Contrasting effects of ammonium and nitrate additions on the biomass of soil microbial communities and enzyme activities in subtropical China

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Abstract

The nitrate to ammonium ratios in nitrogen (N) compounds in wet atmospheric deposits have increased over the recent past, which is a cause for some concern as the individual effects of nitrate and ammonium deposition on the biomass of different soil microbial communities and enzyme activities are still poorly defined. We established a field experiment and applied ammonium (NH_4Cl) and nitrate ($NaNO_3$) at monthly intervals over a period of four years. We collected soil samples from the ammonium and nitrate treatments and control plots in three different seasons, namely spring, summer, and autumn, to evaluate the how the biomass of different soil microbial communities and enzyme activities responded to the ammonium (NH_4Cl) and nitrate ($NaNO_3$) applications. Our results showed that the total contents of phospholipid fatty acids (PLFA) decreased by 24% and 11% in the ammonium and nitrate treatments, respectively. The inhibitory effects of ammonium on gram positive bacteria (G^+) and bacteria, fungi, actinomycetes, and arbuscular mycorrhizal fungi (AMF) PLFA contents ranged from 14% to 40% across the three seasons. We also observed that the absolute activities of C, N, and P hydrolyses and oxidases were inhibited by ammonium and nitrate, but that nitrate had stronger inhibitory effects on the activities of acid phosphatase (AP) than ammonium. The activities of 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 hydrolysis 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 is mostly comprised of 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 degrade labile compounds by excreting hydrolase, while fungi, including arbuscular mycorrhizal fungi (AMF) and saprophytes (SAP), are responsible for degrading recalcitrant compounds by secreting oxidase (Burns et al., 2013;

61 Sinsabaugh et al., 2010; Willers et al., 2015).

62 To date, most studies have considered the influence of organic N on microbial communities (Guo et
63 al., 2010; Hobbie et al., 2012) and few studies have reported how ammonium and nitrate individually
64 influence microbial communities in forest soils. Positively charged ammonium is more easily absorbed
65 by negatively charged soil colloids than nitrate, meaning that ammonium is more available to
66 microorganisms than nitrate. In our previous study, we showed that ammonium promoted the activities
67 of β-1,4-glucosidase (βG) and β-1,4-N-acetylglucosaminidase (NAG) in soil aggregates were strongly
68 than nitrate (Yan et al., 2017). However, the process of nitrification, i.e. where ammonium is rapidly
69 transformed to nitrate when it enters soil, may sterilize microorganisms in the soil (Dail et al., 2001).
70 Ammonium and nitrate have different effects on the microbial decomposition rate and microbial
71 respiration of soil organic matter. For example, substrate respiration in peatlands increased when
72 ammonium was added, but did not change when nitrate was added (Currey et al., 2010). Nitrate additions
73 strongly promoted the decomposition rates of soil organic matter of fir plantations in the early incubation
74 phase (0–15 d; Zhang et al., 2012). However, from a laboratory incubation experiment, Ramirez et al.
75 (2010) showed that nitrate and ammonium had similar inhibitory effects on soil microbial respiration.

76 It is well known that microorganisms and enzymes are sensitive to soil pH. Tian and Niu (2015), from
77 their meta-analysis of soil acidification caused by N additions, suggested that ammonium nitrate
78 (NH_4NO_3) contributed more to soil acidification than ammonium. Further, most studies have not
79 separated the individual effects of additions of different nitrogen forms on PLFAs and microbial biomass
80 carbon (MBC) in forest ecosystems. From their meta-analysis, Treseder et al. (2008) reported that N
81 additions caused MBC to decrease by 15%, and that fungi were more sensitive to N additions than other
82 microbial communities. The responses of microbial biomass to N additions may be influenced by a wide
83 range of factors, including forest type and geographical location. For example, in temperate regions, the
84 total PLFA contents decreased in American beech (*Fagus grandifolia* Ehrh) and yellow birch (*Betula*
85 *alleghaniensis* Britton), but increased in eastern hemlock (*Tsuga Canadensis* (L.) Carr) and red oak
86 (*Quercus rubra* (L.) Britton) forests when NH_4NO_3 was added, with variable responses from bacteria
87 and fungi (Weand et al., 2010). In subtropical forests, NH_4NO_3 additions resulted in an increase in total
88 PLFA contents in a Chinese fir forest (Dong et al., 2015), a decrease in soil MBC contents in an evergreen
89 broad leaved forests, but no change in the pine broad-leaved mixed forest (Wang et al., 2008).

90 Soil enzymes catalyze the decomposition of soil organic matter (Burns et al., 2013). Enzymes involved

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

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

The responses of enzyme activities to N additions are influenced by a range of factors including environmental conditions, plant types, and N background values. For example, in temperate regions, the soil activities of βG, CBH, NAG, and PPO increased in a dogwood forest, decreased in an oak forest, and did not change in a maple forest when NH_4NO_3 was added (Sinsabaugh et al., 2002); The AP activities increased in dogwood and maple forests, but were invariant in an oak forest after NH_4NO_3 additions (Sinsabaugh et al., 2002). However, in acidified temperate regions, the soil βG activities increased in a maple forest, but the soil βG, NAG, and AP activities did not change in yellow birch, oak, hemlock, and beech forests, when NH_4NO_3 was added (Weand et al., 2010). In subtropical and tropical forests, the βG, NAG, and AP activities increased, and oxidase (PPO and PER) activities decreased, after NH_4NO_3 additions (Dong et al., 2015; Guo et al., 2011; Cusack et al., 2011). To date, we are still not sure if ammonium and nitrate additions have different effects on the soil microbial biomass of different communities and on enzyme activities. To support improved predictions of the effects of elevated N

deposition on C, N, and P cycling in soil, we therefore need to evaluate the individual effects of ammonium and nitrate additions on the soil microbial biomass of different communities and enzyme activities.

