK, no N added), low N (N20 or N25, 20 or 25 kg N ha^{-1} $year^{-1}$), medium N (N50, 50 kg N ha^{-1} $year^{-1}$) and high N (N100, 100 kg N ha^{-1} $year^{-1}$). The three common treatments (CK, N50 and N100) at all six sites were used in this study. Ammonium nitrate (NH_4NO_3) was used in all sites expect DLS, where urea was used because of safety regulations in Beijing. N was added monthly by a sprayer during the growing season (note that the sites in the south receive smaller amount of N per month than the sites in the north). More detailed information about the experiment design can be found in Du et al. (2013).

2.3. Sample collection

Soil samples were collected in the growing season (August–early September) in 2015. From GH to JFL, soil cores were taken at the four corners and the center of a plot to a depth of 0–10 cm, 10–20 cm, 20–40 cm and 40–60 cm. We pooled the five soil cores at each soil depth. The soil samples were stored in ice coolers during the transportation. Once in the laboratory, the samples were passed through a 2-mm sieve. Soil samples for the assays of soil enzyme activities were stored at −20 °C for no more than two weeks and samples for the assays of soil total C, N and P were air-dried.

2.4. Laboratory measurements

Soil moisture was measured by drying 20 g fresh soil at 105 °C for 48 h. Soil total C and N contents were determined by elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Soil total P content was determined by the molybdenum blue method with an ultraviolet-visible spectrophotometer (UV-2500, Shimadzu, Kyoto, Japan). Soil pH was measured in a 1:2.5 ratio of fresh soil to deionized water.

We measured the activities of seven soil enzymes that are related to labile-C-cycling (β-1,4-glucosidase (BG) and cellobiohydrolase (CB)), recalcitrant-C-cycling (phenol oxidase (POX) and peroxidase (PER)), N-cycling (β-1,4-*N*-acetyl-glucosaminnidase (NAG) and leucine aminopeptidase (LAP)) and P-cycling (acid phosphatase (AP)) (Table S1).

We used the protocol described by German et al. (2011) and Bach et al. (2013). In brief, we used 96-well micro-plate to conduct the enzyme assays. For each soil sample, we weighted 1.5 g fresh soil and suspended the soil sample in 125 ml of 50 mM sodium acetate buffer (pH = 5.3). For hydrolytic (i.e., BG, CB, NAG, LAP and AP) enzymes, we combined 200 μl of soil slurries and 50 μl of 200 μM enzyme substrate in each well of the micro-plate. Sample control wells received 50 μl of sodium acetate buffer and 200 μl of soil slurries. Quench control wells received 50 μl of the standard substrate of 4-methylumvelliferyl or 7-amino-4-methylcoumarin (10 μM) and 200 μl of soil slurries. Substrate blank wells received 50 μl of 200 μM enzyme specific substrate into 200 μl of sodium acetate buffer. For oxidative (i.e., POX and PER) enzymes, assay wells received 50 μl of 25 mM L-DOPA and 200 μl of soil slurries, sample control received 50 μl of sodium acetate buffer and 200 μl of soil slurries, and substrate control received 50 μl of 25 mM L-DOPA and 200 μl of sodium acetate buffer. We incubated hydrolytic enzymes (BG, CB, LAP, NAG and AP) with 8 replicates for 2.5 h and oxidative enzymes (POX and PER) with 16 replicates for 24 h in the dark at 25 °C.

We measured the quantity of fluorescence (hydrolytic enzymes) at 360 nm excitation and 460 nm emission, and absorbance (oxidative enzymes) at 450 nm in a micro-plate reader (Biotek Synergy 2, Winooski, VT, USA). The units for hydrolytic enzyme activities were nmol g dry $weight^{-1}$ h^{-1} and the units for oxidative enzyme activities were μmol g dry $weight^{-1}$ h^{-1}.

2.5. Statistical analyses

All statistical analyses were performed using R [version 3.3.2] (R Development Core Team, 2016). The R script was deposited at https://github.com/XJingPKU/NEECF. Because the six forest ecosystems shared the same level of N addition at the rates of 0, 50 and 100 kg N ha^{-1} $year^{-1}$, we selected a total of 36 soil samples within the six sampling sites for further statistical analyses (3 blocks × 3 N treatment levels × 4 soil depths) except GH (27 samples were taken, 3 blocks × 3 N treatment levels × 3 soil depths). Ratios of C:N, C:P and N:P were calculated by ln(BG):ln(NAG + LAP), ln(BG):ln(AP) and ln(NAG + LAP):ln(AP). These indices indicate the relative microbial resources demand of organic C, N and P in relation to environmental gradients (Sinsabaugh et al., 2008).

To test the overall effects of site, N addition, soil depth and their interactions on the activities and ratios of soil enzymes, we fitted separate linear mixed-effects (lme) models. For the lme models, site, N addition,

Table 1
General site (ranging from south to north) and soil characteristics (0–10 cm soil depth) in the six forests of NEECF (Nutrient Enrichment Experiments in China's Forests).

Site	Location[a]	Altitude[a] (m)	MAT[a] (°C)	MAP[a] (mm)	Vegetation type[a]	Soil type[b]	STC[c] (mg g^{-1})	STN[c]	STP[c]	STC:STN ratio	STC:STP ratio	STN:STP ratio	pH[c]
JFL	18°43′N, 108°53′E	870	24.7	2265	Tropical montane rain forest	Inceptisols	18.3	1.55	0.13	11.8	140.8	11.9	4.36
WYS	27°39′N, 117°57′E	700	18	1889	Subtropical evergreen broadleaved forest	Ultisols	36.4	2.34	0.27	15.6	134.8	8.7	4.62
GNJ	30°01′N, 117°21′E	375	9.2	1650	Subtropical evergreen broadleaved forest	Alfisols	50.2	3.67	0.55	13.7	91.3	6.7	4.21
DLS	39°58′N, 115°26′E	1300	5.4	505	Temperate deciduous broadleaved forest	Alfisols	37.1	3.09	0.57	12.0	65.1	5.4	6.53
WY	48°07′N, 129°11′E	350	−0.5	654	Temperate broadleaved & conifer mixed forest	Alfisols	104.8	5.96	1.23	17.6	85.2	4.8	5.50
GH	50°56′N, 121°30′E	825	−5.4	481	Temperate forest	Alfisols	323.2	12.72	0.81	25.4	399.0	15.7	5.90

