1 **Contrasting effects of ammonium and nitrate additions on the biomass of soil microbial**
2 **communities and enzyme activities in a slash pine plantation in subtropical China**

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14 **Abstract**
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16 The nitrate to ammonium ratios in nitrogen (N) compounds in wet atmospheric deposits have
17 increased over the recent past, which is a cause for some concern as the individual effects of nitrate and
18 ammonium deposition on the biomass of different soil microbial communities and enzyme activities are
19 still poorly defined. We established a field experiment and applied ammonium (NH_4Cl) and nitrate
20 ($NaNO_3$) at monthly intervals over a period of four years. We collected soil samples from the
21 ammonium and nitrate treatments and control plots in three different seasons, namely spring, summer,
22 and autumn, to evaluate the how the biomass of different soil microbial communities and enzyme
23 activities responded to the ammonium (NH_4Cl) and nitrate ($NaNO_3$) applications. Our results showed
24 that the total contents of phospholipid fatty acids (PLFA) decreased by 24% and 11% in the ammonium
25 and nitrate treatments, respectively. The inhibitory effects of ammonium on gram positive bacteria (G^+)
26 and bacteria, fungi, actinomycetes, and arbuscular mycorrhizal fungi (AMF) PLFA contents ranged
27 from 14% to 40% across the three seasons. We also observed that the absolute activities of C, N, and P
28 hydrolyses and oxidases were inhibited by ammonium and nitrate, but that nitrate had stronger
29 inhibitory effects on the activities of acid phosphatase (AP) than ammonium. The activities of
30 N-acquisition specific enzymes (enzyme activities normalized by total PLFA contents) were about 21%

and 43% lower in the ammonium and nitrate treatments than in the control, respectively. However, the activities of P-acquisition specific enzymes were about 19% higher in the ammonium treatment than in the control. Using redundancy analysis (RDA), we found that the measured C, N, and P hydrolyses and polyphenol oxidase (PPO) activities were positively correlated with the soil pH and ammonium contents, but were negatively correlated with the nitrate contents. The PLFA biomarker contents were positively correlated with soil pH, soil organic carbon (SOC), and total N contents, but were negatively correlated with the ammonium contents. The soil enzyme activities varied seasonally, and were highest in March and lowest in October. In contrast, the contents of the microbial PLFA biomarkers were higher in October than in March and June. Ammonium may inhibit the contents of PLFA biomarkers more strongly than nitrate because of acidification. This study has provided useful information about the effects of ammonium and nitrate on soil microbial communities and enzyme activities.

1. Introduction

Studies have reported increases of 25% in wet atmospheric nitrogen (N) deposition over the past decade (Jia et al., 2014), which has resulted in a range of problems in forest ecosystems, such as induced soil acidification, aggravation of cation and nitrate leaching, and decreased microbial biomass (Liu et al., 2011; Huang et al., 2014; Gao et al., 2015; Liu et al., 2013). While wet atmospheric N deposition mostly comprises ammonium, nitrate deposition has increased over recent years, so that the ratio of ammonium to nitrate has decreased from 5 to 2 (Liu et al., 2013). It is therefore important to study the individual influences of these two forms of N on soil microorganisms to support improved predictions of C, N, and P cycling under increased nitrate deposition.

Soil microorganisms supply nutrients to forests by producing enzymes that catalyze the degradation of soil organic matter, and drive carbon (C), nitrogen (N), and phosphorus (P) cycling, with consequences for forest productivity and sustainability (Heijden et al., 2008). The soil microbial biomass of different communities may be quantified by phospholipid fatty acid (PLFA) biomarkers. Even though the PLFA signature method is not as advanced as genomic technology, it has been used extensively with good results to analyze the biomass and structures of microbial communities (Frostegård et al., 2011). Bacteria, including gram positive (G^+) and negative (G^-) bacteria, generally

61 degrade labile compounds by excreting hydrolase, while fungi, including arbuscular mycorrhizal fungi
62 (AMF) and saprophytes (SAP), are responsible for degrading recalcitrant compounds by secreting
63 oxidase (Burns et al., 2013; Sinsabaugh et al., 2010; Willers et al., 2015).

64 To date, most studies have considered the influence of organic N on microbial communities (Guo et
65 al., 2010; Hobbie et al., 2012) and few studies have reported how ammonium and nitrate individually
66 influence microbial communities in forest soils. Positively charged ammonium is more easily absorbed
67 by negatively charged soil colloids than nitrate, meaning that ammonium is more available to
68 microorganisms than nitrate. In our previous study, we showed that ammonium promoted the activities
69 of BG and NAG in soil aggregates were strongly than nitrate (Yan et al., 2017). However, the process
70 of nitrification, i.e. where ammonium is rapidly transformed to nitrate when it enters soil, may sterilize
71 microorganisms in the soil (Dail et al., 2001). Ammonium and nitrate have different effects on the
72 microbial decomposition rate and microbial respiration of soil organic matter. For example, substrate
73 respiration in peatlands increased when ammonium was added, but did not change when nitrate was
74 added (Currey et al., 2010). Nitrate additions strongly promoted the decomposition rates of soil organic
75 matter of fir plantations in the early incubation phase (0–15 d; Zhang et al., 2012). However, from a
76 laboratory incubation experiment, Ramirez et al. (2010) showed that nitrate and ammonium had similar
77 inhibitory effects on soil microbial respiration.

78 It is well known that microorganisms and enzymes are sensitive to soil pH. Tian and Niu (2015),
79 from their meta-analysis of soil acidification caused by N additions, suggested that ammonium nitrate
80 (NH_4NO_3) contributed more to soil acidification than ammonium. Further, most studies have not
81 separated the individual effects of additions of different nitrogen forms on PLFAs and microbial
82 biomass carbon (MBC) in forest ecosystems. From their meta-analysis, Treseder et al. (2008) reported
83 that N additions caused MBC to decrease by 15%, and that fungi were more sensitive to N additions
84 than other microbial communities. The responses of microbial biomass to N additions may be
85 influenced by a wide range of factors, including forest type and geographical location. For example, in
86 temperate regions, the total PLFA contents decreased in American beech (*Fagus grandifolia* Ehrh) and
87 yellow birch (*Betula alleghaniensis* Britton), but increased in eastern hemlock (*Tsuga Canadensis* (L.)
88 Carr) and red oak (*Quercus rubra* (L.) Britton) forests when NH_4NO_3 was added, with variable
89 responses from bacteria and fungi (Weand et al., 2010). In subtropical forests, NH_4NO_3 additions
90 resulted in an increase in total PLFA contents in a Chinese fir forest (Dong et al., 2015), a decrease in

91 soil MBC contents in an evergreen broad leaved forests, but no change in the pine broad-leaved mixed
92 forest (Wang et al., 2008).