The N-rich subtropical soils in southern China have experienced increased nitrate deposition in the recent past. To facilitate an exploration of the different effects of ammonium and nitrate additions on soil microbial communities and enzyme activities, we established a long-term ammonium and nitrate trial in a slash pine (*Pinus elliottii*) plantation in a subtropical area. We hypothesized that (1) ammonium would have stronger inhibitory effects on total PLFA, fungi PLFA contents, and enzyme activities than nitrate because of its strong negative effect on soil pH; and (2) that ammonium and nitrate additions would result in increased C- and P-hydrolase activities, and decreased N-hydrolase activities in line with the economic theory; and (3) that oxidase activities would be restricted due to their inhibitory effects on fungi.

2. Materials and methods

2.1. Study site

The study was conducted in the Qianyanzhou Experimental Station, in the hilly red soil region of Taihe County, Jiang Xi Province, China (26°44′29.1″N, 115°03′29.2″E, 102 m above sea level). The region has a subtropical monsoon climate, a mean annual temperature of 17.9 °C, and a mean annual precipitation of 1475 mm. The soil formed because of weathering of red sandstone and mudstone, and, based on the US soil taxonomy (Soil Survey Staff, 2010), is classified as a Typical Dystrudepts Udepts Inceptisol. The slash pine (*Pinus elliottii*), one of the dominant species in this hilly red soil region, was planted in 1985 under a vegetation restoration program. *Woodwardia japonica*, *Dicranopteris dichotoma* and *Loropetalum chinense* dominate the understory (Kou et al., 2015).

2.2. Experimental design

As described by Kou et al. (2015), the plots were established in November 2011 using a randomized complete block design. Background atmospheric wet N deposition of about 33 kg N ha^{-1} yr^{-1} comprises 11 kg N ha^{-1} yr^{-1} as ammonium and 8 kg N ha^{-1} yr^{-1} as nitrate (Zhu et al., 2014). Nine 20 × 20 m plots

were established at the experimental sites, including a control, ammonium only and nitrate only treatments with three replicates (3 treatments × 3 replicates). We equally added two types of N to the test plots, i.e. ammonium ($N_{ammonium}$) as ammonium chloride (NH_4Cl) and nitrate ($N_{nitrate}$) as sodium nitrate ($NaNO_3$), at an annual rate of 40 kg N ha^{-1} yr^{-1}. This rate was about double the background N wet deposition. The plots had slope angles of less than 15° and were separated by buffer zones of more than 10 m. The NH_4Cl or $NaNO_3$ were dissolved in 30 L of tap water and evenly sprayed onto the plots once a month, i.e. 12 times per year. The equivalent amount of tap water was sprayed onto the control plots. Nitrogen additions commenced in May 2012 and were applied each month on non-rainy days until March 2015. A total of 113 kg N ha^{-1} was applied over the course of this study.

2.3. Sampling and analysis

We collected soil samples in March, June, and October of 2015, to represent spring, summer, and fall. We removed the surface litter, and extracted soil cores with a diameter of 5 cm from between 0 and 10 cm deep from 5 randomly selected locations in each plot, which we then mixed together as one composite sample. The atmospheric conditions and plant-derived litters differed between the three seasons, and so indirectly affected the soil microbial biomass and enzyme activities of different communities. We collected soils from three seasons so that we could investigate the seasonal responses of soil microbial biomass and enzyme activities to ammonium and nitrate additions and to obtain improved information to support predictions of the effects of elevated N depositions on C, N, and P cycling. Field-fresh samples were sieved through a 2 mm mesh after being mixed evenly. Samples were stored at 4 °C until analysis for PLFA biomarkers, enzyme activities, soil pH, ammonium, nitrate, and soil dissolved organic carbon (DOC). The PLFA biomarker and enzyme activity assays were performed on return to the laboratory. Subsamples of each soil were air-dried, and then sieved through a 0.25 mm mesh before soil organic C (SOC) and total N (TN) concentrations were determined.

The measurement of soil chemical properties was followed the method of Bao (2010). Soil pH was measured in a soil-water suspension by glass electrode at a soil to water ratio of 1g fresh soil:2.5 volume of water. Soil water contents (SWC) were measured by the oven drying method (105 °C). After extraction with 1mol L^{-1} KCl, the ammonium and nitrate concentrations in the fresh soils were measured by a continuous flow auto-analyzer (Bran Lubbe, AA3, Germany). Soil DOC was extracted with distilled

water at a ratio of 1 g soil : 5 ml water, and was measured with an organic element analyzer (Liquid TOC*II,* Elementar, Germany). Soil TN and SOC were measured with a carbon/nitrogen analyzer (Vario Max, Elementar, Germany).