[a] Data are compiled from Du et al., 2013; MAT, mean annual temperature; MAP, mean annual precipitation.
[b] Soil types are classified by USDA soil taxonomy.
[c] Soil properties are determined in the control plots at 0–10 cm soil depth; STC, soil total carbon; STN, soil total nitrogen; STP, soil total phosphorus.

soil depth and the interactions of site, N addition and soil depth were designed as fixed factors, and a plot nested within block term was designed as random factor. The lme models were fitted by the asreml function in the '*ASReml*' package (Butler et al., 2009). Denominator degree of freedom of the lme models were adjusted by Kenward-Roger approximation (Kenward and Roger, 2009). Results were extracted by the test.asreml function in the '*pascal*' package (https://github.com/pascal-niklaus/pascal).

Furthermore, we summarized the mean and standard error of soil enzyme activities at each site × enzyme × N treatment × soil depth combination (Table S2). We then performed Tukey's HSD to test the differences in the activities of soil enzymes among the six forest ecosystems or the four soil depths. The differences between N addition (at a rate of 50 or 100 kg N ha^{-1} $year^{-1}$) and the control were tested by two-tailed paired *t*-tests at each enzyme × site × soil depth combination. In addition, we performed general linear models (GLMs) to assess the effects of latitude and climate (i.e., mean annual temperature and mean annual precipitation) on the activities of soil enzymes. We calculated the R^2 and corrected the *P* values (site is the error term for the effects of latitude and climate, instead of the residuals of the model) of the GLMs using aov.ftest function in '*pascal*' package (https://github.com/pascal-niklaus/pascal). We did the same analyses for enzyme ratios.

To examine the bivariate correlation between the relative change of soil enzyme activities and soil characteristics (total N, total P, pH and moisture), we conducted correlation tests on these variables compiled from the 0–10 cm soil depth. The relative change of soil enzyme activities was calculated as [N treatment − CK] / CK. In addition, correlations were conducted for the climate, soil characteristics and the activities and ratios of soil enzymes in the control plots at the 0–10 cm soil depth. These variables were latitude, mean annual temperature, mean annual precipitation, soil total C, soil total N, soil total P, soil C:N ratio, soil C:P ratio, soil N:P ratio, soil pH, soil moisture, BG, CB, POX, PER, NAG, LAP, AP, and the ratios of enzyme C:N, C:P and N:P. We reported the Pearson correlation coefficients. The *P* values were adjusted by false discovery rate (fdr) (Benjamini and Hochberg, 1995). We performed the correlation tests using corr.test function in '*psych*' package (Revelle, 2014).

Finally, we used principal components analysis (PCA) to assess the N addition effect on the patterns of the activities of the seven soil enzymes. We run the PCA on the entire dataset of soil enzyme activities in the six Chinese forest ecosystems at each soil depth. The activities of each soil enzyme were scaled and centered. We showed the mean and standard error of the first two principal components (PC1 and PC2) at each site × N treatment combination. We also showed the ordination plots of the seven soil enzymes associated with the first two principal components at 0–10 cm, 10–20 cm, 20–40 cm and 40–60 cm soil depths. We performed the PCA using prcomp function in '*stats*' package (R Development Core Team, 2016).

3. Results

3.1. Overall effect of N addition on the activities and ratios of soil enzymes

N addition had no effects on the activities of C-cycling enzymes (BG, CB, POX and PER) and N-cycling enzymes (LAP and NAG) (Table 2). However, we found marginal interactions ($0.05 < P < 0.10$) of N addition and soil depth, and significant interactions ($P < 0.05$) of site, N addition and soil depth on the activities of CB and POX. In addition, N addition and the interaction between N addition and site had significant effect on AP activity (P-cycling) (i.e., N addition generally decreased AP activity at JFL and WYS, increased AP activity at GNJ, and had no effect at DLS, WY and GH). Furthermore, we found that the effect of N addition at the rate of 50 kg N ha^{-1} $year^{-1}$ on the activities of seven enzymes was marginally significant in 7 out of 161 cases and significant in 10 out of 161 cases across all site × enzyme × soil depth combinations (Fig. 1). Meanwhile, the effect of N addition at the rate of 100 kg N ha^{-1} $year^{-1}$ on the activities of the seven enzymes was marginally significant in 9 out of 161 cases and significant in 7 out of 161 cases across all site × enzyme × soil depth combinations (Fig. 1).

N addition had no significant effect on the enzyme ratios of C:N, C:P and N:P (Table 2). Furthermore, the effect of N addition at the rate of 50 kg N ha^{-1} $year^{-1}$ on these enzyme ratios was marginally significant in 2 out of 69 cases, and significant in 3 out of 69 cases across all site × enzyme × soil depth combinations (Fig. 2). Meanwhile, the effect of N addition at the rate of 100 kg N ha^{-1} $year^{-1}$ on these enzyme ratios was marginally significant in 2 out of 69 cases, and significant in 6 out of 69 cases across all site × enzyme × soil depth combinations (Fig. 2).

3.2. Effect of N addition on the activities and ratios of soil enzymes in the six forest ecosystems

We found that the activities of all enzymes significantly varied with site (Table 2). In general, the activities of BG, CB, POX, PER, NAG and LAP were lower in the tropical forest ecosystems (JFL, WYS and GNJ) than in the temperate forest ecosystems (WY and GH) (Fig. 1). In contrast, the activity of AP was higher in the tropical forest ecosystems than in the temperate forest ecosystems (Fig. 1). The latitude trends for these enzymes were diminished with soil depth, while the latitude trends for AP were enhanced with soil depth (Fig. S1). In addition, we found that the activities of BG, CB, LAP and NAG were positively correlated with latitude, soil total C, soil total N, the ratios of soil C:N and C:P and soil moisture, and were negatively correlated with mean annual temperature

Table 2
Summary of the linear mixed-effects models for the effects of site, treatment (N addition), soil depth and their interactions on the activities and ratios of soil enzymes. BG, β-1,4-glucosidase; CB, cellobiohydrolase; POX, phenol oxidase; PER, peroxidase; NAG, β-1,4-*N*-acetyl-glucosaminnidase; LAP, leucine aminopeptidase; AP, acid phosphatase.