93 Soil enzymes catalyze the decomposition of soil organic matter (Burns et al., 2013). Enzymes
94 involved in labile C breakdown that can decompose starch, cellulose, and hemicellulose include
95 α-1,4-glucosidase (αG), β-1,4-glucosidase (βG), cellobiohydrolase (CBH), and β-1,4-xylosidase (βX).
96 β-1,4-N-acetylglucosaminidase (NAG), a nitrogen-degradation enzyme, can decompose
97 oligosaccharides. Acid phosphatase (AP), a phosphorus-degradation enzyme, can decompose chitin
98 lipophosphoglycan (Stone et al., 2014). Recalcitrant C-degradation enzymes that can decompose lignin,
99 and aromatic and phenolic compounds including peroxidase and phenol oxidase (Sinsabaugh et al.,
100 2010). When added to peatland, Currey et al. (2010) found that ammonium and nitrate had different
101 effects on carbon- and phosphorus-enzyme activities (CBH and AP) but had similar effects on
102 polyphenol oxidase (PPO) activities, while Tian et al. (2014) found that the effects of ammonium and
103 nitrate were not significantly different when added to an alpine meadow. To date, few studies have
104 reported how ammonium and nitrate additions individually influence soil enzyme activities in forest
105 ecosystems.

106 In line with the economic theory, microorganisms will allocate enzymes to the resources that are
107 absent, so that N additions will cause C and P-acquisition enzymes to increase, and N-acquisition
108 enzymes to decrease (Burns et al., 2013). It has been reported that, when inorganic N forms were not
109 considered, N additions caused C-degradation enzymes (αG, βG, CBH and βX) and P-degradation
110 enzymes (AP) to increase, restricted oxidase (PPO and PER), but did not inhibit N-degradation
111 enzymes (NAG) (Jian et al., 2016; Marklein and Houlton, 2012), which suggests that the allocation of
112 enzyme activities does not always correspond exactly with the economic theory.

113 The responses of enzyme activities to N additions are influenced by a range of factors including
114 environmental conditions, plant types, and N background values. For example, in temperate regions,
115 the soil activities of BG, CBH, NAG, and PPO increased in a dogwood forest, decreased in an oak
116 forest, and did not change in a maple forest when NH_4NO_3 was added. The AP activities increased in
117 dogwood and maple forests, but were invariant in an oak forest after NH_4NO_3 additions (Sinsabaugh et
118 al., 2002). However, in acidified temperate regions, the soil BG activities increased in a maple forest,
119 but the soil BG, NAG, and AP activities did not change in yellow birch, oak, hemlock, and beech
120 forests, when NH_4NO_3 was added (Weand et al., 2010). In subtropical and tropical forests, the BG,

121 NAG, and AP activities increased, and oxidase (PPO and PER) activities decreased, after NH_4NO_3
122 additions (Dong et al., 2015; Guo et al., 2011; Cusack et al., 2011). To date, we are still not sure if
123 ammonium and nitrate additions have different effects on the soil microbial biomass of different
124 communities and on enzyme activities. To support improved predictions of the effects of elevated N
125 deposition on C, N, and P cycling in soil, we therefore need to evaluate the individual effects of
126 ammonium and nitrate additions on the soil microbial biomass of different communities and enzyme
127 activities.

128 The N-rich subtropical soils in southern China have experienced increased nitrate deposition in the
129 recent past. To facilitate an exploration of the different effects of ammonium and nitrate additions on
130 soil microbial communities and enzyme activities, we established a long-term ammonium and nitrate
131 trial in a slash pine (*Pinus elliottii*) plantation in a subtropical area. We hypothesized that (1)
132 ammonium would have stronger inhibitory effects on total PLFA, fungi PLFA contents, and enzyme
133 activities than nitrate because of its strong negative effect on soil pH; and (2) that ammonium and
134 nitrate additions would result in increased C and P-hydrolase activities, and decreased N-hydrolase
135 activities in line with the economic theory, and (3) that oxidase activities would be restricted due to
136 their inhibitory effects on fungi.
137
138 **2. Materials and methods**
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140 **2.1. Study site**
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142 The study was conducted in the Qianyanzhou (QYZ) Experimental Station, in the hilly red soil
143 region of Taihe County, Jiang Xi Province, China (26°44′29.1″N, 115°03′29.2″E, 102 m a.s.l.). The
144 region has a subtropical monsoon climate, a mean annual temperature of 17.9 °C, and a mean annual
145 precipitation of 1475 mm. The soil formed because of weathering of red sandstone and mudstone, and,
146 based on the US soil taxonomy (Soil Survey Staff, 2010), is classified as a Typical Dystrudepts Udepts
147 Inceptisol. The slash pine (*Pinus elliottii*), one of the dominant species in this hilly red soil region, was
148 planted in 1985 under a vegetation restoration program. *Woodwardia japonica*, *Dicranopteris*
149 *dichotoma* and *Loropetalum chinense* dominate the understory (Kou et al., 2015).
150

151 **2.2. Experimental design**

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153 As described by Kou et al. (2015), the plots were established in November 2011 using a randomized
154 complete block design. Background atmospheric wet N deposition of about 33 kg N ha^{-1} yr^{-1}
155 comprises 11 kg N ha^{-1} yr^{-1} as ammonium and 8 kg N ha^{-1} yr^{-1} as nitrate (Zhu et al., 2014). We
156 established a control and test plots at the experimental sites. We equally added two types of N to the
157 test plots, i.e. ammonium ($N_{ammonium}$) as ammonium chloride (NH_4Cl) and nitrate ($N_{nitrate}$) as sodium
158 nitrate ($NaNO_3$), at an annual rate of 40 kg N ha^{-1} yr^{-1}. This rate was about double the background N
159 wet deposition. Each treatment had three replicates, so the experiment comprised a total of nine plots,
160 which each measured 20 × 20 m. The plots had slope angles of less than 15° and were separated by
161 buffer zones of more than 10 m. The NH_4Cl or $NaNO_3$ were dissolved in 30 L of tap water and evenly
162 sprayed onto the plots once a month, i.e. 12 times per year. The equivalent amount of tap water was
163 sprayed onto the control plots. Nitrogen additions commenced in May 2012 and were applied each
164 month on non-rainy days until March 2015. A total of 113 kg N ha^{-1} was applied over the course of this
165 study.

