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Ammonium and nitrate additions differentially affect soil microbial biomass of different communities and enzyme activities in slash pine plantation in subtropical China

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Abstract

The ratios of nitrate to ammonium in wet atmosphere nitrogen (N) deposition compounds were increasing recently. However, the individual effects of nitrate and ammonium deposition on soil microbial biomass of different communities and enzyme activities are still unclear. We conducted a four-year N addition field experiment to evaluate the responses of soil microbial biomass of different communities and enzyme activities to ammonium (NH_4Cl) and nitrate ($NaNO_3$) additions. Our results showed that (1) the inhibitory effects of ammonium additions on total mass of phospholipid fatty acid (PLFA) were stronger than those of nitrate additions. Both decreased total PLFA mass about 24% and 11% across three sampling time, respectively. The inhibitory effects of ammonium additions on gram positive bacteria (G^+) and bacteria, fungi, actinomycetes (A), and arbuscular mycorrhizal fungi (AMF) PLFA contents ranged from 14%- 40% across three sampling time. (2) Both ammonium and nitrate additions inhibited absolute activities of C, N, and P hydrolyses and oxidases, and nitrate additions had stronger inhibition effects on the acid phosphatase (AP) than ammonium additions. Both ammonium and nitrate additions decreased N-acquisition specific enzyme activities (enzyme activities normalized by total PLFA mass) about 21% or 43%, respectively. However, ammonium additions increased

P-acquisition specific enzyme activities about 19% comparing to control. (3) Redundancy analysis (RDA) showed that the measured C, N, and P hydrolyses and polyphenol oxidase (PPO) activities were positively correlated with soil pH and ammonium contents, but negatively with nitrate contents; the mass of PLFA biomarkers were positively correlated with soil pH, soil organic carbon (SOC), and total N contents, but negatively with ammonium contents. (4) The soil enzyme activities varied seasonally in the order of March > June > October. On the contrary, microbial PLFA mass was higher in October than in March and June. Our results concluded that inhibition of mass of PLFA biomarkers and enzyme activities might be contributed to acidification caused by ammonium addition. Soil absolute enzyme activities were inhibited indirectly by acidification and nitrification, but specific enzyme activities normalized by PLFA were directly affected by N additions. It was meaningful to separate the effects of ammonium and nitrate additions on soil microbial communities and enzyme activities.

Copyright statement

1. Introduction

Wet atmospheric nitrogen (N) deposition had increased 25% in the past decade (Jia et al., 2014), which caused a series of problems in forest ecosystems, such as induced soil acidification, aggravated the leaching of cation and nitrate, and decreased microbial biomass (Liu et al., 2011; Huang et al., 2014; Gao et al., 2015; Liu et al., 2013). Although most of wet atmospheric N deposition was ammonium,

nitrate had improved over years, which the ratio of ammonium to nitrate decreased from 5 to 2 (Liu et al., 2013). Study the differential effects of the two forms of N additions on soil microorganisms could improve our ability to predict the cycling of C, N and P under nitrate deposition increasing scenario.

Soil microorganism supplies nutrients to forests by producing enzymes to catalyze the degradation of soil organic matter, drives the cycling of carbon (C), nitrogen (N) and phosphorus (P), and therefore, influences the forest productivity and sustainability (Heijden et al., 2008). Soil microbial biomass of different communities could be quantified by phospholipid fatty acid (PLFA) biomarkers. Although the use of PLFA signature to evaluate microbial diversity was not as advanced as genomics technology, PLFA method was widely applied to analyze the biomass and microbial community structures (Frostegård et al., 2011). Usually, bacteria (B), including gram positive (G^+) and negative (G^-) bacteria, are liable to degrade labile compound by excreting hydrolase. And fungi (F), including arbuscular mycorrhizal fungi (AMF) and saprophyte (SAP), are liable to degrade recalcitrant compound by secreting oxidase (Burns et al., 2013; Sinsabaugh et al., 2010; Willers et al., 2015).

However, only few field studies reported individual effects of ammonium and nitrate additions on microbial communities in forest soils. Most studies paid more attention to the influence of organic N to microbial communities (Guo et al., 2010; Hobbie et al., 2012). Compared to nitrate, ammonium with positive charge could be more easily absorbed by soil colloid with negative charge. Thus, ammonium would be more available to microorganism than nitrate. However, the process of nitrification, i.e. ammonium transforming rapidly to nitrate when entering into soil, would sterilize microorganisms in soil (Dail et al., 2001). There were mechanisms caused different effects of ammonium and nitrate additions on soil microbial biomass of different communities and enzyme activities. Ammonium and nitrate additions had different effects on microbial decomposition rate and microbial respiration of soil organic matter. For example, ammonium additions increased substrate respirations, while nitrate additions had no influence on substrate respirations in peatland (Currey et al., 2010); Nitrate additions had strong promotion effects on the decomposition rate of soil organic matter for fir plantation in the early incubation phase (0-15d; Zhang et al., 2012). However, the inhibition effect of nitrate additions on soil microbial respiration was similar to ammonium additions in a laboratory incubation experiment (Ramirez et al., 2010). It was unclear whether ammonium and nitrate additions had a different influence on soil microbial biomass of different communities.