	Site (S)	Treatment (T)	Depth (D)	S × T	S × D	T × D	S × T × D
BG	$F_{5}^{a},_{35.1}^{b} = 27.6^{***}$	$F_{2,35.1} = 0.4$	$F_{3,100.1} = 86.9^{***}$	$F_{10,35.1} = 0.7$	$F_{14,100.1} = 18.8^{***}$	$F_{6,100.1} = 1.0$	$F_{28,100.2} = 0.7$
CB	$F_{5,11.4} = 23.8^{***}$	$F_{2,22.8} = 0.7$	$F_{3,99.3} = 41.5^{***}$	$F_{10,22.8} = 1.1$	$F_{14,99.3} = 11.8^{***}$	$F_{6,99.3} = 1.8^{\#}$	$F_{28,99.3} = 2.1^{**}$
POX	$F_{5,34.3} = 37.0^{***}$	$F_{2,34.1} = 0.1$	$F_{3,99.5} = 38.2^{***}$	$F_{10,34.3} = 0.7$	$F_{14,99.5} = 19.3^{***}$	$F_{6,99.5} = 2.1^{\#}$	$F_{28,99.5} = 1.8^{*}$
PER	$F_{5,11.8} = 58.1^{***}$	$F_{2,23.0} = 2.1$	$F_{3,99.8} = 10.0^{***}$	$F_{10,23.2} = 1.1$	$F_{14,99.8} = 4.7^{***}$	$F_{6,99.8} = 1.4$	$F_{28,99.9} = 1.0$
NAG	$F_{5,34.0} = 55.8^{***}$	$F_{2,33.9} = 0.6$	$F_{3,99.0} = 74.2^{***}$	$F_{10,34.0} = 1.3$	$F_{14,99.0} = 29.3^{***}$	$F_{6,99.0} = 0.7$	$F_{28,99.1} = 0.4$
LAP	$F_{5,11.4} = 8.7^{**}$	$F_{2,22.3} = 0.3$	$F_{3,98.9} = 73.8^{***}$	$F_{10,22.4} = 0.6$	$F_{14,98.9} = 11.2^{***}$	$F_{6,98.9} = 0.2$	$F_{28,99.0} = 0.5$
AP	$F_{5,11.8} = 44.8^{***}$	$F_{2,124.8} = 8.9^{***}$	$F_{3,124.8} = 122.9^{***}$	$F_{10,124.8} = 5.4^{***}$	$F_{14,124.9} = 7.7^{***}$	$F_{6,124.9} = 1.7$	$F_{28,124.9} = 1.1$
C:N ratio[c]	$F_{5,11.0} = 0.6$	$F_{2,23.2} = 2.1$	$F_{3,83.6} = 2.4^{\#}$	$F_{10,23.6} = 1.1$	$F_{14,84.0} = 1.4$	$F_{6,83.5} = 1.4$	$F_{28,84.3} = 1.9^{*}$
C:P ratio	$F_{5,133} = 45.6^{***}$	$F_{2,133} = 0.5$	$F_{3,133} = 11.6^{***}$	$F_{10,133} = 0.9$	$F_{14,133} = 1.4$	$F_{6,133} = 0.3$	$F_{28,133} = 0.4$
N:P ratio	$F_{5,36.2} = 33.5^{***}$	$F_{2,35.9} = 0.7$	$F_{3,98.8} = 22.0^{***}$	$F_{10,36.2} = 0.7$	$F_{14,98.8} = 3.2^{***}$	$F_{6,98.8} = 0.4$	$F_{28,99.0} = 1.5^{\#}$

[a] Nominator degree of freedom.
[b] Denominator degree of freedom is adjusted by Kenward-Roger approximations.
[c] Ratios of ln(BG):ln(NAG + LAP), ln(BG):ln(AP) and ln(NAG + LAP):ln(AP) were calculated to represent C:N, C:P and N:P ratio.
$^{\#}$ $P < 0.10$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