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167 **2.3. Sampling and analysis**

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169 We collected soil samples in March, June, and October of 2015, to represent spring, summer, and
170 fall. We removed the surface litter, and extracted soil cores with a diameter of 5 cm from between 0
171 and 10 cm deep from 5 randomly selected locations in each plot, which we then mixed together as one
172 composite sample. The atmospheric conditions and plant-derived litters differed between the three
173 seasons, and so indirectly affected the soil microbial biomass and enzyme activities of different
174 communities. We collected soils from three seasons so that we could investigate the synthetic
175 responses of soil microbial biomass and enzyme activities to ammonium and nitrate additions and to
176 obtain improved information to support predictions of the effects of elevated N depositions on C, N,
177 and P cycling. Field-fresh samples were sieved through a 2 mm mesh after being mixed evenly.
178 Samples were stored at 4 °C until analysis for PLFA biomarkers, enzyme activities, soil pH,
179 ammonium, nitrate, and soil dissolved organic carbon (DOC). The PLFA biomarker and enzyme
180 activity assays were performed on return to the laboratory. Subsamples of each soil were air-dried, and

181 then sieved through a 0.25 mm mesh before soil organic C (SOC) and total N (TN) concentrations were
182 determined.

183 Soil pH was measured in a soil-water suspension by glass electrode at a soil to water ratio of 1g fresh
184 soil:2.5 volume of water. Soil water contents (SWC) were measured by the oven drying method
185 (105 ℃). After extraction with 1mol L^{-1} KCl, the ammonium and nitrate concentrations in the fresh
186 soils were measured by a continuous flow auto-analyzer (Bran Lubbe, AA3, Germany). Another
187 portion of the soil sample was extracted with soil and distilled water at a ratio of 1:5 and soil DOC
188 concentrations were measured with an organic element analyzer (Liquid TOC*II,* Elementar, Germany).
189 Soil TN and SOC were measured with a carbon/nitrogen analyzer (Vario Max, Elementar, Germany).

190 Phospholipid fatty acid (PLFA) biomarkers were measured as outlined by Bossio and Scow (1998).
191 In brief, field-fresh soil equal to 8 g of dry soil was subjected to mild alkaline methanolusis to form
192 fatty acid methyl esters (FAMEs). The extracted PLFAs were dissolved in hexane and measured by gas
193 chromatography (Agilent 6890N) with MIDI peak identification software (version 4.5; MIDI Inc.
194 Newark, DE) and a DB-5 column. The abundances of the PLFA biomarkers were calculated as nmol
195 PLFA g^{-1} dry soil. The total amounts of the different PLFA biomarkers were used to represent different
196 groups of soil microorganisms, i.e. gram-positive bacteria (G^+) by i14:0, i15:0, a15:0, i16:0, i17:0,
197 a17:0; gram-negative bacteria (G^-) by 16:1ω7c, cy17:0, 18:1ω7c, cy19:0; arbuscular mycorrhizal fungi
198 (AMF) by 16:1ω5; saprophytic fungi (SAP) by 18:1ω9c, 18:2ω6c, 18:2ω9c 18:3ω6c; actinomycete (A)
199 by 10Me16:0, 10Me17:0, 10Me18:0 (Bradley et al., 2007; Denef et al., 2009). Bacterial biomass was
200 calculated as the sum of G^+ and G^-, and fungi biomass were calculated as the sum of AMF and SAP,
201 respectively.

202 We measured four C-acquisition hydrolases (i.e. αG, βG, CBH, and βX), one N-acquisition
203 hydrolase (NAG), and one P-acquisition hydrolase (AP) following the methods of Saiya-Cork et al.
204 (2002), and have provided information about their corresponding substrates and functions in Table S1.
205 In brief, 1 g of field-fresh soil was homogenized in a sodium acetate buffer (125 ml). We then added
206 200 μl of homogenate and 50 μl of substrate to black microplates with 96 wells with eight replicates for
207 each soil sample. The microplates were then incubated at 20 °C for 4 h. After incubation, 10 μl of 1 mol
208 L^{-1} NaOH was added to each well to terminate the reactions, and fluorescence values were measured at
209 an excitation of 365 nm and emission of 450 nm with a microplate fluorometer (Synergy H4, BioTek).
210 The absolute hydrolase activities were expressed in units of nmol g^{-1} soil h^{-1}. We compared the

211 stoichiometry of C and P to N-acquisition enzyme activities by ln(aG+BG+CBH+BX) and lnaP to
212 lnNAG, respectively (n=27).
213 Two oxidases, i.e. PER and PPO, were measured using 96-well transparent microplates as outlined
214 by Saiya-Cork et al. (2002). We added 600 μl of homogenate and 150 μl of substrate to deep
215 microplates with 96 wells. To measure the PER activities, we added 10 μl of 0.3% H_2O_2 to the
216 homogenate and substrates mixtures. After incubation at 20 °C for 5 h, the microplates were
217 centrifuged at 3000 r for 3 minutes, then 250 μl of liquid supernatant was transferred to a 96-well
218 transparent microplate. The absorbance values were measured at 460 nm by microplate
219 spectrophotometer (Synergy H4, BioTek). We calculated the specific activities of the enzymes by
220 dividing the enzyme activities by the PLFA values to normalize the activity to the size of the microbial
221 active biomass (Cusack et al. 2011).
222
223 **2.4. Statistical analyses**
224
225 We used a two factor randomized block analysis of variance and Duncan's multiple comparisons to
226 test the differences between the treatments and sampling time (n=9). To evaluate the effects of
227 ammonium and nitrate additions, the treatment differences of time-dependent indexes were tested by
228 one-way analysis of variance (ANOVA) and Duncan's multiple comparisons for each sampling event or
229 season (n=3). Analyses were performed with SPSS 17.0. Relationships among the soil physical and
230 chemical properties, soil PLFA biomarker contents, and the soil enzyme activities were tested by
231 redundancy analysis (RDA) in CANOCO 4.5 (n=27). Results were statistically significant when $P <$
232 0.05. The figures were plotted in Sigmaplot 10.0.
233
234 **3. Results**
235
236 **3.1. Soil physical and chemical properties**
237
238 The soil pH and ammonium contents were either treatment- or time-independent. There were
239 interaction effects between the treatments and the sampling time on the soil DOC and nitrate contents
240 (P<0.01, Table 1). The soil pH decreased by 0.7 of a unit across the three sampling events in the