It was well known that microorganisms and enzymes were sensitive to soil pH. A meta analysis of

91 soil acidification caused by N additions suggested that ammonium nitrate (NH_4NO_3) additions
92 contributed more to soil acidification than ammonium additions (Tian and Niu, 2015). Most studies did
93 not differentiate the individual effects of nitrogen addition forms on PLFAs and MBC in forest
94 ecosystems. A meta analysis reported that N additions decreased MBC by 15%, and fungi were more
95 sensitive to N additions than the other microbial communities (Treseder et al., 2008). A wide range of
96 factors could influence the response of microbial biomass to nitrogen additions, including forest type
97 and geographical location. For example, in temperate forests, NH_4NO_3 additions decreased microbial
98 total PLFAs contents in American beech (*Fagus grandifolia* Ehrh) and yellow birch (*Betula*
99 *alleghaniensis* Britton), but increased in eastern hemlock (*Tsuga Canadensis* (L.) Carr) and red oak
100 (*Quercus rubra* (L.) Britton), and the responses of bacteria and fungi were variable (Weand et al.,
101 2010). In subtropical forest, NH_4NO_3 additions increased microbial total PLFAs contents in Chinese fir
102 (Dong et al., 2015), but decreased soil MBC contents in evergreen broad leaved forests, and no
103 influence on the pine broad-leaved mixed forest (Wang et al., 2009). To date, the effects of N on soil
104 microbial communities were inconsistent and it was still unclear how ammonium and nitrate additions
105 influenced microbial communities, individually.

106 Soil enzymes catalyze the decomposition of soil organic matter (Burns et al., 2013). The common
107 labile C-degradation enzymes included α-1,4-glucosidase (αG), β-1,4-glucosidase (βG),
108 cellobiohydrolase (CBH) and β-1,4-xylosidase (βX) that can decompose starch, cellulose and
109 hemicellulose. Nitrogen-degradation enzyme includes β-1,4-N-acetylglucosaminidase (NAG) that can
110 decompose oligosaccharides. Phosphorus-degradation enzyme included acid phosphatase (AP) that can
111 decompose chitin lipophsphoglycan (Stone et al., 2014). Recalcitrant C-degradation enzymes included
112 peroxidase and phenol oxidase that can decompose lignin, aromatic and phenolic compounds
113 (Sinsabaugh et al., 2010). While few study reported the differential effects of ammonium and nitrate
114 additions on soil enzyme activities in forest ecosystem. In other ecosystem, eg. peatland and alpine
115 meadow, it showed different effect (Currey et al., 2010; Tian et al., 2014). For example, ammonium
116 and nitrate additions had an obvious different effect on carbon-, phosphorus-enzyme activities (CBH,
117 AP) but not for PPO in peatland (Currey et al., 2010). While no significant effects were found in alpine
118 meadow (Tian et al., 2014).

119 According to the economic theory, the microorganisms will allocate enzymes to the resources that
120 were absent for microorganisms, thus N additions relatively increased C, P-acquisition enzymes and

decrease N-acquisition enzymes (Burns et al., 2013). However, a meta analysis reported that N additions without considering inorganic N forms not only increased the C-degradation enzymes (αG, βG, CBH and βX) and P-degradation enzymes (AP), and restricted oxidase (PPO and PER), but did not inhibited N-degradation enzymes (NAG) (Jian et al., 2016; Marklein and Houlton, 2012). It suggested that allocation of enzyme activities did not completely follow the economic theory.

The response of enzyme activities to N additions were influenced by a series of factors including environmental conditions, plant types and N background values. For example, in temperate region, the soil activities of BG, CBH, NAG and PPO were improved by NH_4NO_3 additions in dogwood forest, but were decreased in oak, and were invariant in maple forest. The AP activities were increased in dogwood and maple forests, but were invariant in oak forest response to NH_4NO_3 additions (Sinsabaugh et al., 2002). However, in acidification temperate region, NH_4NO_3 additions increased soil BG activities in maple forest, but had no influence on soil BG, NAG and AP in yellow birch, oak, hemlock and beech forests (Weand et al., 2010). In subtropical and tropical forests, NH_4NO_3 additions increased BG, NAG, AP activities, and decreased oxidase (PPO and PER) activities (Dong et al., 2015; Guo et al., 2011; Cusack et al., 2011). In general, it was still unclear how N addition affected on enzyme activities and whether there were different effects of ammonium and nitrate additions on enzyme activities. To better predict the effect of elevated N deposition on soil cycling of C, N, and P, it was necessary to evaluate the individual effects of ammonium and nitrate additions on soil microbial biomass of different communities and enzyme activities.

The subtropical soils were thought to be N-rich and undergone increasing nitrate deposition in southern China. We established a long-term nitrate and ammonium additions experiment in the slash pine (*Pinus elliottii*) plantations in subtropical area. We aimed to explore the differential effects of ammonium and nitrate on soil microbial communities and enzyme activities, respectively. We hypothesized that (1) ammonium additions would have a stronger inhibitive ability to total PLFAs, fungi PLFA contents, and enzyme activities due to its strong negative effect on soil pH; and (2) ammonium and nitrate additions would increase C, P-hydrolase, but decreased N-hydrolase activities according to the economic theory, and inhibited oxidase activities due to their effects on fungi.