Phospholipid fatty acid (PLFA) biomarkers were measured as outlined by Bossio and Scow (1998). In brief, field-fresh soil equal to 8 g of dry soil was subjected to mild alkaline methanolusis to form fatty acid methyl esters (FAMEs). The extracted PLFAs were dissolved in hexane and measured by gas chromatography (Agilent 6890N) with MIDI peak identification software (version 4.5; MIDI Inc. Newark, DE) and a DB-5 column. The abundances of the PLFA biomarkers were calculated as nmol PLFA g^{-1} dry soil. The total amounts of the different PLFA biomarkers were used to represent different 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). Bacterial biomass was 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 hydrolases (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), and have provided information about their corresponding substrates and functions in Table S1. In brief, 1 g of field-fresh soil was homogenized in a 50 mmol L^{-1} sodium acetate buffer (125 ml). We then added 200 µl of homogenate and 50 µl of substrate to black microplates with 96 wells with eight replicates for each soil sample. The microplates were then incubated at 20 ℃ for 4 h. After incubation, 10 µl of 1 mol L^{-1} NaOH was added to each well to terminate the reactions, and fluorescence values were measured at an excitation of 365 nm and emission of 450 nm with a microplate fluorometer (Synergy H4, BioTek). The absolute hydrolase activities were expressed in units of nmol g^{-1} soil h^{-1}. We compared the stoichiometry of C and P to N-acquisition enzyme activities by ln(αG+βG+CBH+βX) and lnaP to lnNAG, respectively (n=27).

Two oxidases, i.e. PER and PPO, were measured using 96-well transparent microplates as outlined by Saiya-Cork et al. (2002). We added 600 µl of homogenate and 150 µl of substrate to deep microplates with 96 wells. To measure the PER activities, we added 10 µl of 0.3% H_2O_2 to the homogenate and substrates mixtures. After incubation at 20 ℃ for 5 h, the microplates were centrifuged at 3000 r for 3

minutes, then 250 μl of liquid supernatant was transferred to a 96-well transparent microplate. The absorbance values were measured at 460 nm by microplate spectrophotometer (Synergy H4, BioTek). We calculated the specific activities of the enzymes by dividing the enzyme activities by the PLFA values to normalize the activity to the size of the microbial active biomass (Cusack et al. 2011).

2.4. Statistical analyses

We used a two factor randomized block analysis of variance and Duncan's multiple comparisons to test the differences between the treatments and sampling time (n=9). To evaluate the effects of ammonium and nitrate additions, the treatment differences of time-dependent indexes were tested by one-way analysis of variance (ANOVA) and Duncan's multiple comparisons for each sampling event or season (n=3). Analyses were performed with SPSS 17.0. Relationships among the soil physical and chemical properties, soil PLFA biomarker contents, and the soil enzyme activities were tested by redundancy analysis (RDA) in CANOCO 4.5 (n=27). Results were statistically significant when $P < 0.05$. The figures were plotted in Sigmaplot 10.0.

3. Results

3.1. Soil physical and chemical properties

The soil pH and ammonium contents were either treatment- or time-independent. There were interaction effects between the treatments and the sampling time on the soil DOC and nitrate contents ($P<0.01$, Table 1). The soil pH decreased by 0.7 of a unit across the three sampling events in the ammonium-treated plots, but did not change significantly in the nitrate-treated plots (Fig. 1a). The soil nitrate contents were 165% and 129% higher (Fig. 2b), and the soil ammonium contents were 31% and 38% lower in the ammonium and nitrate treatments (Fig. 1b) than in the control for the three sampling events. Compared with the control, the soil DOC concentrations were 17% higher in the nitrate-treated plots across the three sampling events, but did not change significantly in the ammonium-treated plots (Fig. 2a). Ammonium contents were higher in March than in June and October (Table S2), while DOC and nitrate concentrations were highest in October and lowest in March (Fig. 2a,b).

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242 **3.2. Soil microbial biomass of different communities**

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244 Both the treatment and the time of sampling significantly influenced the soil microbial biomass of the
245 different communities ($P<0.01$). Total PLFAs, bacteria, G^-, and G^+/G^- were either treatment- or time-
246 independent. There were also interaction effects between treatments and sampling time on fungi,
247 actinomycetes, G^+, AMF, SAP, and the fungi/bacteria ratio (Table 1). The inhibition effects of ammonium
248 additions on total PLFA contents were stronger than those of nitrate additions and the total PLFA contents
249 were 24% and 11% less in the ammonium- and nitrate-treated plots across the three sampling events than
250 in the control (Fig. 3a). The PLFA contents of G^+, AMF, bacteria, fungi and actinomycetes were between
251 14% and 40%, and 7% and 24%, lower in the plots treated with ammonium and nitrate, respectively, than
252 in the control across the three sampling events (Fig. 3b,c and Fig. 4a,b,c,d,e). The soil PLFA contents
253 also showed seasonal variation (Table 1). Total PLFA biomarker contents and bacterium, fungi, G^+, G^-,
254 AMF, and SAP PLFA biomarker contents were highest in March and lowest in October, while
255 actinomycete PLFA biomarker contents were highest in June and lowest in October (Fig. 4a,b,c,d,e, Table
256 S2).