241 ammonium-treated plots, but did not change significantly in the nitrate-treated plots (Fig. 1). The soil
242 nitrate contents were 165% and 129% higher, and the soil ammonium contents were 31% and 38%
243 lower, respectively, in the ammonium and nitrate treatments (Fig. 1 & 2) than in the control for the
244 three sampling events. Compared with the control, the soil DOC concentrations were 17% higher in the
245 nitrate-treated plots across the three sampling events, but did not change significantly in the
246 ammonium-treated plots (Fig. 2). Ammonium contents were higher in March than in June and October
247 (Fig. 2, Table S2), while DOC and nitrate concentrations were highest in October and lowest in March
248 (Table 2).
249
250 **3.2. Soil microbial biomass of different communities**
251
252 Both the treatment and the time of sampling significantly influenced the soil microbial biomass of
253 the different communities ($P<0.01$). Total PLFAs, bacteria, G^{-}, and G^{+}/G^{-} were either treatment- or
254 time-independent. There were also interaction effects between treatments and sampling time on fungi,
255 actinomycetes, G^{+}, AMF, SAP, and the fungi/bacteria ratio (Table 1). The inhibition effects of
256 ammonium additions on total PLFA contents were stronger than those of nitrate additions and the total
257 PLFA contents were 24% and 11% less in the ammonium- and nitrate-treated plots across the three
258 sampling events than in the control. The PLFA contents of G^{+}, AMF, bacteria, fungi, actinomycetes
259 were between 14% and 40%, and 7% and 24%, lower in the plots treated with ammonium and nitrate,
260 respectively, than in the control across the three sampling events (Fig. 3 and 4). The soil PLFA contents
261 also showed seasonal variation (Table 1). Total PLFA biomarker contents and bacterium, fungi, G^{+}, G^{-},
262 AMF, and SAP PLFA biomarker contents were highest in March and lowest in October, while
263 actinomycete PLFA biomarker contents were highest in June and lowest in October (Fig. 4, Table S2).
264 The microbial communities shifted from G^{-} to G^{+} in the ammonium-treated plots, meaning that the
265 G^{+}/G^{-} ratios were higher in the ammonium-treated plots than in the control or nitrate-treated plots. The
266 fungi/bacteria ratios were lower in both the ammonium- and nitrate-treated plots than in the control, but
267 were much lower in the nitrate-treated plots than in the ammonium-treated plots (Figs. 3 and 4).
268
269 **3.3. Soil enzyme activities**
270

271 There were significant influences from both treatment and sampling time on the measured absolute
272 enzyme activities ($P<0.01$). Activities of BG, AP, and PPO were either treatment- or time-independent,
273 and there were interaction effects between the treatments and sampling time on activities of aG, BX,
274 CBH, NAG, and PER (Table 1). Ammonium and nitrate had similar inhibition effects on aG, BG, BX,
275 CBH, NAG, PPO, and PER activities, which decreased by between 6% and 50% across the three
276 sampling events (Table 2). The AP absolute activities were about 9% lower in the nitrate treatment than
277 in the ammonium treatment (Table 2). When compared to control, the ratios of C to N-acquisition
278 enzyme activities were about 0.2 higher, the ratios of N to P acquisition enzyme activities were about
279 0.1 lower, and there were no obvious differences in the ratios of C to P acquisition enzyme activities in
280 the ammonium and nitrate treatments. The measured enzyme activities varied seasonally (Table 2).
281 Activities of BG, BX, CBH, NAG, AP, and PPO were lowest in March and highest in October; aG
282 activities were highest in March and lowest in June, and PER activities were highest in March and
283 lowest in October (Table 2).
284 The treatments had a significant influence on the activities of N- and P-acquisition specific enzymes
285 ($P<0.01$), but not on the activities of C and oxidase specific enzymes (Table 1). The inhibitory effects
286 of nitrate on the activities of N-acquisition specific enzymes were stronger (about 43%) than those of
287 ammonium (about 21%). When compared with the control, the AP specific activities were about 19%
288 higher in the ammonium-treated plots across the three sampling events (Fig. 5).
289
290 **3.4. Redundancy analyses**
291
292 The results of RDA between soil properties, absolute enzyme activities, and PLFA biomarker
293 contents showed that the first ordination RDA axis explained 72.0% and 67.5%, respectively, and the
294 second axis explained 11.5% and 14.3%, respectively, of the variation. The RD1 for soil absolute
295 enzyme activities and PLFA biomarkers was correlated with DOC/SOC, DOC, ammonium, and SOC.
296 However, nitrate was only correlated with the RD1 of the absolute enzyme activities but not the PLFA
297 biomarker contents. Most of the measured absolute soil enzyme activities and the PLFA biomarker
298 contents were positively correlated with soil pH, but G^+/G^- and F/B were negatively correlated with
299 soil pH. Ammonium and DOC contents were positively correlated with all the soil absolute enzyme
300 activities except PER, but were negatively correlated with PLFA biomarker contents. Nitrate contents

301 were negatively correlated with soil absolute enzyme activities, but were barely correlated with the
302 PLFA biomarker contents. SWC were positively correlated with soil PLFA biomarker contents, but
303 were not correlated with the absolute enzyme activities (Fig. 6).
304
305 **4. Discussion**
306
307 Our results agree with our first hypothesis and show that the inhibition effects on soil PLFA contents
308 of bacteria, fungi, and actinomycetes across the three sampling events or seasons were stronger when
309 ammonium was added than when nitrate was added (Figures 3 and 4, Table 1). Results from RDA
310 suggest that acidification because of the ammonium additions triggered the decrease in the microbial
311 biomarkers-PLFA contents (Fig. 6). Soil microbial biomass may be inhibited by resource availability
312 and acidification (Sinsabaugh et al., 2014; Moorhead et al., 2006). However, C and N availability (sum
313 of the ammonium and nitrate concentrations) either increased or stayed the same over the three
314 sampling events when ammonium and nitrate were added (Figs. 1 and 2). Ammonium additions may
315 aggravate nitrification in subtropical soils (Tang et al. 2016), and nitrification may be toxic to
316 microorganisms (Dail et al., 2001), which may then lead to a decrease in the microbial PLFA contents.
317 The soil pH did not change when nitrate was added (Fig. 1), which may explain why nitrate had
318 weaker inhibition effects on PLFA biomarker contents than ammonium. Nitrate additions may inhibit
319 the PLFA biomarker contents because of accelerated leaching of Ca^{2+} and Mg^{2+} (Qian et al., 2007),
320 increases in the soil osmotic potential, and activation of Al^{3+} absorbed by soil colloids (Treseder et al.,
321 2008). The PER activity was lower when ammonium and nitrate were added (Table 2), which may
322 eventually result in polyphenol accumulation in soil. Accumulated polyphenol may be toxic to
323 microorganisms (Sinsabaugh et al., 2010) and may have contributed to the decrease in the contents of
324 the PLFA biomarkers. Moreover, the higher soil DOC concentrations observed in the nitrate-addition
325 treatments (Fig. 2) may be attributed to changes in the diversity of the composition of saprophytic
326 bacteria (Freedman and Zak, 2014; Freedman et al., 2016).
327 In our study, the fungi /bacteria ratios were lower in the ammonium and nitrate treatments than in the
328 control, which suggests that fungi were more sensitive to N additions than bacteria. In an earlier study,
329 we found that the fine root biomass decreased after N additions (Kou et al., 2015), which suggests that
330 N might upset the symbiosis between AMF and plants, thereby restricting the AMF-PLFA contents.