2. Materials and methods

151 **2.1. Study site**

152

153 The study was conducted in Qianyanzhou (QYZ) Experimental site in hilly red soil region, Taihe
154 country, Jiang Xi province (26°44′29.1″N, 115°03′29.2″E, 102 m a. s. l). The region was subtropical
155 monsoon climate with mean annual temperature and precipitation of 17.9 ºC and 1475 mm,
156 respectively. Atmospheric wet N deposition was about 33 kg N ha^{-1} yr^{-1} consisting of 11 kg N ha^{-1} yr^{-1}
157 ammonium and 8 kg N ha^{-1} yr^{-1} nitrate, respectively (Zhu et al., 2014). The soil is weathered from red
158 sandstone and mud stone, and is classified as Typical Dystrudepts Udepts Inceptisols according to US
159 soil taxonomy (Soil Survey Staff, 2010). The slash pine (*Pinus elliottii*) was planted in 1985 and was
160 one of the dominant species using vegetation restoration in this hilly red soil region. The dominant
161 understory vegetation is *Woodwardia japonica*, *Dicranopteris dichotoma* and *Loropetalum chinense*
162 (Kou et al., 2015).

163

164 **2.2. Experimental design**

165

166 As described by Kou et al. (2015), the plots were established in November 2011 using a randomized
167 complete block design. There were two forms of N treatments, i.e. ammonium additions ($N_{ammonium}$)
168 using ammonium chloride (NH_4Cl) and nitrate additions ($N_{nitrate}$) using sodium nitrate ($NaNO_3$), with a
169 dosage of 40 kg N ha^{-1} $yr^{-1}$and a Control (CK). Each treatment had three replicates, totally nine plots
170 (20 m × 20 m, slope <15 °). The plots were separated with more than 10 m buffer zone between plots.
171 The NH_4Cl or $NaNO_3$ were dissolved in 30 L tap water and evenly sprayed onto the plots once per
172 month, i.e. 12 times per year. The equivalent amount of tap water was sprayed onto each Control plot.
173 Nitrogen additions started on 01-May-2012 and proceeded at a month interval on non-rainy days, and
174 totally 140 kg N ha^{-1} was inputted when soils were collected.

175

176 **2.3. Sampling and analysis**

177

178 We collected soil samples at March, June and October of 2015 after removing surface litters, and
179 mixed 5 cm diameter cores from five randomly selected locations together as one composite sample.
180 Soil samples were taken from 0-10 cm depth from each plot, then field- fresh samples were sieved

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through a 2 mm sieve after mixed evenly. Samples were kept at 4 °C for PLFA biomarkers, enzyme activities, soil pH, ammonium and nitrate, and soil dissolved organic carbon (DOC) analyses. The assays of PLFA biomarkers and enzyme activities were performed at once after back to laboratory. A subsample was air dried, and then sieved through a 0.25 mm mesh for soil organic C (SOC), total N (TN), and Total P (TP) analyses.

Soil pH was measured in a 1g fresh soil : 2.5 v:v soil-water suspension by glass electrode. Fresh soils were extracted by 1mol L^{-1} KCl, shaken for 2 hours, and measured by a continuous flow auto-analyzer (Bran Lubbe, AA3, Germany) to determine ammonium and nitrate contents. Soil DOC was extracted with 1:5 (v:v) soil : distilled water, and measured by Liquid TOC*II* (Elementar, Germany). Soil TN and SOC were measured by CN Analyzer (Vario Max, Elementar, Germany).

Phospholipid fatty acid (PLFA) biomarkers were measured according to the methods of Bossio and Scow (1998). In brief, field-fresh soil equal to 8 g dry soil was undergone mild alkaline methanolusis to form fatty acid methyl eaters (FAMEs). Then the extraction of PLFA dissolved in hexane was measured by Agilent 6890N Gas Chromatograph, with MIDI peak identification software (version 4.5; MIDI Inc. Newark, DE) with a DB-5 column. The abundances of PLFA biomarkers were calculated as nmol PLFA g^{-1} dry soil. Total amount of the different PLFA biomarkers were used to represent differnt groups of soil microorganisms, i.e. gram-positive bacteria (G^+) by i14:0, i15:0, a15:0, i16:0, i17:0, a17:0; gram-negative bacteria (G^-) by 16:1ω7c, cy17:0, 18:1ω7c, cy19:0; arbuscular mycorrhizal fungi (AMF) by 16:1ω5; saprophytic fungi (SAP) by 18:1ω9c, 18:2ω6c, 18:2ω9c 18:3ω6c; actinomycete (A) by 10Me16:0, 10Me17:0, 10Me18:0 (Bradley et al., 2007; Denef et al., 2009). Bacteria biomass were calculated as the sum of G^+ and G^-, and fungi biomass were calculated as the sum of AMF and SAP, respectively.

We measured four C-acquisition hydrolase (i.e. αG, βG, CBH and βX), one N-acquisition hydrolase (NAG) and one P-acquisition hydrolase (AP) following the methods of Saiya-Cork et al. (2002). Their corresponding substrates and functions see Table 1. In brief, 1 g field-fresh soil was homogenized in 125 ml of sodium acetate buffer. The buffer was adjusted to 4.5 of pH based on the ambient soil pH. 200 µl homogenate and 50 µl substrate was added to 96-well black microplates, then incubated at 20 °C for 4 h. After incubation, 10 µl 1 mol L^{-1} NaOH was added to each well to terminate the reactions, and fluorescence values were measured with 365 nm excitation and 450 nm emission filters by a microplate fluorometer (Synergy H4，BioTek). Totally, there were eight replicates per soil sample.