257 The microbial communitieswas dominated by G^+ in the ammonium-treated plots, meaning that the
258 G^+/G^- ratios were higher in the ammonium-treated plots than in the control or nitrate-treated plots (Fig.
259 3d). The fungi/bacteria ratios were lower in both the ammonium- and nitrate-treated plots than in the
260 control, but were much lower in the nitrate-treated plots than in the ammonium-treated plots (Fig. 4f).

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262 **3.3. Soil enzyme activities**

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264 There were significant influences from both treatment and sampling time on the measured absolute
265 enzyme activities ($P<0.01$). Activities of βG, AP, and PPO were either treatment- or time-independent,
266 and there were interaction effects between the treatments and sampling time on activities of αG, βX,
267 CBH, NAG, and PER (Table 1). Ammonium and nitrate had similar inhibition effects on αG, βG, βX,
268 CBH, NAG, PPO, and PER activities, which decreased by between 6% and 50% across the three
269 sampling events. The AP absolute activities were about 9% lower in the nitrate treatment than in the
270 ammonium treatment (Table 2). When compared to control, the ratios of C to N-acquisition enzyme

activities were about 0.2 higher, the ratios of N to P acquisition enzyme activities were about 0.1 lower, and there were no obvious differences in the ratios of C to P acquisition enzyme activities in the ammonium and nitrate treatments. The measured enzyme activities varied seasonally (Table 2). Activities of βG, βX, CBH, NAG, AP, and PPO were lowest in March and highest in October; αG activities were highest in March and lowest in June, and PER activities were highest in March and lowest in October (Table 2).

The treatments had a significant influence on the activities of N- and P-acquisition specific enzymes ($P<0.01$), but not on the activities of C and oxidase specific enzymes (Table 1). The inhibitory effects of nitrate on the activities of N-acquisition specific enzymes were stronger (about 43%) than those of ammonium (about 21%, Fig.5a). When compared with the control, the AP specific activities were about 19% higher in the ammonium-treated plots across the three sampling events (Fig. 5b).

3.4. Redundancy analyses

The results of RDA between soil properties and absolute enzyme activities showed that the first axis explained 72.0% of the variability (Fig. 6a), while the results of RDA between soil properties and microbial community structures showed that the first axis explained 67.5% of the variability (Fig. 6b). The RD1 for soil absolute enzyme activities and PLFA biomarkers was correlated with DOC/SOC, DOC, ammonium, and SOC. However, nitrate was only correlated with the RD1 of the absolute enzyme activities but not the PLFA biomarker contents (Fig. 6 a, b). Most of the measured absolute soil enzyme activities and the PLFA biomarker contents were positively correlated with soil pH, but G^+/G^- and F/B were negatively correlated with soil pH. Ammonium and DOC contents were positively correlated with all the soil absolute enzyme activities except PER, but were negatively correlated with PLFA biomarker contents. Nitrate contents were negatively correlated with soil absolute enzyme activities, but were barely correlated with the PLFA biomarker contents. SWC were positively correlated with soil PLFA biomarker contents, but were not correlated with the absolute enzyme activities (Fig. 6 a, b).

4. Discussion

Our results agree with our first hypothesis and show that the inhibition effects on soil PLFA contents of

bacteria, fungi, and actinomycetes across the three sampling events or seasons were stronger when ammonium was added than when nitrate was added (Fig. 3b and Fig. 4a.b, Table 1). Results from RDA suggest that acidification because of the ammonium additions triggered the decrease in the microbial biomarkers-PLFA contents (Fig. 6b). Soil microbial biomass may be inhibited by resource availability and acidification (Sinsabaugh et al., 2014; Moorhead et al., 2006). However, C and N availability either increased or stayed the same over the three sampling events when ammonium and nitrate were added (Fig. 1b and Fig. 2a,b). Ammonium additions may aggravate nitrification in subtropical soils (Tang et al. 2016), and nitrification may be toxic to microorganisms (Dail et al., 2001), which may then lead to a decrease in the microbial PLFA contents.

The soil pH did not change when nitrate was added (Fig. 1a), which may explain why nitrate had weaker inhibition effects on PLFA biomarker contents than ammonium. Nitrate additions may inhibit the PLFA biomarker contents because of accelerated leaching of Ca^{2+} and Mg^{2+} (Qian et al., 2007), increases in the soil osmotic potential, and activation of Al^{3+} absorbed by soil colloids (Treseder et al., 2008). The PER activity was lower when ammonium and nitrate were added (Table 2), which may eventually result in polyphenol accumulation in soil. Accumulated polyphenol may be toxic to microorganisms (Sinsabaugh et al., 2010) and may have contributed to the decrease in the contents of the PLFA biomarkers. Moreover, the higher soil DOC concentrations observed in the nitrate-addition treatments (Fig. 2a) may be attributed to changes in the diversity of the composition of saprophytic bacteria (Freedman and Zak, 2014; Freedman et al., 2016).

In our study, the fungi /bacteria ratios were lower in the ammonium and nitrate treatments than in the control, which suggests that fungi were more sensitive to N additions than bacteria. In an earlier study, we found that the fine root biomass decreased after N additions (Kou et al., 2015), which suggests that N might upset the symbiosis between AMF and plants, thereby restricting the AMF-PLFA contents.

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

plots than in untreated plots (Allison et al., 2005).