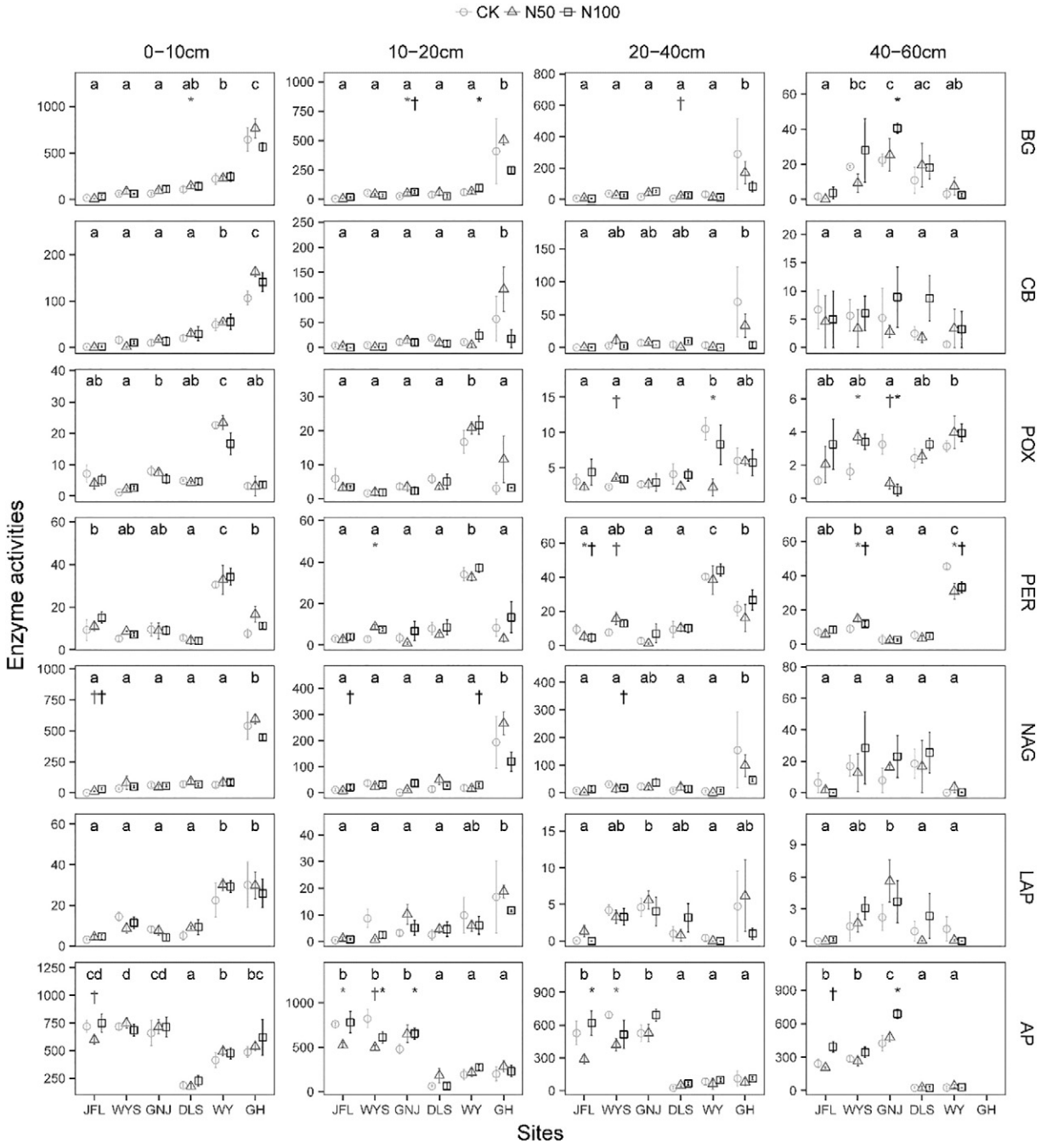

Fig. 1. Soil enzyme activities in the six Chinese forests. Values of enzyme activities are the mean of each site × enzyme × N treatment × soil depth combination ± 1 standard error ($n = 3$). Enzyme activities among sites (JFL, WYS, GNJ, DLS, WY and GH) are compared using the Tukey post-hoc tests at each soil depth. Values sharing the same letter are not significantly different among sites ($P > 0.05$). Daggers and stars indicate significant differences in enzyme activities in comparison to control at $0.05 < P < 0.10$ and $P < 0.05$, respectively. Circle, triangle and squared points are N addition rate at 0, 50 and 100 kg N ha^{-1} $year^{-1}$, respectively. BG, β-1,4-glucosidase; CB, cellobiohydrolase; POX, phenol oxidase; PER, peroxidase; NAG, β-1,4-*N*-acetyl-glucosaminnidase; LAP, leucine aminopeptidase; AP, acid phosphatase. Units for BG, CB, NAG, LAP and AP are nmol g dry $weight^{-1}$ h^{-1}. Units for POX and PER are μmol g dry $weight^{-1}$ h^{-1}. JFL, Jianfengling; WYS, Wuyishan; GNJ, Guniujiang; DLS, Donglingshan; WY, Wuying; GH, Genhe.

and precipitation (Table 3, Figs. S1, S2 and S3). In contrast, the activity of AP was negatively correlated with latitude and pH, and was positively correlated with mean annual temperature and precipitation (Table 3, Figs. S1, S2 and S3). Finally, the activities of POX and PER were not related to the site, climate and soil variables except soil total P (Table 3). Although we observed significant site patterns in the activities of soil enzymes involved in C-, N- and P-cycling (Fig. 1), we did not find significant interaction between N addition and site on enzyme activities ($P > 0.05$) except AP (Table 2). Furthermore, we did not observe significant correlation between the relative change of enzyme activities and soil characteristics across the six forest ecosystem at 0–10 cm soil depth (Table 2). The PCA analysis showed that there were no significant changes in the patterns of soil enzyme activities when N added across the six forest ecosystems in eastern China (Fig. 3).

We observed that enzyme C:N ratio did not vary with site, but the site effect on C:P and N:P ratios were significant (Table 2). Although the enzyme C:N ratio was correlated with latitude and climate (i.e., mean annual temperature and precipitation), these bivariate correlations were not significant when they were corrected for site (Figs. S4, S5 and S6). In addition, enzyme C:N ratio did not show significant correlations with soil characteristics (Table S3). However, the C:P and N:P ratios were generally lower in the tropical forest ecosystems than in the