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Two oxidases (i.e. PER and PPO were measured using 96-well transparent microplates according to the methods of Saiya-Cork et al. (2002). 600 µl homogenate and 150 µl substrate were added to 96-well deep microplates. When measuring PER activities, 10 µl of 0.3% H_2O_2 was added to homogenate and substrates mixtures. After incubated at 20 °C for 5 h, the microplates were centrifuged at 3000 r for 3 minutes, then transferred 250 µl liquid supernatant to 96-well transparent microplate. Absorbance values were measured at 460 nm by microplate spectrophotometer (Synergy H4, BioTek). The corresponding substrates and their functions of the measured enzymes were shown in S 2.

After correcting for homogenate control, substrate control and quenching, absolute activities were expressed in units of nmol g^{-1} soil h^{-1}. We calculated the specific activities of the enzymes by dividing enzyme activities by PLFA values to normalize the activity to the size of the microbial active biomass (Cusack et al. 2011).

2.4. Statistical analyses

Two factors randomized block variance of analyses and Duncan analyses were applied to test the differences between treatments and sampling time. One-way analysis of variance (ANOVA) and Duncan analyses were applied to test the difference of the treatments in individual sampling time. Analyses were performed using SPSS 17.0. Relationships among the soil physical-chemical properties, soil PLFA biomarkers and the soil enzyme activities were tested by redundancy analysis (RDA) using CANOCO 4.5. Statistical significance was determined as $P < 0.05$. The figures were drawn by sigmaplot 10.0.

3. Results

3.1. Soil physical and chemical properties

Totally, treatments have a significant influence on soil pH ($F=12.43$, $P<0.01$), DOC ($F=23.53$, $P<0.01$), nitrate ($F=43.19$, $P<0.01$) and ammonium ($F=11.84$, $P<0.01$) (Table 2). Ammonium additions decreased soil pH by 0.7 unit across three sampling time, while nitrate additions did not affect soil pH significantly. Nitrate additions increased soil DOC by 17% across three sampling time, while

ammonium additions did not affect soil DOC significantly. Ammonium and nitrate additions increased soil nitrate contents by 165% and 129%, respectively, but they all decreased soil ammonium contents by 31% and 38% across three sampling time, respectively (Fig.1).

The sampling time have a significant influence on DOC ($F= 561.25$, $P<0.01$), nitrate ($F=7.96$, $P<0.01$) and ammonium ($F= 65.46$, $P<0.01$), but not on soil pH (Table 2). DOC contents were in order of March < June < October. In contrast to nitrate contents, ammonium contents were in order of March > June and October.

3.2. Soil microbial biomass of different communities

Both treatments and sampling time had a significant influence on soil microbial biomass of different communities ($P<0.01$, Table 2). Totally, ammonium and nitrate additions decreased total PLFAs contents, and the effects of ammonium additions on the different PLFA biomarkers were stronger than those of nitrate additions across three sampling time. Ammonium additions decreased total PLFA contents by 24 %, and decreased G^+, AMF, B, F, A PLFA contents by 14 % - 40 % across three sampling time. Nitrate additions decreased total PLFA contents by 11%, and decreased G^+, AMF, B, F, A PLFA contents by 7% - 24% across three sampling time. Only ammonium additions shifted the microbial communities from G^- to G^+, i.e. increased the ratio of G^+/G^- comparing to CK or nitrate additions. Additionally, both ammonium and nitrate additions decreased the ratios of F/B, but the effects of nitrate additions were stronger than those of ammonium additions (Fig.2).

Additionally, the measured soil PLFA biomarkers exhibited seasonal variations (Table 2). Total PLFA and PLFA contents of B, F, G^+, G^-, AMF, SAP were in order of March > June > October. PLFA contents of A were in order of June > March > October.

3.3. Soil enzyme activities

Both treatments and sampling time had a significant influence on the measured absolute enzyme activities ($P<0.01$, Table 2), i.e. ammonium inhibited by 15%-43% and nitrate by 6%-50% across three sampling time, respectively (Table 3). The AP absolute activities were about 9% lower under nitrate than under ammonium additions (Table 3).

271 The treatments had a significant influence on N, P-acquisition specific enzyme activities ($P<0.01$),
272 but not on C and oxidase specific enzyme activities (Table 2). The inhibition effects of nitrate additions
273 on the N-acquisition specific enzyme activities (about 43%) were stronger than those under the
274 ammonium additions (about 21%) across three sampling time. And only ammonium additions
275 increased the AP specific activities (about 19%) compared to the CK across three sampling time
276 (Fig.3).
277 Additionally, the measured enzyme activities exhibited seasonal variations (Table 2). BG, BX, CBH,
278 NAG, AP and PPO activities were in order of March < June < October. aG activities were in order of
279 March > October > June, and PER activities were in order of March > June > October (Table 3).
280
281 **3.4. Redundancy analyses**
282
283 The RDA between soil properties, enzyme activities, and PLFA biomarkers showed that the first
284 ordination RDA axis explained 72.0% and 66.8%, respectively, the second axis explained 11.5% and
285 13.2%, respectively. The RD1 for soil enzyme activities and PLFA biomarkers were correlated with
286 DOC/SOC, DOC, ammonium, and SOC. However, nitrate was only correlated with the RD1 of enzyme
287 activities but not that of PLFA biomarkers. Most of the measured soil enzyme activities and the PLFA
288 biomarkers were positively correlated with soil pH, but G^+/G^- and F/B were negatively correlated with
289 soil pH. Ammonium and DOC were positively correlated with the soil enzyme activities except PER,
290 but negatively with PLFA biomarkers. Nitrate was negatively correlated with soil enzyme activities, but
291 hardly with PLFA biomarkers (Fig. 4).
292
293 **4. Discussion**
294
295 In agreement with our first hypothesis, our results showed that both ammonium and nitrate additions
296 significantly decreased soil total mass of PLFA biomarkers, bacteria, fungi, actinomycetes, G^+, AMF,
297 SAP-PLFA contents, and ammonium additions had stronger inhibition effects on PLFA biomarkers
298 across three sampling time (Figure 2, Table 2). Soil microbial biomass was negatively influenced by
299 resource availability and acidification (Sinsabaugh et al., 2014; Moorhead et al., 2006). However, N
300 additions tended to increase soil DOC contents, and available N (sum of ammonium and nitrate