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

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

The N treatments also varied significantly on a seasonal basis and there were interaction effects between N treatments and seasons on the contents of some PLFA biomarkers and enzyme activities (Table 2). Climate conditions, plant growth, the amount of litter returned, and plant-microorganism competitive relationship varied across the three seasons. The temperature ranged from 13.5 to 27.6 °C, and precipitation ranged from 88.2 to 176.6 mm, across the three seasons (Fig. S1), and did not limit the growth of microorganisms. The positive relationships between PLFA biomarker contents and soil moisture contents indicate that soil moisture had a strong influence on soil microbial community biomass. There may be interaction effects between plant growth, the mass and quality of litter, plant-microbe competition, and soil nutrient dynamics. For example, compared with the control plots, the soil DOC contents were lower, and soil nitrate contents stayed the same in June (the growing season) in the ammonium treatment, but the soil DOC and nitrate contents were higher in the ammonium and nitrate treatments in March and October (non-growing season, Fig. 2a). This indicates that there was stronger competition between plants and microbes for available C and N in June than in March and October, and that there were interaction effects between plants and microbes on soil C and N availability. This might explain the interaction effects between N additions and seasons on the activities of C and N-acquisition

enzymes. The effects of interactions between N additions and season on the AMF PLFA contents, along with available C and N dynamics, may result from plant growth as plant-AMF symbiotic systems may be influenced by fine root biomass.

5. Conclusions

The results showed that soil bacteria, fungi, and actinomycetes- PLFA biomarker contents decreased after ammonium and nitrate additions. Ammonium inhibited the biomass of different soil microbial communities except SAP more strongly than nitrate, perhaps because of acidification caused by ammonium. The microbial communities were dominated by G^+ and bacteria after ammonium additions, and were dominated by bacteria under nitrate additions.

The absolute activities of C, N, and P-acquisition hydrolases and oxidase decreased after additions of ammonium and nitrate, and nitrate had a stronger inhibition effects on P-acquisition absolute enzyme activities than ammonium. However, ammonium improved the P-demand per unit of microbial biomass. C and P-acquisition absolute enzyme activities were higher than N-acquisition absolute enzyme activities under ammonium and nitrate additions. Because of the positive correlation between the measured absolute enzyme activities and soil pH, the decreases in the absolute hydrolase and oxidase activities reflected abiotic restrictions, i.e. acidification and nitrification caused by ammonium additions, rather than biotic restrictions.

Ammonium and nitrate additions had a range of effects on soil microbial communities and the activities of specific enzymes. Our results show that the effects of ammonium and nitrate need to be discussed separately to provide the information that we need to predict the effects of elevated N deposition on soil microbial biomass and enzyme activities.

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

Competing interests: The authors declare no conflict of interest.

391 *Acknowledgments*

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393 This study was jointly financed by the General, State Key and Major Programs of National Natural
394 Science Foundation of China (Nos. 41571251, 41571130043, 31130009)

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524 **Figure Legends**

525

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

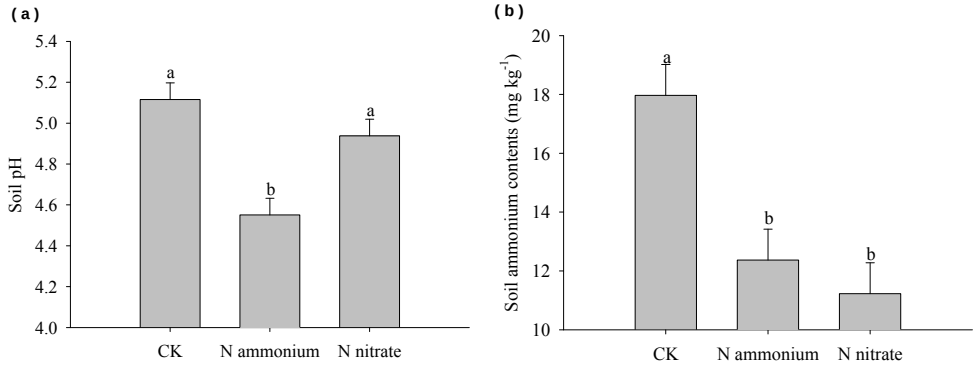
529 **Fig. 2.** The effects of ammonium and nitrate additions on soil nitrate and soil dissolved organic carbon
530 contents for each sampling event. Capital letters represent significant differences between the treatments
531 (P <0.05), and small letters represent significant differences between the sampling events (P <0.05), error
532 bars represent means ± standard errors (n=3).

533 **Fig. 3.** The effects of ammonium and nitrate additions on Total PLFAs, PLFA contents of bacteria, G^-
534 and G^+/G^-. Small letters represent significant differences between treatments (P <0.05), error bars
535 represent means ± standard errors (n=9). The abbreviations are the same as Table 1.

536 **Fig. 4.** The effects of ammonium and nitrate additions on PLFA contents of fungi, actinomycetes, AMF,
537 SAP, G^+, and fungi/bacteria ratio for each sampling event. Capital letters represent significant differences
538 between the treatments (P <0.05), and small letters represent significant differences between the
539 sampling time (P <0.05), error bars represent means ± standard errors (n=3). The abbreviations are the
540 same as Table 1.