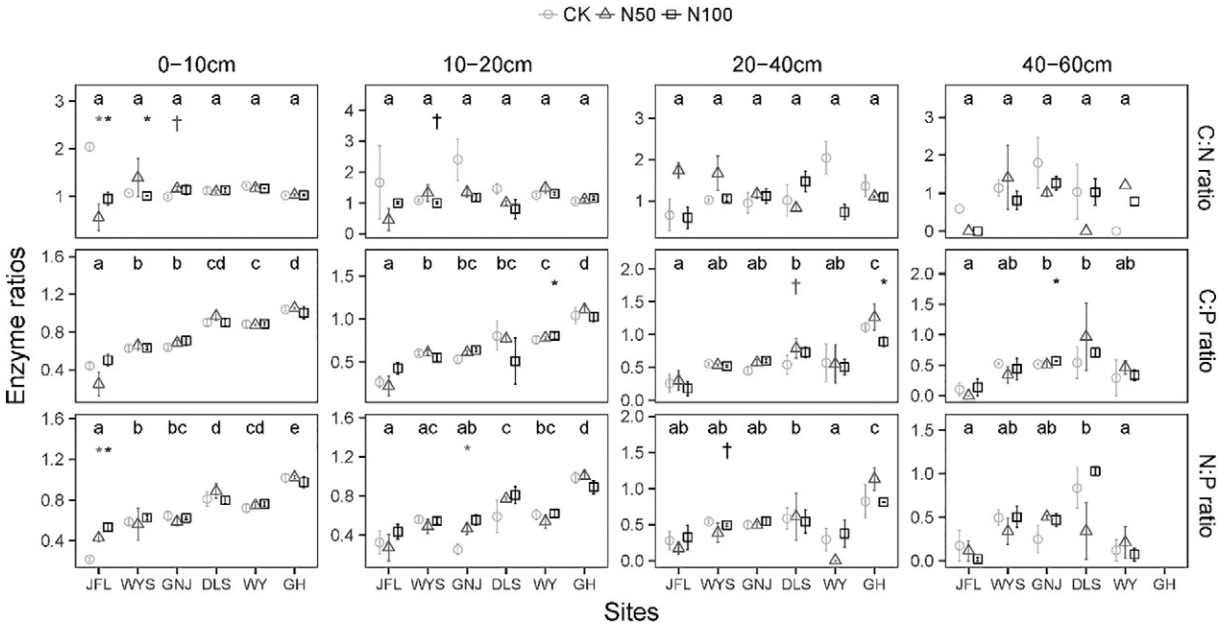

Fig. 2. Soil enzyme ratios in the six Chinese forests. Ratios of C:N, C:P and N:P were calculated by ln(BG):ln(NAG + LAP), ln(BG):ln(AP) and ln(NAG + LAP):ln(AP). Values of enzyme ratios are the mean of each site × enzyme × N treatment × soil depth combination ± 1 standard error ($n = 3$). Enzyme ratios among sites are compared using the Tukey post-hoc tests in the six sampling sites within the four soil depths. Values sharing the same letter are not significantly different among sites ($P > 0.05$). Daggers and stars indicate significant differences in enzyme activities in comparison to control at $0.05 < P < 0.10$ and $P < 0.05$, respectively. Circle, triangle and squared points are N addition rate at 0, 50 and 100 kg N ha^{-1} $year^{-1}$, respectively. Enzyme ratios are unitless. BG, β-1,4-glucosidase; NAG, β-1,4-*N*-acetyl-glucosaminnidase; LAP, leucine aminopeptidase; AP, acid phosphatase. JFL, Jianfengling; WYS, Wuyishan; GNJ, Guniujiang; DLS, Donglingshan; WY, Wuying; GH, Genhe.

temperate forest ecosystems (Fig. 2), and the latitude trends were diminished with soil depth (Fig. S4). Finally, we found that the C:P and N:P ratios were positively correlated with latitude, soil C, N and P, soil pH and soil moisture, but were negatively correlated with mean annual temperature and precipitation (Table S3, Figs. S4, S5 and S6). We did not find significant interaction between N addition and site on enzymatic ratios ($P > 0.05$; Table 2).

3.3. Effect of N addition on the activities and ratios of soil enzymes with soil depth

The activities of all enzymes significantly varied with soil depth (Table 2). In general, most of the enzyme activities decreased with soil depth, and the depth trends were more significant in the temperate forest ecosystems than in the tropical forest ecosystems (Fig. S7). In addition, we found that the drivers of these patterns in soil enzyme activities changed with soil depth (Fig. 4). The activities of AP, POX and PER were the main drivers of PC2 (Fig. 4 (a), (b) and (c)), but in subsoil, AP, POX and PER were most correlated with PC1 and became one of the major drivers in the patterns of soil enzymes (Fig. 4 (d)). Moreover, BG, CB, NAG and LAP were highly correlated with PC1 throughout the soil profile (Fig. 4). Finally, we did not find significant interaction between N addition and soil depth on enzyme activities ($P > 0.05$; Table 2).

Enzyme C:N ratio did not vary with soil depth, yet the effect of soil depth on enzyme C:P and N:P ratios were significant (Table 2). Specifically, we found that enzyme C:P ratio significantly decreased with soil depth at JFL, WYS, GNJ and WY, and enzyme N:P ratio decreased with soil depth at JFL, GNJ and WY (Fig. S8). We did not find significant interaction between N addition and soil depth on enzyme ratios ($P > 0.05$; Table 2).

4. Discussion

We originally expected that N addition would increase the activities of soil enzymes involved in C- and P-cycling, and would decrease the activities of soil N-cycling enzymes and soil oxidative enzymes. In fact, the results of this study showed that N addition did not affect soil enzymes involved in C- and N-cycling in most cases. We only observed that N addition had effect on the activity of AP, and the effect varied with site (Table 2). In addition, we expected that the effect of N addition would be greater in the temperate forest ecosystems than in the tropical forest ecosystems. In contrast to our hypothesis, we did not observe significant interaction effect between site and N addition on both activities and ratios of soil enzymes except AP. Finally, we had hypothesized that the effect of N addition would be greater in topsoil than in subsoil. Our results did not support this hypothesis because the interaction effect between

Table 3
Correlations between the relative change of soil enzyme activities and soil characteristics at 0–10 cm soil depth. The relative change of soil enzyme activities is calculated as [N50 − CK] / CK or [N100 − CK] / CK. The Pearson correlation coefficients and adjusted *P* values (in the parenthesis) are reported. STN, soil total nitrogen; STP, soil total phosphorus; SM, soil moisture. BG, β-1,4-glucosidase; CB, cellobiohydrolase; POX, phenol oxidase; PER, peroxidase; NAG, β-1,4-*N*-acetyl-glucosaminnidase; LAP, leucine aminopeptidase; AP, acid phosphatase.

