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ORIGINAL PAPER

Enhanced deposition of nitrate alters microbial cycling of N in a subtropical forest soil

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Abstract In China, atmospheric deposition of NO_3^- is increasing rapidly. However, information on how microbial N cycling in forest soils may respond to increasing deposition of NO_3^- is currently lacking. Determination of process- and pool-specific N transformation rates can provide additional insights into the controls on the production and consumption of inorganic N, and microbial function. Here, we present results from a laboratory ^{15}N tracing study with a soil (0–10 cm) from a subtropical forest receiving fertilization for more than 2.5 years at a rate of 0, 40, and 120 kg NO_3^-–N ha^{-1} $year^{-1}$. The process- and pool-specific N transformation rates were quantified with a ^{15}N tracing model. The directions of changes in microbial mineralization of labile and recalcitrant organic N were opposite under increased NO_3^- additions. Microbial mineralization of labile organic N first decreased, then increased, while microbial mineralization of recalcitrant organic N showed the opposite in response to increasing NO_3^- additions. Ammonium immobilization into labile and recalcitrant organic N was not changed by increased NO_3^- additions. Nitrate additions did not affect heterotrophic and gross nitrification, but stimulated autotrophic nitrification. Nitrate immobilization decreased under increased NO_3^- additions, with a greater reduction under low NO_3^- addition treatment compared to high NO_3^- addition treatment. Overall, our results reflect a contrasting change in microbial mineralization of liable and recalcitrant organic N under increased NO_3^- additions, and a contrasting effect of low and high NO_3^- additions on microbial mineralization of liable and recalcitrant organic N. It also has implications for our understanding of NO_3^- deposition-induced nonlinear changes in net production and loss of NO_3^- in subtropical/tropical forest soils.

Keywords N deposition · Gross N transformations · ^{15}N tracing · Model · Subtropical forest

Introduction

In China, nitrate (NO_3^-) deposition is increasing rapidly, although NH_4^+ is currently the dominant form of N in bulk deposition (Liu et al. 2013). Enhanced deposition of NO_3^- may fundamentally change microbial cycling of N in forest soils. In vegetated soils, additions of NO_3^- can stimulate more root-derived respiration (Gavrichkova and Kuzyakov 2008) and soil CO_2 flux (Wang et al. 2015) than NH_4^+, partly because the reduction of NO_3^- to NH_4^+ prior to assimilation requires oxidation of carbohydrates (Aslam 1982; Ninomiya and Sato 1984). More recently, NO_3^- has been shown as an inhibitor of several fungal lignolytic enzyme production and activity, e.g., fungal phenol oxidases and peroxidases of white-rot basidiomycetes (Ekberg et al. 2007; Kaiser et al. 2010; Waldrop and Zak 2006).

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The soil internal N cycling exerts a strong control over N availability, ultimately affecting net primary productivity, plant growth, microbial function, and N loss of an ecosystem (Schimel and Bennett 2004). Many factors influence the rates of soil N cycling, including soil moisture, soil pH, soil organic matter (SOM), and land use and management (Cheng et al. 2014; Gao et al. 2015; Lang et al. 2016). Soil organic matter is the largest soil N pool (Huygens et al. 2008), and its composition and concentration influence the production and fate of mineral N (Booth et al. 2005; Laungani and Knops 2012). Microbial communities play key roles in decomposition and utilization of organic substrates (Nannipieri et al. 2003). Fungi, compared to bacteria, are more efficient decomposers of recalcitrant SOM (Hodge et al. 2000), and gram-negative bacteria are typically linked to the degradation and utilization of protein and chitin (liable, N-contained compounds) (Koranda et al. 2014).

Global change drivers such as N deposition are likely to feed back on forest soil N cycling through the effects on SOM composition and concentration, and microbial activity, biomass, and composition. In the short term, low additions of N to forest soils generally stimulated fungal activity and growth, decomposition of low N-wood substrates, and microbial respiration (Allison et al. 2009; Koranda et al. 2014), while it reduced protein- and chitin-degrading enzyme activities and the abundance of gram-negative bacteria (Allison et al. 2008; Koranda et al. 2014), and resulted in a depletion of labile SOM pool (Cusack et al. 2011). These observations indicate that at low N deposition condition, a shift of N mineralization from fast to slow turnover pools might occur. However, a shift towards fast turnover organic N pools may occur at excessive N deposition, due to either enhanced formation of recalcitrant compounds that are resistant to microbial decay, shifts in microbial enzyme synthesis, and activity towards preferential decomposition of labile and energy-rich compounds, or both (Janssens et al. 2010; Maaroufi et al. 2015). Detailed investigations of these responses are central to understanding how forest ecosystems may maintain their functional stability under anthropogenic N inputs, but are currently lacking.