301 contents) did not change in response to N additions in our study. It suggested that PLFA biomarkers
302 contents were inhibited by some other factors except soil availability of C and N in the subtropical
303 slash pine (*pinus elliottii*) forest. The RDA analysis showed the positive correlations between PLFA
304 biomarkers contents and soil pH (Fig. 4). Acidification caused by ammonium additions might be
305 attributed to decrease of mass of microbial PLFA. Ammonium additions could aggravate nitrification
306 in subtropical soils (Tang et al. 2016), and nitrification might be toxic to microorganism (Dail et al.,
307 2001), which would decrease microbial PLFA contents. Nitrate additions had no influence on soil pH
308 (Fig 1), which would explain why nitrate addition had weaker inhibition effects on mass of PLFA
309 biomarkers. The possible reasons that nitrate addition inhibited the mass of PLFA biomarkers might be
310 as follows, nitrate additions could accelerate leaching of Ca^{2+}, Mg^{2+} (Qian et al., 2007), increase soil
311 osmotic potential, and activate Al^{3+} absorbed by soil colloid (Treseder et al., 2008). Additionally, N
312 additions decreased the PER activity, which would cause polyphenol accumulation in soil.
313 Accumulated polyphenol might also be toxic to microorganism (Sinsabaugh et al., 2010) and
314 contributed to decrease the contents of PLFA biomarkers.

315 In our study, both ammonium and nitrate additions decreased the ratios of fungi /bacteria, suggesting
316 that fungi were more sensitive to N additions. We found that N additions decreased fine root biomass in
317 our previous study (Kou et al., 2015), and N additions could destroy symbiotic system between AMF
318 and plants, so that restrict AMF-PLFA contents.

319 Our study showed that both ammonium and nitrate additions inhibited the absolute activities of C, N,
320 P-hydrolase and oxidase across three sampling time (Table 2, Table 3). It agrees with our hypothesis
321 and the economic theory that N additions decreased the absolute activities of N-acquisition enzyme
322 (NAG). However, it does not agree with our hypothesis that N additions will increase the C- or
323 P-acquisition enzymes. We found positive correlations between soil pH, ammonium contents and the
324 measured enzyme activities except PER, and negative correlations between nitrate contents and most of
325 the measured enzyme activities (Figure 4), indicating that acidification and nitrification could restrict
326 enzyme activities. Microorganisms were main producers of soil enzymes, the decrease of microbial
327 biomass would reduce soil absolute enzyme activities (Allison et al., 2005).

328 We found that the specific enzyme activities of N, P-acquisition were different after ammonium and
329 nitrate additions (Figure 3, Table 2). The specific enzyme activities of C-hydrolase and oxidase
330 maintained constant under N additions, although N additions restricted the absolute activities of

C-acquisition enzymes. Microorganisms tended to preferentially allocate energy resource (C) to meet their growth demanding (Schimel and Schaeffer, 2012). Nitrate additions had stronger inhibition effects on the specific enzyme activities of N-acquisition than under ammonium additions. It is indicated that N addition decreased the N-demanding of unit-microbial biomass. Analogously, increase of P-acquisition specific enzyme activities under ammonium additions suggests the increase of P-demanding of unit-microbial biomass in the P-limited subtropical region. Acidification due to ammonium additions might aggravate P-deficiency by reactivating Al^{3+} reaction with available P (Vitousek et al., 2010; Mohren et al., 1986), which would improve the demanding of P. Additionally, soil absolute enzyme activities would be influenced more strongly by abiotic, i.e. soil pH, than biotic conditions (Kivlin et al., 2016). Declines of C, P-acquisition absolute enzyme activities might be attributed to the edaphic variations such as acidification and nitrification.

We also found significant seasonal variations in mass of PLFA biomarkers and enzyme activities (Table 2). Microbial PLFA contents were higher in October, which may be explained by litter increase in October. Fresh litter inputs could promote decomposition of old recalcitrant compounds (Blagodatskaya and Kuzyzakov, 2008), which might be confirmed by the high PER activities in October. Additionally, we also found there were interactive effects of N treatments and different sampling time on soil enzyme activities and PLFA contents of biomarkers (Table 2). It suggested that soil microbial biomass and enzyme activities were simultaneously influenced by a series of factors, such as atmosphere conditions, precipitation, and sequent change of soil variables.