	N50				N100			
	STN	STP	pH	SM	STN	STP	pH	SM
BG	−0.17 (0.83)	−0.23 (0.83)	−0.17 (0.83)	−0.20 (0.83)	−0.28 (0.60)	−0.10 (0.82)	−0.13 (0.75)	−0.36 (0.52)
CB	−0.05 (0.95)	−0.01 (0.98)	−0.23 (0.83)	−0.10 (0.93)	−0.15 (0.71)	−0.15 (0.71)	−0.32 (0.56)	−0.15 (0.71)
POX	−0.06 (0.95)	−0.21 (0.83)	−0.17 (0.83)	0.01 (0.98)	−0.16 (0.71)	−0.55 (0.18)	−0.20 (0.69)	−0.05 (0.93)
PER	0.47 (0.24)	−0.06 (0.95)	−0.07 (0.95)	0.52 (0.20)	0.28 (0.60)	−0.09 (0.85)	−0.18 (0.71)	0.35 (0.52)
NAG	−0.17 (0.83)	−0.17 (0.83)	−0.01 (0.98)	−0.12 (0.91)	−0.27 (0.60)	−0.11 (0.82)	−0.07 (0.87)	−0.29 (0.60)
LAP	−0.04 (0.96)	0.17 (0.83)	0.43 (0.35)	−0.14 (0.86)	−0.15 (0.71)	−0.01 (0.98)	0.40 (0.46)	−0.22 (0.64)
AP	0.05 (0.96)	0.19 (0.83)	−0.01 (0.98)	0.06 (0.95)	0.25 (0.60)	0.22 (0.64)	0.33 (0.55)	0.16 (0.71)

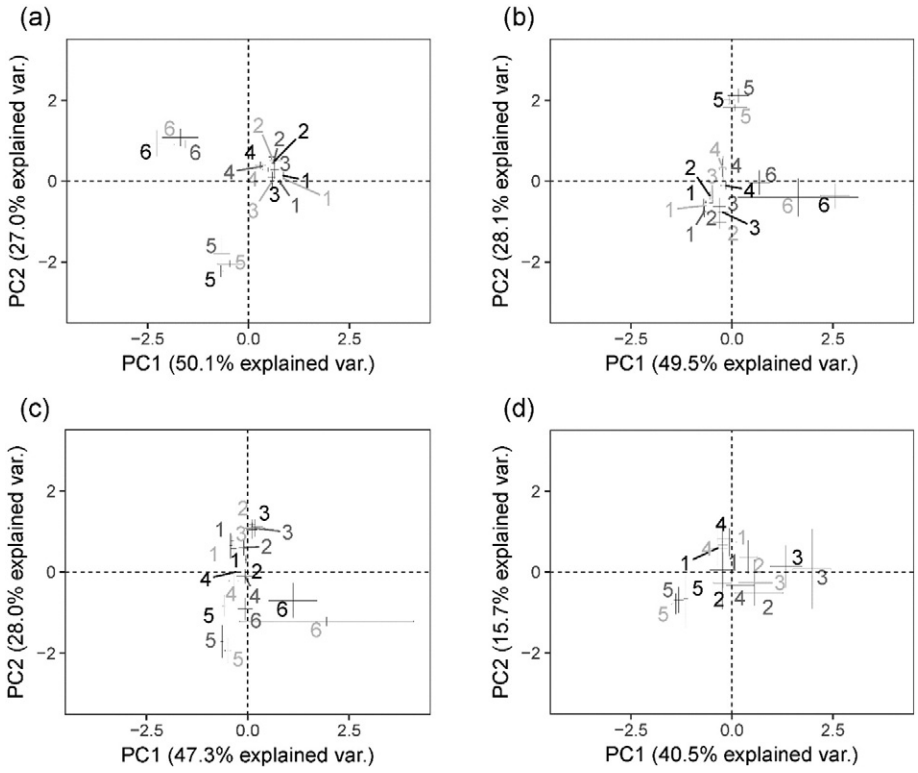

Fig. 3. Principal components analysis (PCA) of soil enzyme activities at (a) 0–10 cm, (b) 10–20 cm, (c) 20–40 cm and (d) 40–60 cm soil depths in the six Chinese forest ecosystems. Points represent mean ± 1 standard error ($n = 3$). Numbers with color from light to dark indicate N addition rate at 0, 50 and 100 kg N ha^{-1} $year^{-1}$, respectively. 1, Jianfengling; 2, Wuyishan; 3, Guniujiang; 4, Donglingshan; 5, Wuying; 6, Genhe.

soil depth and N addition on the activities and ratios of soil enzymes was not significant. Taken together, these results suggested that 4–5 years of simulated N deposition (50 and 100 kg N ha^{-1} $year^{-1}$) had minor effect on soil extracellular enzyme activities across the four soil depth intervals in the six Chinese forest ecosystems.

4.1. N addition effect on soil enzymes

In this study, we examined N addition effect on seven soil enzymes involved in C-, N- and P-cycling across all combinations of 6 sites × 4 soil depths from the 4–5 years of N deposition field experiments. We did not observe significant differences in activities and ratios for these enzymes in most cases except AP. The absence of N addition effect on the activities of soil enzymes was unexpected, given that we sampled soils at 0–60 cm soil depth ranging from the tropical forest ecosystems to the temperate forest ecosystems. Tian et al. (2017) found that the overall effect of N addition on soil total C, N and P, and soil microbial biomass C and N is not significant in these forests, which may partly explain the minor effect of N addition on soil enzymes in this study.

The duration of N addition (4 or 5 years) may be a major explanatory factor that leads to these unexpected non-significant responses of soil enzymes to N addition in our field experiments. In fact, numerous studies reported that long-term microbial responses may be more significant than short-term microbial responses to N addition. For instance, Ajwa et al. (1999) reported that eight years of N fertilization increases the activities of BG and AP, but decreases urease activity in tallgrass prairie. Olander and Vitousek (2000) observed that N addition elicits a negative effect on the NAG activity after four years of N fertilization in a tropical montane rainforest. In contrast, Ma et al. (2013) observed that two years of N treatment has no effect on the activities of BG and CB in a temperate forest ecosystem. Jing et al. (2016) also found that two years of N addition has no effect on the activities of BG, NAG, AP and POX in an alpine grassland. Therefore, the long-term effect of N addition on soil enzymes in these forest ecosystems awaits further research.