Despite presence of widespread evidence for N addition-induced nonlinear increase in net production and loss of nitrate (NO_3^-) from soils, it remains uncertain to what extent this increase results from stimulation of either autotrophic or heterotrophic nitrifiers, or from the saturation of N uptake by plants, heterotrophic microbes, mycorrhizae, and abiotic reactions (Perakis et al. 2005). In acid soils of subtropical/tropical forests, heterotrophic nitrification dominates over autotrophic nitrification in NO_3^- production, and NO_3^- immobilization is an effective N retention mechanism (Zhang et al. 2013; Zhu et al. 2013). In these soils, microbial assimilation of NO_3^- depends likely on heterotrophic nitrification for substrate generation, and both processes are functionally linked (Huygens et al. 2008; Zhang et al. 2013; Zhu et al. 2013). Acidic forest soils with high soil C/N ratio and high fungal biomass generally show a high rate of heterotrophic nitrification (Zhang et al. 2015; Zhu et al. 2013). There is increasing evidence of stimulated effects of low N additions on fungal growth or activity (Koranda et al. 2014; Rousk and Baath 2007), suggesting that there would be an increase in heterotrophic nitrification rates (Zhu et al. 2013). However, soil C/N ratio (Corre et al. 2010; Templer et al. 2012) and fungal biomass (Demoling et al. 2008; Frey et al. 2004, 2014) decreased at forest sites receiving long-term or high N deposition, which might inhibit heterotrophic nitrification and also NO_3^- immobilization.

In this paper, we report results from a ^{15}N tracing study with soil fertilized for more than 2.5 years at a rate of 0, 40, and 120 kg NO_3^-–N ha^{-1} $year^{-1}$, respectively, in a slash pine (*Pinus elliottii*) forest of subtropical China. Our objective was to determine if and how enhanced deposition of NO_3^- would affect microbial cycling of N in the soil. We hypothesized that (1) mineralization of slow turnover organic N would increase, and mineralization of fast turnover organic N would decrease under low NO_3^- additions, and vise versa under high NO_3^- additions; (2) heterotrophic nitrification, would first increase, then decrease with increasing NO_3^- additions; and (3) NO_3^- immobilization would show a similar change as heterotrophic nitrification in response to increasing NO_3^- additions. To test our hypotheses, process- and pool-specific N transformation rates were quantified via a process-based ^{15}N model (Müller et al. 2007; Rütting and Müller 2007).

Materials and methods

Site description, experimental design, and sampling

The study site is a 28-year-old subtropical slash pine (*P. elliottii*) forest at Qianyanzhou (QYZ) Red Soil Hilly Station, Jiangxi, Southern China (115° 03′ 29.2″ E, 26° 44′ 29.1″ N, 102 m a.s.l.). The mean annual temperature is 17.9 °C, and the mean annual precipitation is 1475 mm (Wen et al. 2010). The understory is dominated by *Woodwardia japonica*, *Loropetalum chinense*, and *Dicranopteris dichotoma* (Wang et al. 2012). The soils are classified as Typic Dystrudepts (USDA Soil Taxonomy). Annual precipitation deposition of dissolved inorganic N (DIN) equals 12.6 ± 1.5 kg N hm^{-2} a^{-1}, with a NH_4^+–N/NO_3^-–N ratio of 1.9:1 (Zhan et al. 2014).

To investigate the influence of atmospheric NO_3^- deposition on microbial N cycling in the soil, we established a simulated NO_3^- deposition experiment. Starting in May 2012, we fertilized 20 m × 20 m plots with 0, 40, and 120 kg N ha^{-1} $year^{-1}$ in a randomized block design

(three replicates per treatment, totaling 9 plots). Nitrogen was added as sodium nitrate ($NaNO_3$). The three blocks were laid out across 40 ha, and there were at least 10-m buffer zones between plots within each block. N solutions were sprayed monthly to the forest floors with a backpack sprayer. Each month, fertilizer was weighed and mixed with 30-L water for each plot, and each control plot received 30 L of water without fertilizer.

In late November 2014 (roughly 2.5 years after N additions), 8 subsamples were taken from the 0–10 cm in each plot using a PVC tube (inner diameter of 7 cm; length of 15 cm). Subsamples were pooled together by plot and were not mixed for the same treatment (totaling to nine composited samples). Each composited sample was sieved (2-mm), homogenized, and subsequently divided into two subsamples: one for analysis of soil properties, and another for the laboratory ^{15}N tracing experiments. The tracing experiments were carried out within 2 weeks, and soil samples were stored at 4 °C before analyses.