331 Our study showed that the absolute activities of C, N, and P-hydrolases and oxidase were inhibited
332 by ammonium and nitrate in the three seasons (Table 2). This agrees with our second and third
333 hypothesis, i.e., that N additions caused the absolute activities of the N-acquisition enzyme (NAG) to
334 decrease, in line with the microbial economic theory; and that N additions reduced the absolute
335 activities of the oxidase by decreasing the PLFA contents of fungi. However, we did not expect the C-
336 or P-acquisition enzymes to decrease. As main producers of soil enzymes, the microbial biomass would
337 decrease in response to ammonium and nitrate additions, resulting in lower absolute enzyme activities
338 in the treated plots than in untreated plots (Allison et al., 2005).

339 The ratios of C or P to N acquisition enzyme activities were higher in the ammonium and nitrate
340 treatments than in the control plots, and the N-acquisition enzyme activities per unit of microbial
341 biomass were lower in the ammonium and nitrate treatments than in the control (Fig. 5), indicating that
342 microorganisms secreted enzymes in line with the economic theory. Measured absolute enzyme
343 activities were positively correlated with soil pH and ammonium contents, and negatively correlated
344 with nitrate contents (Fig. 6). The inhibitory effects of N on the soil absolute enzyme activities may be
345 more closely related to abiotic factors, i.e. soil pH and nitrification, than biotic factors (Kivlin et al.,
346 2016).

347 We also found that ammonium and nitrate additions inhibited AP activities (Table 2). However,
348 P-acquisition enzyme activities per unit of microbial biomass increased in the ammonium treatments
349 (Fig. 5). Li et al. (2016) reported that N applications aggravated the P-limitations on biomass
350 production (Li et al., 2016). In line with the microbial economic theory, when the P-availability was
351 low, the activities of P-acquisition enzymes were higher. The decreased AP activities that resulted from
352 ammonium additions may be more strongly related to abiotic inhibition caused by the ammonium, such
353 as acidification, aggravated nitrification, and leaching of cations and nitrate, than biotic inhibition.

354 The N treatments also varied significantly on a seasonal basis and there were interaction effects
355 between N treatments and seasons on the contents of some PLFA biomarkers and enzyme activities
356 (Table 2). Climate conditions, plant growth, the amount of litter returned, and plant-soil-microorganism
357 systems varied across the three seasons. The temperature ranged from 13.5 to 27.6 ℃, and precipitation
358 ranged from 88.2 to 176.6 mm, across the three seasons (Fig. S1), and did not limit the growth of
359 microorganisms. The positive relationships between PLFA biomarker contents and soil moisture
360 contents indicate that soil moisture had a strong influence on soil microbial community biomass. There

361 may be interaction effects between plant growth, the mass and quality of litter, plant-microbe
362 competition, and soil nutrient dynamics. For example, compared with the control plots, the soil DOC
363 contents were lower, and soil nitrate contents stayed the same in June (the growing season) in the
364 ammonium treatment, but the soil DOC and nitrate contents were higher in the ammonium and nitrate
365 treatments in March and October (non-growing season, Fig. 2). This indicates that there was stronger
366 competition between plants and microbes for available C and N in June than in March and October, and
367 that there were interaction effects between plants and microbes on soil C and N availability. This might
368 explain the interaction effects between N additions and seasons on the activities of C and N-acquisition
369 enzymes. The effects of interactions between N additions and season on the AMF PLFA contents,
370 along with available C and N dynamics, may result from plant growth as plant-AMF symbiotic systems
371 may be influenced by fine root biomass.
372
373 **5. Conclusions**
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375 The results showed that soil bacteria, fungi, and actinomycetes- PLFA biomarker contents decreased
376 after ammonium and nitrate additions. Ammonium inhibited the biomass of different soil microbial
377 communities except SAP more strongly than nitrate, perhaps because of acidification caused by
378 ammonium. The microbial communities were dominated by G^+ and bacteria after ammonium additions,
379 and were dominated by bacteria under nitrate additions.
380 The absolute activities of C, N, and P-acquisition hydrolases and oxidase decreased after additions of
381 ammonium and nitrate, and nitrate had a stronger inhibition effects on P-acquisition absolute enzyme
382 activities than ammonium. However, ammonium improved the P-demand per unit of microbial biomass.
383 C and P-acquisition absolute enzyme activities were higher than N-acquisition absolute enzyme
384 activities under ammonium and nitrate additions. Because of the positive correlation between the
385 measured absolute enzyme activities and soil pH, the decreases in the absolute hydrolase and oxidase
386 activities reflected abiotic restrictions, i.e. acidification and nitrification caused by ammonium
387 additions, rather than biotic restrictions.
388 Ammonium and nitrate additions had a range of effects on soil microbial communities and the
389 activities of specific enzymes. Our results show that the effects of ammonium and nitrate need to be
390 discussed separately to provide the information that we need to predict the effects of elevated N

391 deposition on soil microbial biomass and enzyme activities.

392

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

396

397 *Competing interests*: The authors declare no conflict of interest.

398

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400

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403

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532 **Figure Legends**

533

534 **Fig. 1.** The effects of ammonium and nitrate additions on soil pH and ammonium contents. Small
535 letters represent significant differences between treatments (*P* <0.05), error bars represent means ±
536 standard errors (n=9).