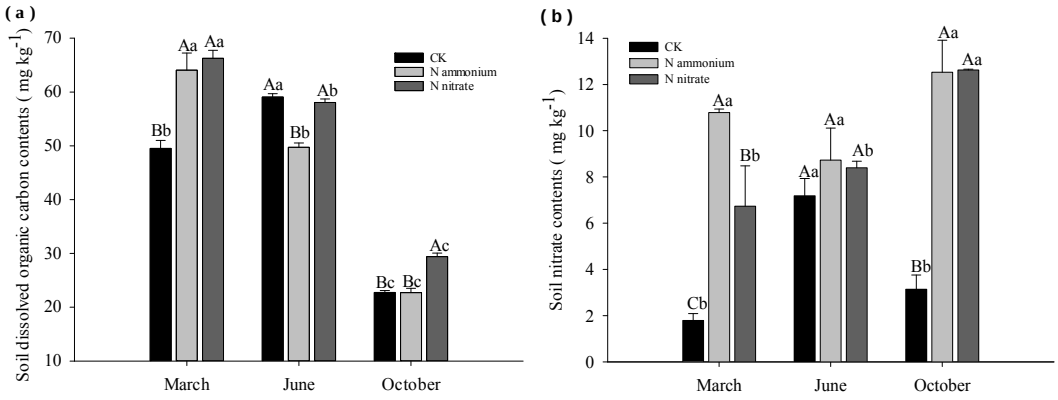
541 **Fig. 5.** The effects of ammonium and nitrate additions on N, P-acquisition specific enzyme activities for
542 each sampling event. Capital letters represent significant differences between the treatments (P <0.05),
543 and small letters represent significant differences between the sampling time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

545 **Fig. 6.** Redundancy analyses between (a) soil properties and enzyme activities, and (b) soil properties
546 and PLFA-biomarker contents. The abbreviations are the same as Table 1. SOC: soil organic matter; TN:
547 total nitrogen; C/N: the ratio of soil organic matter to total nitrogen; SWC: soil water contents.


(a)
Soil pH
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
a
b
a
CK
N ammonium
N nitrate
(b)
Soil ammonium contents (mg kg-1)
20
18
16
14
12
10
a
b
b
CK
N ammonium
N nitrate

548

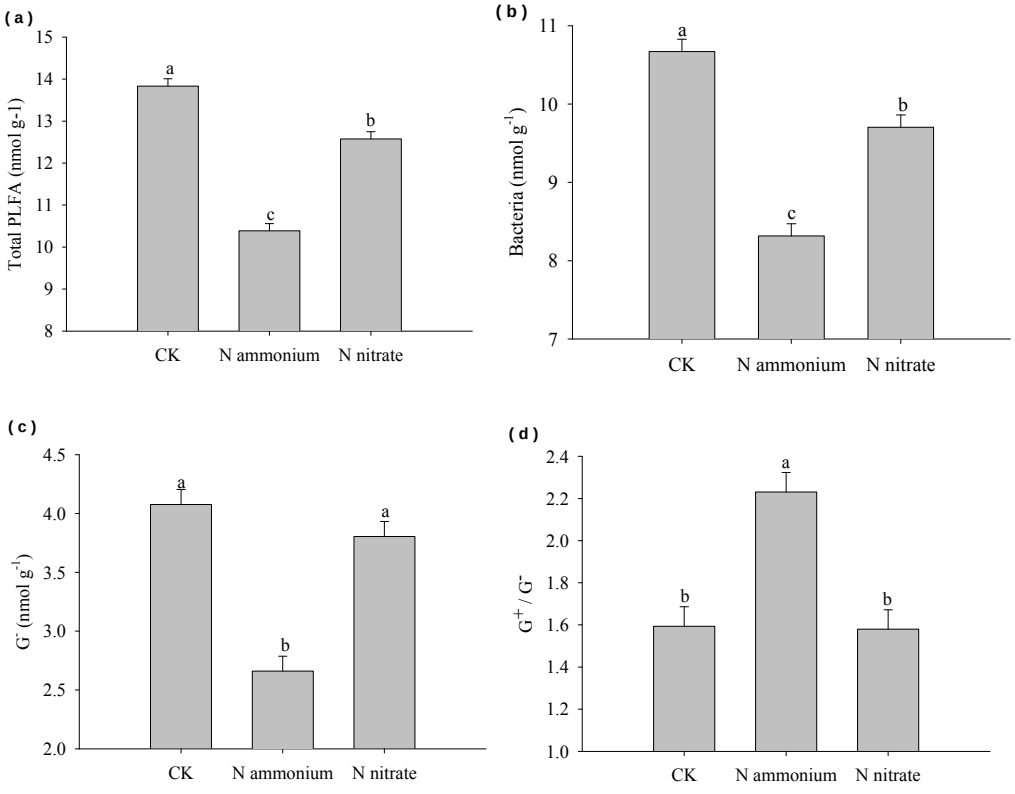
549 **Fig.1**


(a)
Soil dissolved organic carbon contents (mg kg-1)
70
60
50
40
30
20
10
Aa
Aa
Aa
Ab
Bb
Bb
Bc
Bc
Ac
CK
N ammonium
N nitrate
March
June
October
(b)
Soil nitrate contents (mg kg-1)
14
12
10
8
6
4
2
0
Cb
Aa
Bb
Aa
Aa
Ab
Bb
Aa
Aa
CK
N ammonium
N nitrate
March
June
October