5. Conclusions

The results showed that both ammonium and nitrate additions decreased soil total microbial PLFA mass, and PLFA mass of bacteria, fungi, actinomycetes, G^+, G^-, AMF, SAP. The inhibitive effects on the biomass of different soil microbial communities except SAP were more significant under ammonium additions than under nitrate additions. It might be attributed to acidification caused by ammonium additions since PLFA biomarkers were positively correlated with pH. Ammonium additions shifted microbial communities to G^+ and bacteria-dominated, and nitrate additions shifted microbial communities to bacteria-dominated.

Although ammonium and nitrate additions reduced absolute enzyme activities of C, N, and

P-acquisition, the specific enzyme activities of P-acquisition were increased under ammonium additions, and specific enzyme activities of C-acquisition maintained constant. It suggested that ammonium and nitrate additions increased the microbial demanding of C and P. Soil absolute enzyme activities were inhibited indirectly by acidification and nitrification, but specific enzyme activities normalized by PLFA were directly affected by N additions.

In general, the effects of ammonium and nitrate additions on soil microbial communities and specific enzyme activities was various. In order to better predict the elevated N deposition on soil microbial functions and enzyme activities, it was necessary to discuss the effect of ammonium and nitrate, separately.

Author contribution: Xin-yu Zhang, Xue-Fa Wen, Sheng-Gong Li, Hui-Min Wang, and Xiao-Min Sun designed research; Liang Kou performed research; Chuang Zhang, Yang Yang and Xin-yu Zhang analyzed data; and Chuang Zhang wrote the paper.

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509 **Figure Legends**

510

511 **Fig. 1.** The effects of ammonium and nitrate additions on soil pH, DOC, nitrate and ammonium
512 contents in individual sampling time. Capital letters represent significant differences between the
513 treatments (P <0.05), and small letters represent significant between the different sampling time (P
514 <0.05). Error bars represent standard errors, the same below.

515 **Fig. 2.** The effects of ammonium and nitrate additions on PLFA biomarkers in different sampling time.

516 **Fig. 3.** The effects of ammonium and nitrate additions on C, N, P-acquisition specific enzyme and
517 oxidase specific activities in different sampling time.

518 **Fig. 4.** Redundancy analyses between (a) soil properties and enzyme activities; (b) soil properties and
519 PLFA-biomarkers.

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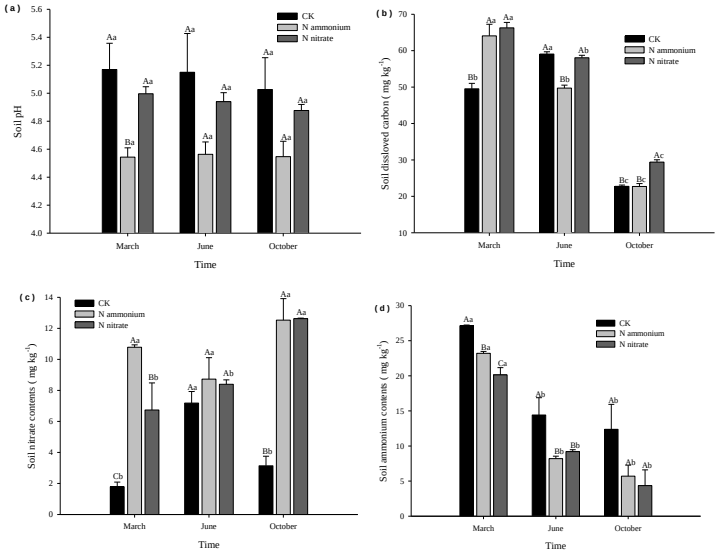

Fig.1

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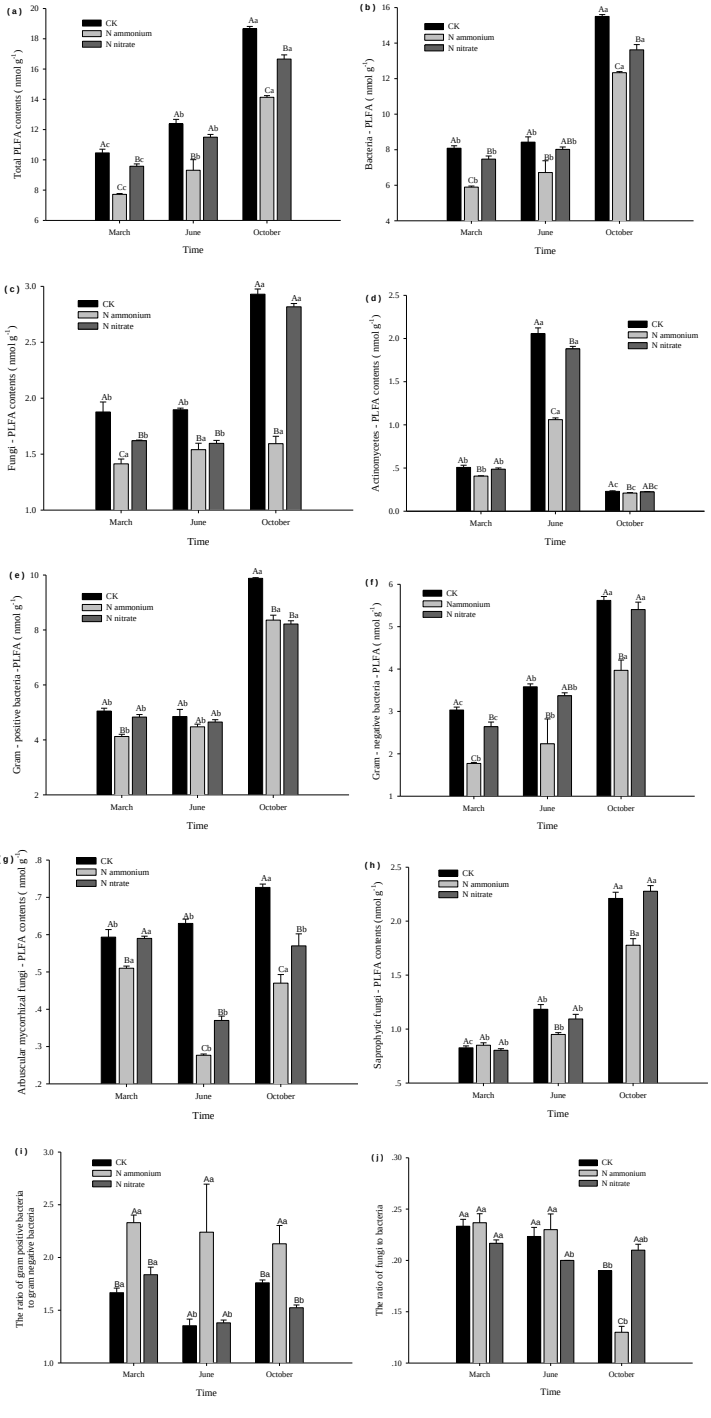