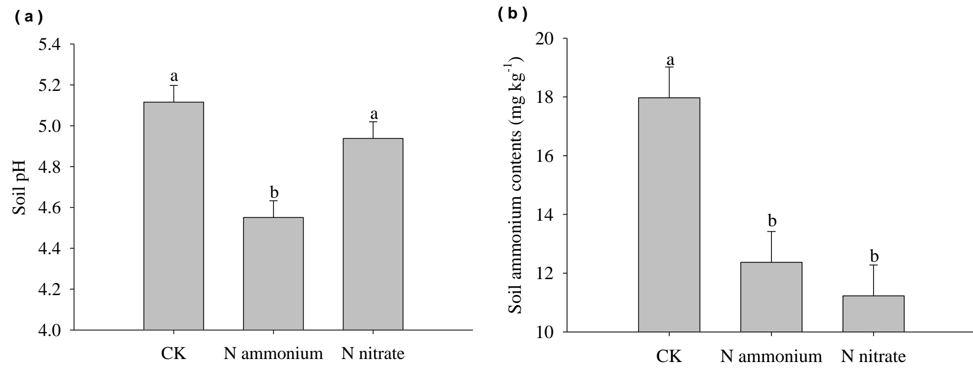
537 **Fig. 2.** The effects of ammonium and nitrate additions on soil nitrate and DOC contents for each
538 sampling event. Capital letters represent significant differences between the treatments (*P* <0.05), and
539 small letters represent significant differences between the sampling events (*P* <0.05), error bars
540 represent means ± standard errors (n=3).

541 **Fig. 3.** The effects of ammonium and nitrate additions on Total PLFAs, PLFA contents of bacteria, G^-
542 and G^+/G^-. Small letters represent significant differences between treatments (*P* <0.05), error bars
543 represent means ± standard errors (n=9). G^+ represents gram positive bacteria and G^- represents gram
544 negative bacteria.

545 **Fig. 4.** The effects of ammonium and nitrate additions on PLFA contents of fungi, actinomycetes, AMF,
546 SAP, G^+, and fungi/bacteria ratio for each sampling event. Capital letters represent significant
547 differences between the treatments (*P* <0.05), and small letters represent significant differences
548 between the sampling time (*P* <0.05), error bars represent means ± standard errors (n=3). G^+ is gram
549 positive bacteria, AMF is arbuscular mycorrhizal fungi, and SAP is saprophytic fungi.

550 **Fig. 5.** The effects of ammonium and nitrate additions on N, P-acquisition specific enzyme activities
551 for each sampling event. Capital letters represent significant differences between the treatments (*P*
552 <0.05), and small letters represent significant differences between the sampling time (*P* <0.05), error
553 bars represent means ± standard errors (n=3).

554 **Fig. 6.** Redundancy analyses between (a) soil properties and enzyme activities, and (b) soil properties
555 and PLFA-biomarker contents.



556

557 **Fig.1**

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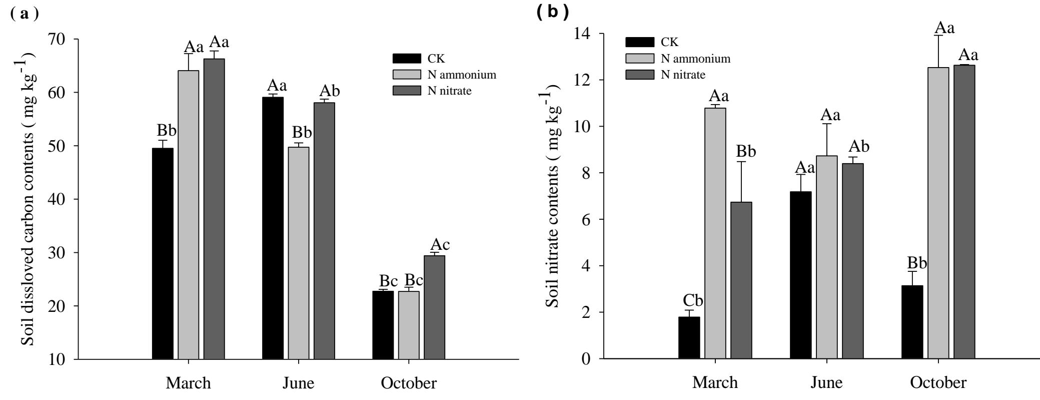
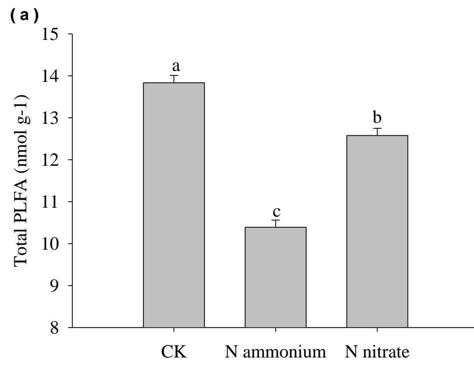
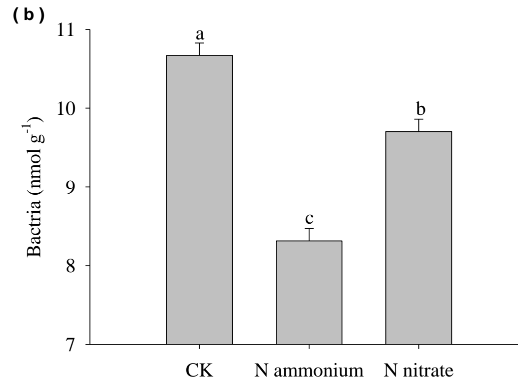


559 **Fig.2**





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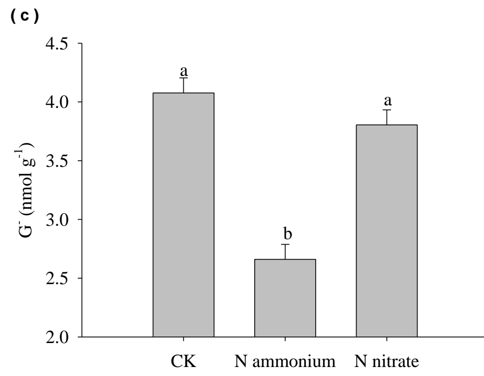