Furthermore, Du et al. (2014) observed contrasting effects of inorganic (NH_4NO_3) and organic N (mixed urea and glycine) addition on cellulase and POX activity after two years of field treatment. Their findings suggest that the effect of N addition may be dependent on N type (organic vs. inorganic). A meta-analysis provided strong evidence that mixed organic and inorganic N addition tends to significantly increase glycosidase activity (Chen et al., 2017). Although urea added at DLS (a temperate forest ecosystem) did not influence the activities and ratios of soil enzymes, we expected that soil enzymes may be more responsive to complex nutrient treatment, because soil microbes tend to use complex substrates to acquire limiting nutrients (Allison and Vitousek, 2005; Stone et al., 2013). However, we still lack evidence on the relative responses of soil enzymes to complex vs. simple nutrient addition in forest ecosystems (but see Weintraub et al., 2013).

Unlike other enzymes, AP activity was significantly affected by N addition, and the effect varied with site (significant N and site interaction, Table 2). For instance, N addition generally decreased AP activity at JFL and WYS, and increased AP activity at GNJ, while had no effect in the temperate forest ecosystems (Fig. 1). These findings did not support our expectation that N addition would increase P-cycling enzymes (i.e., AP), and are not consistent with Marklein and Houlton (2012) which showed that N addition enhances soil phosphatase activity. Although the underlying mechanisms on the responses of AP to N addition are unclear, soil depth is likely to affect the activity of AP and its response to N addition. For example, a positive effect of N addition on AP activity was observed at JFL (tropical forest) in 40–60 cm soil depth (Fig. 1). This indicates that N addition may increase the production of phosphatase to satisfy the needs of microbial P demand in deeper soils of P-limited tropical ecosystems. The ratios of enzyme C:P and N:P decreasing with soil depth provide additional evidence of microbial production of phosphatase to acquire P in deeper soils (Fig. S8). However, AP did not respond to N addition in temperate forests, which suggest

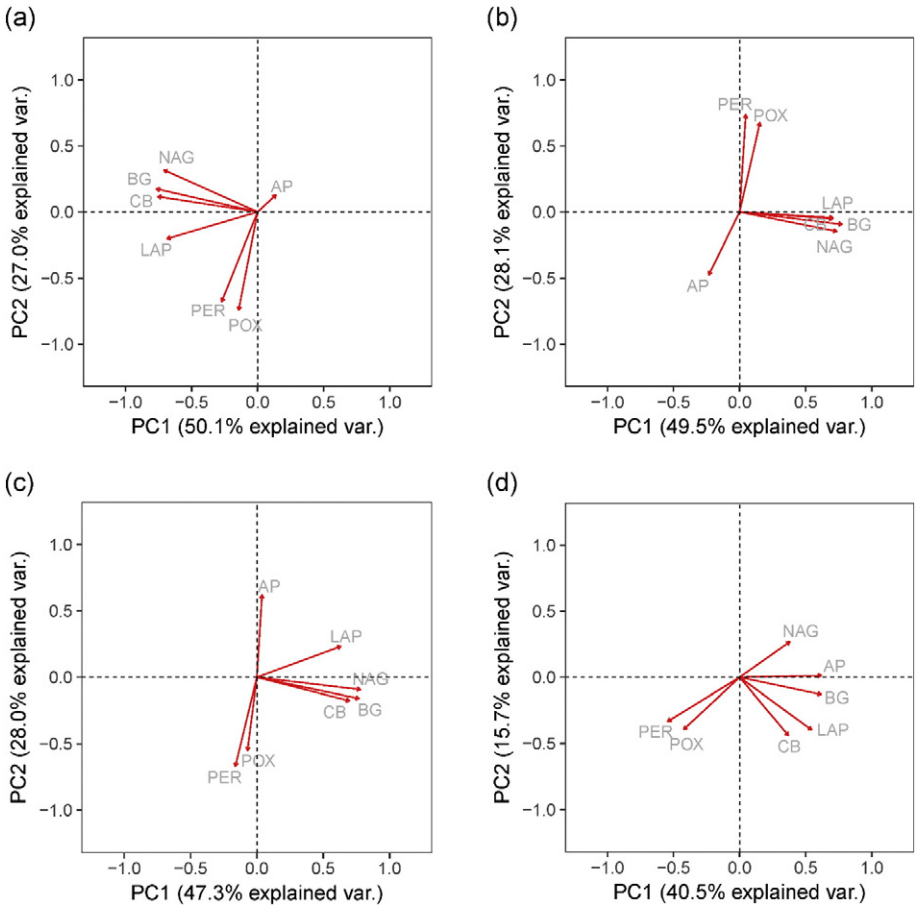

Fig. 4. Ordination plots show soil enzymes associated with the first two principle components at (a) 0–10 cm, (b) 10–20 cm, (c) 20–40 cm and (d) 40–60 cm soil depths. BG, β-1,4-glucosidase; CB, cellobiohydrolase; NAG, β-1,4-*N*-acetyl-glucosaminnidase; LAP, leucine aminopeptidase; AP, acid phosphatase; POX, phenol oxidase; PER, peroxidase.

that P may not be a limited nutrient when N was added in these temperate ecosystems. Therefore, we expect that atmospheric N deposition will increase P cycling in P-limited tropical forests, but not in N-limited temperate forests.

4.2. Interaction between site and N addition on soil enzymes

Given that P is most limited in tropical ecosystems, and N is most limited in temperate ecosystems (Vitousek and Howarth, 1991; Vitousek et al., 2010), we expected that the effect of N addition on the activities of soil enzymes would be greater in the northern temperate forests than in the southern tropical forests. However, our results did not support this hypothesis. We found that soil enzymes related to C- and N-cycling enzymes did not respond to N addition, and the interaction between site and N addition on soil enzymes is not significant across the six forests. Furthermore, we did not find any correlation between the effect size of N treatment on enzyme activities and soil characteristics (i.e., total N and P content, moisture and pH) (Table 3). These unexpected results do not support the initial hypothesis, and are inconsistent with most previous studies which showed that the effect of N addition on soil enzymes is dependent on the types of enzymes and ecosystems. For instance, Saiya-Cork et al. (2002) found that soil enzymes can be enhanced (BG, NAG and AP), unresponsive (PER) or depressed (LAP and POX) by N addition in a temperate hardwood forest. Cusack et al. (2011) found that N addition increases hydrolytic enzyme activities, but decreases oxidative enzyme activities at a low-elevation tropical forest.