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528 **Fig. 2**

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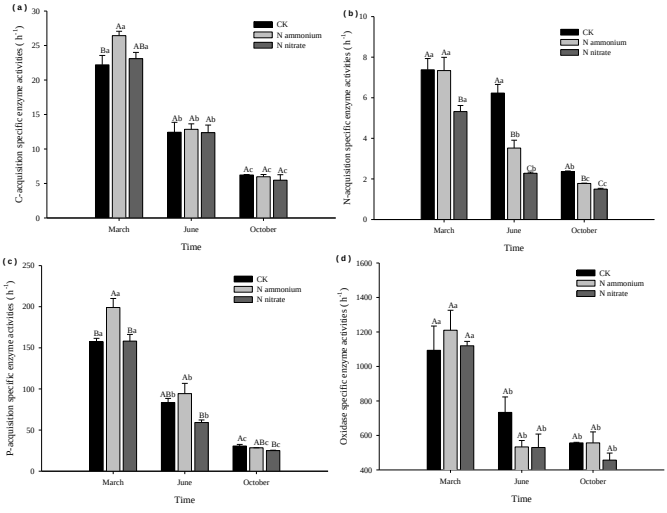

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531 **Fig.3**

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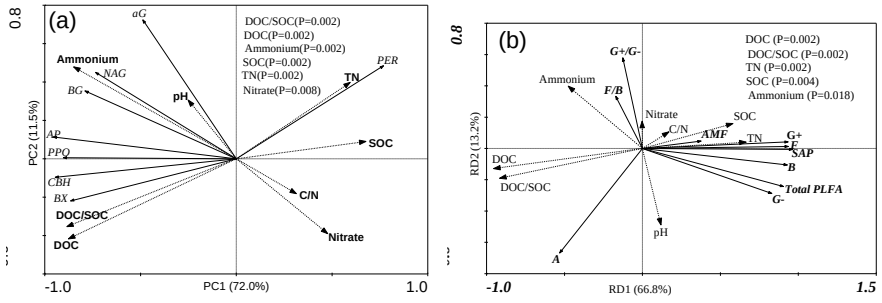

Fig. 4

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534 **Table 1** Enzymes and their corresponding substrates and functions

Enzyme	Ec	Abbreviation	Substrate	Function
Peroxidase	1.11.1.7	PER	L-DOPA	Oxidize lignin and aromatic compounds using H_2O_2 or secondary oxidants as an electron acceptor
Phenol oxidase	1.10.3.2	PPO	L-DOPA	Oxidize phenolic compounds using oxygen as an electron acceptor
α-1,4-glucosidase	3.2.1.20	aG	4-MUB-α-D-glucoside	Releases glucose from starch
β-1,4-glucosidase	3.2.1.21	BG	4-MUB-β-D-glucoside	Releases glucose from cellulose
Cellobiohydrolase	3.2.1.91	CBH	4-MUB-β-D-cellobioside	Releases disaccharides from cellulose
β-1,4-xylosidase	3.2.1.37	BX	4-MUB-β-D-xyloside	Releases xylose from hemicellulose
β-1,4-N-acetylglucosaminidase	3.2.1.14	NAG	4-MUB-N-acetyl-β-D-glucosaminide	Releases N-acetyl glucosamine from oligosaccharides
Acid phosphatase	3.1.3.1	AP	4-MUB-phosphate	Releases phosphate groups

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536 **Table 2** Summary statistics (F ratio, P value) for two factors randomized block variance of analyses (ANOVA) and Duncan analyses
537 applied to soil variables, enzyme activities and PLFA biomarkers. P value that are significant level ($P < 0.05$)