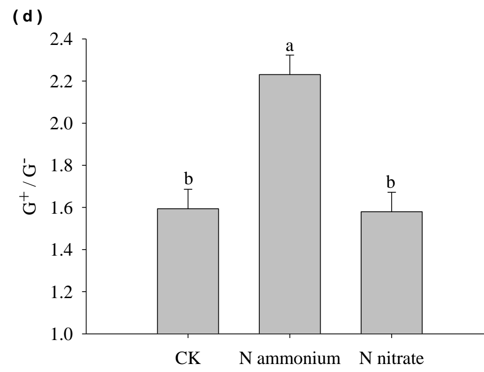

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562 **Fig.3**

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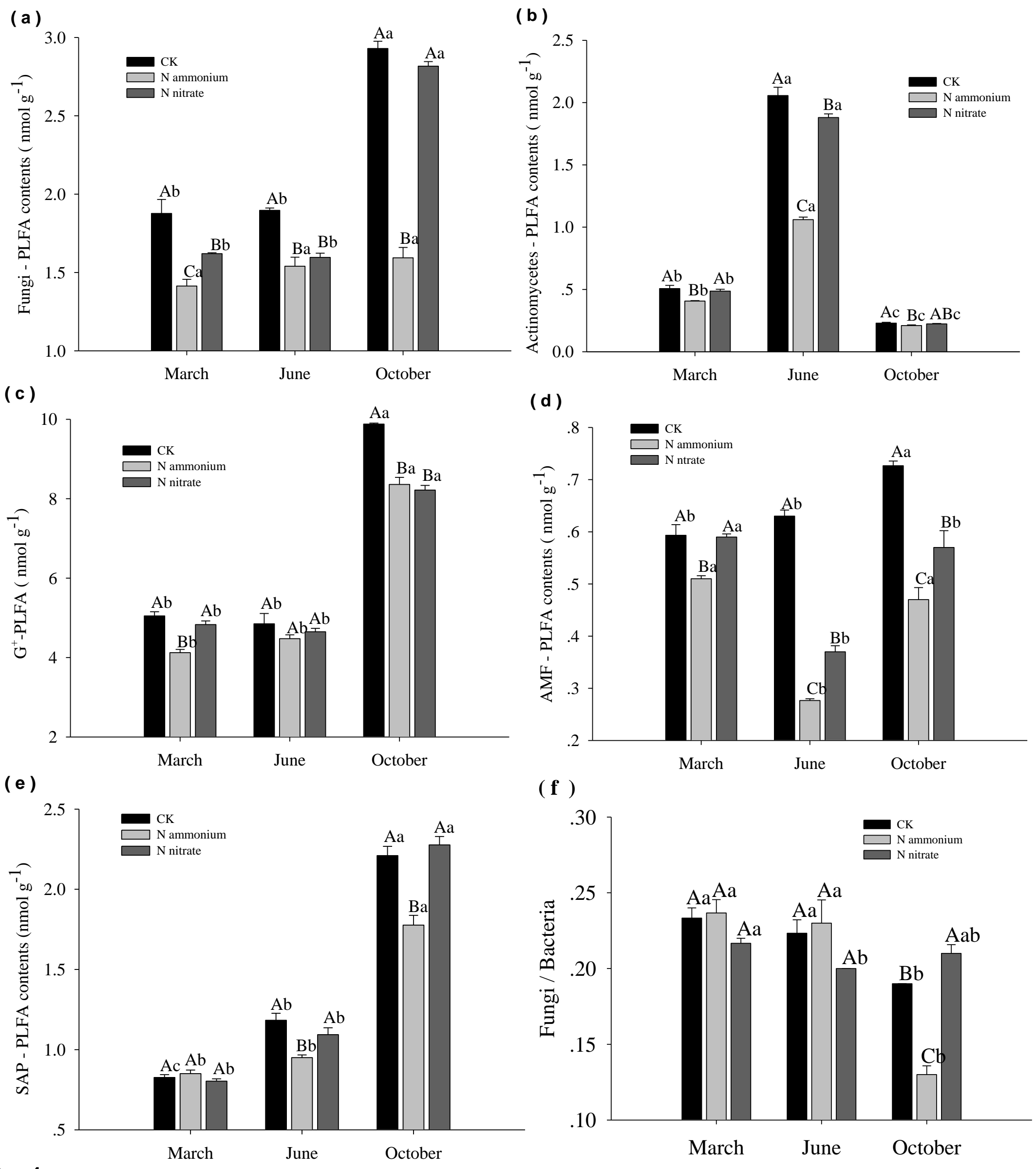
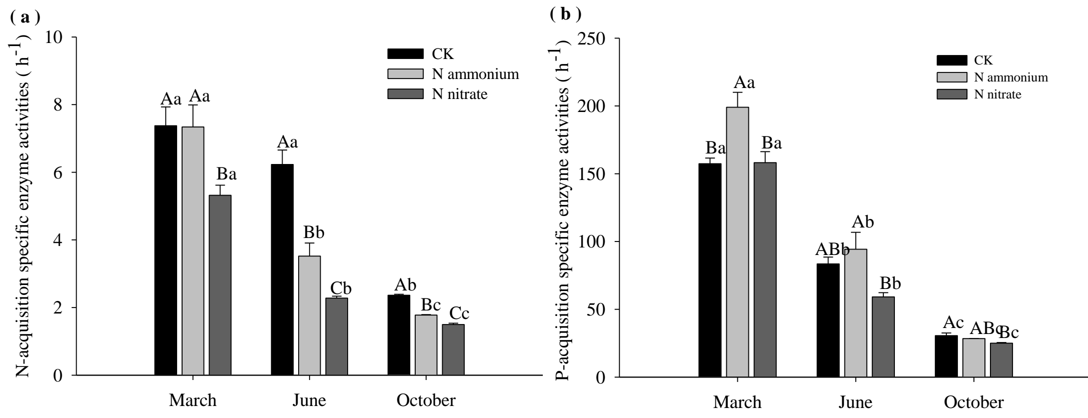


566 **Fig. 4**



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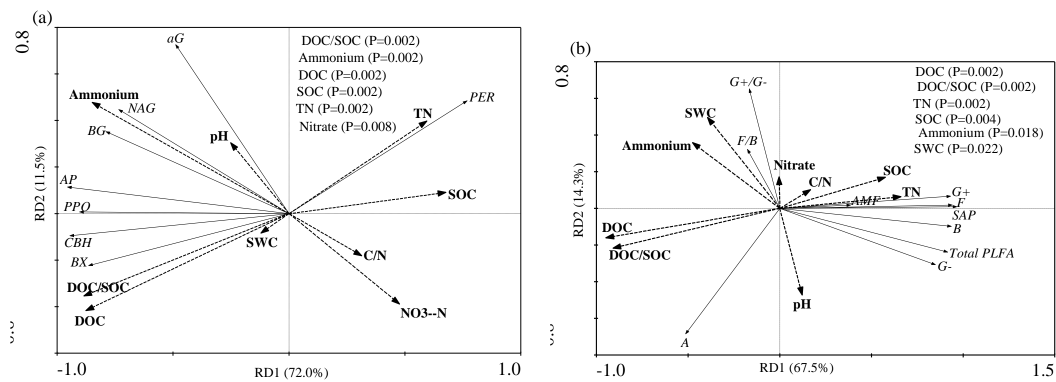
568 **Fig.5**