We observed significant biogeographical patterns in the enzymes involved in C-, N- and P-cycling, and showed that there are different drivers for such patterns. For instance, the activities of BG, CB, NAG and LAP were lower in the tropical forest ecosystems than in the temperate forest ecosystems, while the AP activity showed the opposite pattern. These results indicate that P may be one of the limiting nutrients in tropical ecosystems (Olander and Vitousek, 2000; Cusack et al., 2010; Cusack et al., 2011; Xu et al., 2017). In addition, AP activity was negatively correlated with latitude, soil total P and pH, and was positively correlated with climate (MAT and MAP). These results further indicate that the low P availability in tropical forest ecosystems could mediate the production of soil enzymes (Turner and Wright, 2014). However, we still have limited knowledge to directly quantify the relative importance of enzyme production vs. enzyme turnover on the pool size of soil enzymes (Wallenstein and Weintraub, 2008), which can determine the intrinsic response of soil enzyme to nutrient addition.

4.3. Interaction between soil depth and N addition on soil enzymes

There are numerous studies to investigate the responses of soil enzymes in litter layer and mineral soil. For instance, Saiya-Cork et al. (2002) reported that the effect size of N addition on the activities of soil enzymes involved in C-, N- and P-cycling is greater in litter than in soil. Ma et al. (2013) found that N addition depresses NAG activity in litter, while has no effect on NAG activity in soil. Furthermore, Keeler et al. (2008) found that N addition has no effect on the activity of lignin degrading enzyme, but increases cellulose degrading and N- and P-acquiring enzymes in both litter and soil. However, it is still not clear whether the effect of N addition on soil enzymes varies with soil depth. We found that the interaction between soil depth and N addition is not significant. This is most likely due to the non-significant responses of soil enzyme activities and soil microbial biomass (Tian et al., 2017) to N addition in most cases in our study. Our results are not consistent with

Heitkötter et al. (2017) who reported that N addition has significant effect on soil enzymes involved in C-, N- and P-cycling in topsoil and subsoil, except phosphatase enzyme in the lower subsoil and both α-1,4-glucosidase and xylosidase enzymes in topsoil. These results indicate that soil microorganisms show differential demands for N or P along the soil profile (Peng and Wang, 2016; Heitkötter et al., 2017). Given enough time, we may expect to see significant effect of N addition on soil enzymes in the studied forest ecosystems, particularly in the surface soils.

We observed that the latitudinal trends in the activities of BG, CB, NAG and LAP are diminished along with soil depth. One possible explanation is that the available substrates for soil enzymes involved in C- and N-cycling decrease with soil depth, and thus the energy and resource availability in subsoil are lower than in topsoil (Stone et al., 2014). Meanwhile, in the subsoil, enzyme production is mainly from constitutive enzyme production, and enzyme turnover is relatively slower (Stone et al., 2014). Therefore, the abiotic drivers (temperature and precipitation) might be weakly correlated with the activities of soil enzymes involved in C- and N-cycling in subsoil at broad biogeographical scales (Sinsabaugh et al., 2008; Peng and Wang, 2016). In contrast, we found that the latitudinal trends for AP activity are enhanced along with soil depth. This finding is consistent with Stone et al. (2014) who found that microbial allocation to P-cycling enzyme increases with depth. Their results further indicate that P is limited, and is in most demand for soil microorganisms in subsoil. Another possible explanation is that shift in microbial C and nutrient demand and community composition are drivers of microbial allocation to P-cycling enzymes along the soil profile (Stone et al., 2014).

4.4. Limitations

There are several limitations related to our current study. First, soil samples were collected based on one-time sampling in the growing season, which cannot reflect the seasonal variation in the enzyme activities and the temporal responses of soil enzymes to N addition (DeForest et al., 2004; Ma et al., 2013). Second, soil enzyme measurements are the potential enzyme activities (i.e., without considering diffusion limitation, substrate depletion, physical protection and biological interactions), which cannot directly provide insight into *in situ* conditions of soil characteristics (Wallenstein and Weintraub, 2008; Henry, 2012). Finally, the different background N deposition and large geographic variation in plant, soil and microbial characteristics may play an important role in modulating the responses of soil enzymes to N addition. Therefore, future studies should sample soils in different seasons, extend the study to longer term, and consider site-specific plant, soil and microbial characteristics to further investigate the responses of soil enzyme activity to N addition in these diverse forest ecosystems.

5. Conclusions

At global scale, increased inputs of atmospheric N into forest ecosystems can potentially affect soil microbial activity. In this study, we systematically investigated the effect of N addition on the activities of soil enzymes at four soil depths across six Chinese forest ecosystems. Although we observed shifts in soil enzyme activities with soil depth across the six forest ecosystems, we did not find significant effect of N addition on soil enzyme activities in most cases, and the interactions between site and N addition, and soil depth and N addition on soil enzymes were not significant. Our results suggested that soil enzymes involved in C- and N-cycling (except acid phosphatase) generally do not track 4–5 years of simulated N deposition in the six forest ecosystems. As the fertilization experiment is ongoing, future studies that include more frequent sampling (seasonally), longer treatment duration (6–10 years) and better linkage to plant, soil and microbial characteristics may further elucidate the responses of soil enzymes to nitrogen deposition and their feedback to soil carbon and nutrient cycling in these forest ecosystems.

Acknowledgements

We thank Dr. Weixin Cheng and Dr. Chao Liang for kindly allowing us to use their lab equipment and microplate reader, Dr. Bernhard Schmid for kindly providing us with the ASReml license, and three anonymous reviewers for giving constructive comments on this manuscript. This study was financially supported by National Natural Science Foundation of China [grant numbers 31670525, 31622013 and 31600428], and China Postdoctoral Science Foundation [grant numbers 2015M580918 and 2016T90012].

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.07.060.

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