Factors	Treatments	Months	Treatments × Months
pH	**12.43, 0.00**	0.31, 0.74	0.09 , 0.99
DOC	**23.53, 0.00**	**561.25, 0.00**	**20.11, 0.00**
Nitrate	**43.19, 0.00**	**7.96, 0.00**	**8.21, 0.00**
Ammonium	**11.84, 0.00**	**65.46, 0.00**	0.42, 0.79
TPLFA	**102.51, 0.00**	**477.77, 0.00**	2.68, 0.07
B	**56.94, 0.00**	**555.14, 0.00**	2.73, 0.07
F	**180.49, 0.00**	**277.81, 0.00**	**52.16, 0.00**
A	**172.23, 0.00**	**2627.61, 0.00**	**123.12, 0.00**
G^+	**50.30, 0.00**	**1221.19, 0.00**	**14.39, 0.00**
G^-	**34.33, 0.00**	**105.59, 0.00**	0.45, 0.77
AMF	**147.77, 0.00**	**83.55, 0.00**	**21.64, 0.00**
SAP	**24.70, 0.00**	**781.67, 0.00**	**13.08, 0.00**
G^+/G^-	**16.24, 0.00**	2.38, 0.12	0.94, 0.46
F/B	**3.82, 0.04**	**56.42, 0.00**	**21.67, 0.00**
aG	**30.24, 0.00**	**53.17, 0.00**	**3.47, 0.03**
BG	**3.26, 0.07**	**72.90, 0.00**	0.58, 0.68
BX	**9.86, 0.00**	**79.08, 0.00**	**3.86, 0.02**
CBH	**28.51, 0.00**	**194.75, 0.00**	**4.39, 0.01**
NAG	**100.42, 0.00**	**67.49, 0.00**	**8.47, 0.00**
AP	**22.81, 0.00**	**467.77, 0.00**	1.73, 0.19
PPO	**6.87, 0.01**	**64.40, 0.00**	1.98, 0.15
PER	**6.27, 0.01**	**194.30, 0.00**	**3.07, 0.05**
C-acquisition specific enzyme	2.82, 0.09	**334.41, 0.00**	2.07, 0.13
N-acquisition specific enzyme	**29.10, 0.00**	**128.31, 0.00**	**6.36, 0.00**
P-acquisition specific enzyme	**13.42, 0.00**	**397.19, 0.00**	**4.53, 0.00**
Oxidase specific enzyme	1.68, 0.22	**89.04, 0.00**	1.84, 0.17

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538 **Table 3** Summary statistics (mean ± standard error) for One way analyses (ANOVA) and Duncan analyses applied to soil absolute
539 enzyme activities. Capital letters represent significant differences between the treatments (P <0.05), and small letters represent
540 significant between the different sampling time (P <0.05).

Months	Treatments	aG nmol g^{-1} h^{-1}	BG nmol g^{-1} h^{-1}	BX nmol g^{-1} h^{-1}	CBH nmol g^{-1} h^{-1}	NAG nmol g^{-1} h^{-1}	AP nmol g^{-1} h^{-1}	PPO μmol g^{-1} h^{-1}	PER μmol g^{-1} h^{-1}
March	CK	7.0±0.1 Aa	160.9±15.6 Aa	36.4±3.4 Aa	30. ±2.1 Aa	77.5±4.7 Aa	1658.7±59.1 Aa	7.9±0.9 Aa	1.4±0.1 Ab
	$N_{ammonium}$	4.5±0.2 Ba	143.5±4.0A a	26.8±3.2 Aa	27.3±1.5 Aa	56.1±5.2 Ba	1520.7±78.2 Aa	8.9±0.0 Aa	1.5±0.1 Ab
	$N_{nitrate}$	4.5±0.2 Ba	157.1±10.9 Aa	33.4±1.0 Aa	21.0±0.8 Ba	49.7±2.6 Ba	1475.2±53.2 Aa	9.9±1.4 Aa	1.6±0.1 Ab
June	CK	4.0±0.9 Ab	83.2±13.0A b	37.2±1.6 Aa	28.6±2.5 Aa	77.0±4.7 Aa	1030.3±41.2 Ab	7.7±1.2 Aa	1.4±0.1 Ab
	$N_{ammonium}$	2.2±0.1 ABc	70.6±0.9 Ab	25.9±1.8 Ba	17.9±0.2 Bb	31.8±1.7 Bb	848.5±62.1 Bb	4.0±0.0 Bb	0.9±0.1 Bb
	$N_{nitrate}$	1.7±0.3 Bb	89.4±10.3A b	28.7±1.2 Bb	19.8±0.2 Ba	25.7±0.6 Bb	667.8±26.5 Cb	4.8±0.9 ABb	1.2±0.1 Ab
October	CK	3.7±0.4 Ab	89.1±0.9 Ab	15.2±0.4 ABb	9.7±0.3A b	44.7±0.2 Ab	578.0±38.1A c	2.9±0.2 Ab	7.6±0.1 Aa
	$N_{ammonium}$	3.7±0.1 Ab	64.0±4.2 Ab	16.2±0.9 Ab	5.2±0.1 Bc	26.5±0.2 Bb	423.4±1.6 Bc	2.8±0.1 Ab	5.5±0.8 Aa
	$N_{nitrate}$	2.2±0.0 Bb	68.3±11.5A b	13.5±0.1 Bc	5.3±0.1 Bb	24.5±0.2 Cb	409.8±4.7 Bc	1.9±0.1 Bc	5.6±0.8 Aa

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