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570 **Fig. 6**

571 **Table 1** Summary statistics (F ratio) for the two factor randomized block analysis of variance (ANOVA)
572 applied to soil variables, enzyme activities and PLFA biomarkers. The bold numbers are significant (*P*
573 < 0.05)

Factors	Treatments	Months	Treatments × Months
pH	**12.43**	0.31	0.09
DOC	**23.53**	**561.25**	**20.11**
Nitrate	**43.19**	**7.96**	**8.21**
Ammonium	**11.84**	**65.46**	0.42
TPLFA	**102.51**	**477.77**	2.68
B	**56.94**	**555.14**	2.73
F	**180.49**	**277.81**	**52.16**
A	**172.230**	**2627.61**	**123.12**
G^+	**50.30**	**1221.19**	**14.39**
G^-	**34.33**	**105.59**	0.45
AMF	**147.77**	**83.55**	**21.64**
SAP	**24.70**	**781.67**	**13.08**
G^+/G^-	**16.24**	2.38	0.94
F/B	**3.82**	**56.42**	**21.67**
aG	**30.24**	**53.17**	**3.47**
BG	**3.26**	**72.90**	0.58
BX	**9.86**	**79.08**	**3.86**
CBH	**28.51**	**194.75**	**4.39**
NAG	**100.42**	**67.49**	**8.47**
AP	**22.81**	**467.77**	1.73
PPO	**6.87**	**64.40**	1.98
PER	**6.27**	**194.30**	**3.07**
C-acquisition specific enzyme	2.82	**334.41**	2.07
N-acquisition specific enzyme	**29.10**	**128.31**	**6.36**
P-acquisition specific enzyme	**13.42**	**397.19**	**4.53**
Oxidase specific enzyme	1.68	**89.04**	1.84

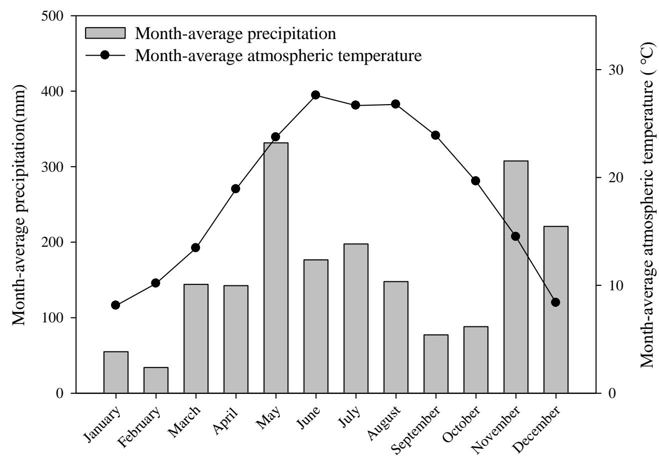
574 **Table 2** Summary statistics (means ± standard errors, n=3) for one way analyses of variance (ANOVA) and Duncan multiple 575 comparisons applied to soil absolute enzyme activities. Capital letters represent significant differences between the treatments (*P* 576 <0.05), and small letters represent significant differences between the sampling events (*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±0.1Aa	160.9±15.6 Aa	36.4±3.4Aa	30.±2.1A a	77.5±4.7 Aa	1658.7±59.1 Aa	7.9±0.9Aa	1.4±0.1 Ab
	$N_{ammonium}$	4.5±0.2Ba	143.5±4.0A a	26.8±3.2Aa	27.3±1.5 Aa	56.1±5.2 Ba	1520.7±78.2 Aa	8.9±0.0Aa	1.5±0.1 Ab
	$N_{nitrate}$	4.5±0.2Ba	157.1±10.9 Aa	33.4±1.0Aa	21.0±0.8 Ba	49.7±2.6 Ba	1475.2±53.2 Aa	9.9±1.4Aa	1.6±0.1 Ab
June	CK	4.0±0.9Ab	83.2±13.0A b	37.2±1.6Aa	28.6±2.5 Aa	77.0±4.7 Aa	1030.3±41.2 Ab	7.7±1.2Aa	1.4±0.1 Ab
	$N_{ammonium}$	2.2±0.1A Bc	70.6±0.9Ab	25.9±1.8Ba	17.9±0.2 Bb	31.8±1.7 Bb	848.5±62.1B b	4.0±0.0Bb	0.9±0.1B b
	$N_{nitrate}$	1.7±0.3Bb	89.4±10.3A b	28.7±1.2Bb	19.8±0.2 Ba	25.7±0.6 Bb	667.8±26.5C b	4.8±0.9A Bb	1.2±0.1 Ab
October	CK	3.7±0.4Ab	89.1±0.9Ab	15.2±0.4A Bb	9.7±0.3A b	44.7±0.2 Ab	578.0±38.1A c	2.9±0.2Ab	7.6±0.1 Aa
	$N_{ammonium}$	3.7±0.1Ab	64.0±4.2Ab	16.2±0.9Ab	5.2±0.1B c	26.5±0.2 Bb	423.4±1.6Bc	2.8±0.1Ab	5.5±0.8 Aa
	$N_{nitrate}$	2.2±0.0Bb	68.3±11.5A b	13.5±0.1Bc	5.3±0.1B b	24.5±0.2 Cb	409.8±4.7Bc	1.9±0.1Bc	5.6±0.8 Aa

577

578 **Supplementary materials**

579



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581 Fig S1. Average monthly atmospheric temperature and precipitation at the study site during 2015.

582 **Table S 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

583 **Table S2** Time-independent seasonal variations in ammonium and PLFAs. Small letters represent
584 significant differences between the sampling time (P <0.05), error bars represent means ± standard
585 errors (n=9).

Months	Ammonium mg kg^{-1}	Total PLFA nmol g^{-1}	Bacteria nmol g^{-1}	G^- nmol g^{-1}
March	23.5±1.0a	9.2±0.2c	7.1±0.2c	2.5±0.1c
June	10.6±1.0b	11.0±0.2b	7.7±0.2b	3.1±0.1b
October	7.5±1.0b	16.7±0.2a	13.8±0.2a	5.0±0.1a

586