550

551 **Fig.2**

552



553

554 **Fig.3**

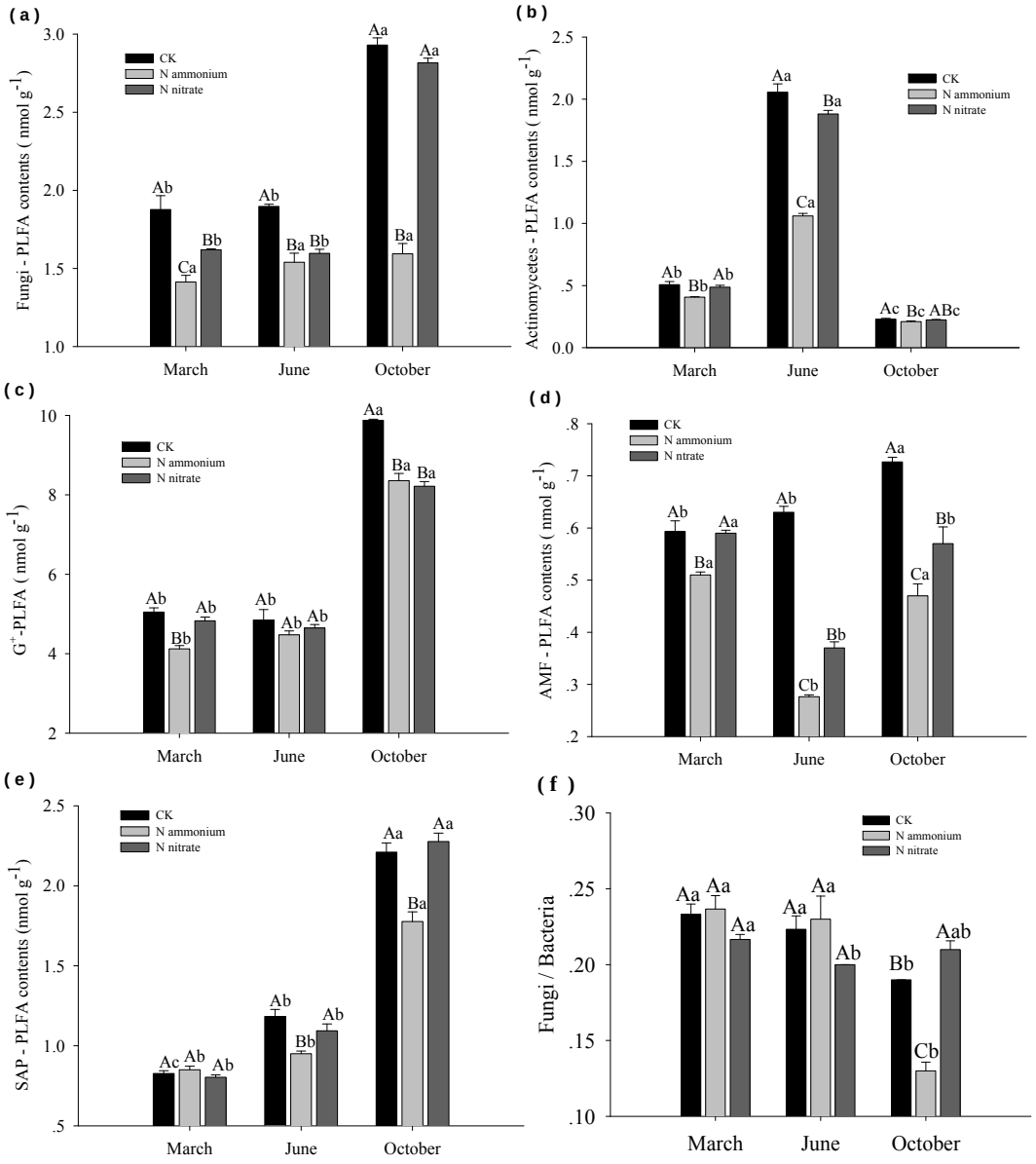
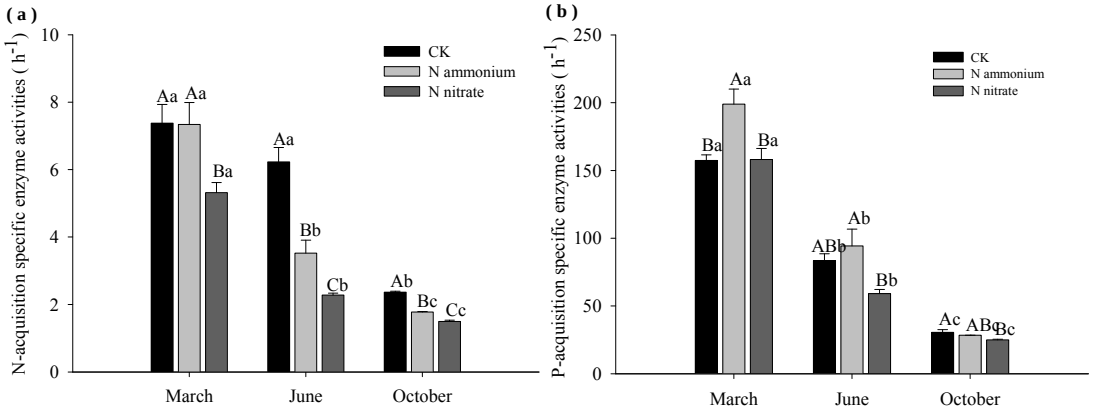
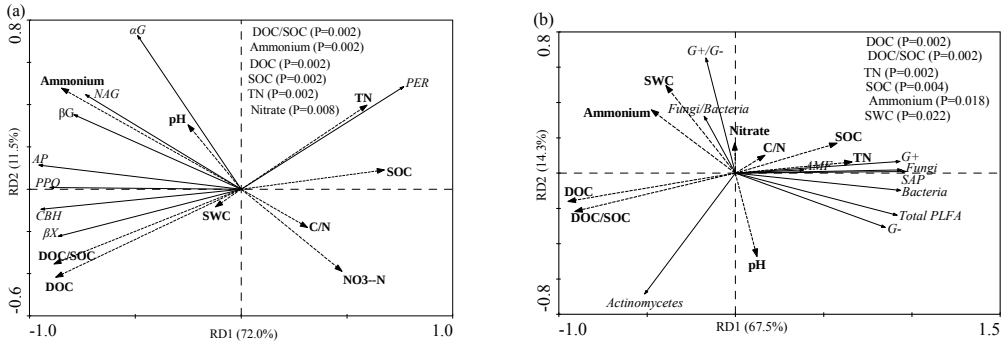

(a)
Fungi - PLFA contents (nmol g-1)
CK
N ammonium
N nitrate
March
June
October
(b)
Actinomycetes - PLFA contents (nmol g-1)
(c)
G+ -PLFA (nmol g-1)
(d)
AMF - PLFA contents (nmol g-1)
N ntrate
(e)
SAP - PLFA contents (nmol g-1)
(f)
Fungi / Bacteria

Fig. 4


(a)
N-acquisition specific enzyme activities (h-1)
CK
N ammonium
N nitrate
Aa Aa Ba
Aa Bb Cb
Ab Bc Cc
March
June
October
(b)
P-acquisition specific enzyme activities (h-1)
CK
N ammonium
N nitrate
Ba Aa Ba
ABb Ab Bb
Ac ABc Bc
March
June
October

559
560 **Fig.5**


(a)
αG
DOC/SOC (P=0.002)
Ammonium (P=0.002)
DOC (P=0.002)
SOC (P=0.002)
TN (P=0.002)
Nitrate (P=0.008)
Ammonium
NAG
βG
pH
TN
PER
AP
SOC
PPO
CBH
SWC
βX
C/N
DOC/SOC
NO3--N
DOC
0.8
-0.6
RD2 (11.5%)
-1.0
RD1 (72.0%)
1.0
(b)
G+/G-
DOC (P=0.002)
DOC/SOC (P=0.002)
TN (P=0.002)
SOC (P=0.004)
Ammonium (P=0.018)
SWC (P=0.022)
SWC
Fungi/Bacteria
Ammonium
Nitrate
SOC
C/N
TN
G+
AMF
Fungi
SAP
DOC
Bacteria
DOC/SOC
Total PLFA
G-
pH
Actinomycetes
0.8
-0.8
RD2 (14.3%)
-1.0
RD1 (67.5%)
1.5

561

562 **Fig. 6**

563 **Table 1** Summary statistics (F ratio) for the two factor randomized block analysis of variance (ANOVA) applied

564 to soil variables, enzyme activities and PLFA biomarkers. The bold numbers are significant ($P < 0.05$).

Factors (Abbreviation)	Treatments	Months	Treatments × Months
Soil acidity(pH)	**12.43**	0.31	0.09
Soil dissolved organic carbon (DOC)	**23.53**	**561.25**	**20.11**
Nitrate	**43.19**	**7.96**	**8.21**
Ammonium	**11.84**	**65.46**	0.42
Total phospholipid fatty acid (TPLFA)	**102.51**	**477.77**	2.68
Bacteria	**56.94**	**555.14**	2.73
Fungi	**180.49**	**277.81**	**52.16**
Actinomycetes	**172.230**	**2627.61**	**123.12**
Gram positive bacteria (G^+)	**50.30**	**1221.19**	**14.39**
Gram negative bacteria (G^-)	**34.33**	**105.59**	0.45
Arbuscular mycorrhizal fungi (AMF)	**147.77**	**83.55**	**21.64**
Saprophytic fungi (SAP)	**24.70**	**781.67**	**13.08**
G^+/G^-	**16.24**	2.38	0.94
Fungi/Bacteria	**3.82**	**56.42**	**21.67**
α-1,4-glucosidase (αG)	**30.24**	**53.17**	**3.47**
β-1,4-glucosidase (βG)	**3.26**	**72.90**	0.58
β-1,4-xylosidase (βX)	**9.86**	**79.08**	**3.86**
Cellobiohydrolase (CBH)	**28.51**	**194.75**	**4.39**
β-1,4-N- acetylglucosaminidase (NAG)	**100.42**	**67.49**	**8.47**
Acid phosphatase (AP)	**22.81**	**467.77**	1.73
Peroxidase (PPO)	**6.87**	**64.40**	1.98
Phenol oxidase (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

565

Table 2 Summary statistics (means ± standard errors, n=3) for one way analyses of variance (ANOVA) and Duncan multiple comparisons applied to soil absolute enzyme activities.

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

Note: Capital letters represent significant differences between the treatments (P <0.05), and small letters represent significant differences between the sampling events (P <0.05). The abbreviations are the same as Table 1.