^{15}N tracing experiment

There were two ^{15}N treatments (each with three replications), of which either ammonium ($^{15}NH_4NO_3$) or nitrate ($NH_4{}^{15}NO_3$) were labeled with ^{15}N at 10 atom percent excess. For each plot, the sieved soil was placed in four sets of conical flasks (six conical flasks per sets, three of the six conical flasks for $^{15}NH_4NO_3$ labeling, and the remaining three for $NH_4{}^{15}NO_3$ labeling; each conical flask containing fresh soil with the equivalent of 20 g of dry soil). These conical flasks were preincubated in the dark for 24 h at 25 °C prior to ^{15}N labeling after sealing with parafilm with five pin holes for gas exchange. $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ was added at a rate of 2.86 μmol N g^{-1} dry soil (20 μg NH_4^+–N g^{-1} dry soil and 20 μg NO_3^-–N g^{-1} dry soil) in 3-ml per conical flask. The conical flask was incubated in the dark for 144 h at 25 °C after adjusting the soil to 60 % water holding capacity (WHC) and sealing with parafilm (with five pin holes for air exchange). Soil extractions were carried out at 0.5, 48, 96, and 144 h after label addition to determine the concentrations and isotopic compositions of NH_4^+ and NO_3^-. A detailed description of laboratory ^{15}N tracing study on each soil sample can be found in Fig. S1 (see supporting information).

Analyses

Soil moisture, soil pH, and contents of total C, total N, exchangeable NH_4^+, and NO_3^- were determined. Soil moisture was measured by drying at 105 °C for 48 h. Total C and N contents were analyzed by an elemental analyzer (Europa Scientific Integra, UK) using air-dried, finely ground soil. Soil pH was determined in a soil (air-dried)/water ratio of 1:2.5 by a DMP-2 mV/pH detector (Quark Ltd., Nanjing, China). Soil exchangeable NH_4^+ and NO_3^- were extracted with 2 M KCl at a soil/extractant ratio of 1:5 after shaking for 60 min at 250 rpm and 25 °C. The concentrations of exchangeable NH_4^+ and NO_3^- were assayed with a continuous-flow analyzer (Skalar, Breda, the Netherlands). The soil properties are presented in Table 1.

The isotopic compositions of exchangeable NH_4^+ and NO_3^- were determined using an automated C–N analyzer coupled to an isotope ratio mass spectrometer (Europa Scientific Integra, UK). For isotopic analysis, NH_4^+ and NO_3^- were separated by distillation with magnesium oxide (MgO) and Devarda's alloy. A detailed description of this method can be found in previous studies (Zhang et al. 2012, 2013).

^{15}N tracing model

Gross rates of N transformations were quantified with a process-based ^{15}N tracing model (Fig. 1) (Müller et al. 2007). The model considered 10 simultaneously occurring gross N transformations: M_{Nrec} mineralization of recalcitrant organic N to NH_4^+, M_{Nlab} mineralization of labile organic N to NH_4^+, I_{NH4_Nrec} immobilization of exchangeable NH_4^+ to recalcitrant organic N, I_{NH4_Nlab} immobilization of exchangeable NH_4^+ to labile organic N, R_{NH4ads} release of adsorbed NH_4^+, A_{NH4} adsorption of NH_4^+ on cation exchange sites, O_{NH4}, oxidation of NH_4^+ to NO_3^- (autotrophic nitrification), O_{Nrec} oxidation of recalcitrant organic N to NO_3^- (heterotrophic nitrification), $I_{NO3\text{-}Nrec}$ immobilization of NO_3^- to recalcitrant organic N, and D_{NO3} dissimilatory NO_3^- reduction to NH_4^+. The transformation rates were calculated either by zero (M_{Nrec} and O_{Nrec}) or first-order (M_{Nlab}, I_{NH4_Nrec}, I_{NH4_Nlab}, O_{NH4}, $I_{NO3\text{-}Nrec}$, and D_{NO3}) kinetics. Based on available N cycling parameters, gross N mineralization ($M_{tot} = M_{Nlab} + M_{Nrec}$), gross nitrification/gross NO_3^- production ($O_{NH4} + O_{Nrec}$),

Table 1 Soil properties at 0–10-cm depth after 2.5 years of NO_3^- additions

N addition	Soil pH 1:2.5 (H_2O)	Exchangeable NH_4^+ mg N kg^{-1} soil	NO_3^- mg N kg^{-1} soil	Soil C g C kg^{-1} soil	Soil N g N kg^{-1} soil	Soil C/N
Control	5.0 ± 0.2	11.69 ± 2.10	8.27 ± 1.04	14.46 ± 0.48	0.91 ± 0.12	16.3 ± 1.9
Low N	4.9 ± 0.0	12.41 ± 0.56	7.31 ± 0.34	16.81 ± 2.76	1.02 ± 0.13	16.4 ± 0.6
High N	5.2 ± 0.2	9.86 ± 1.61	15.74 ± 0.67	19.08 ± 0.42	1.01 ± 0.12	19.3 ± 1.8

Values are means with standard errors (SE; $n = 3$ plots)

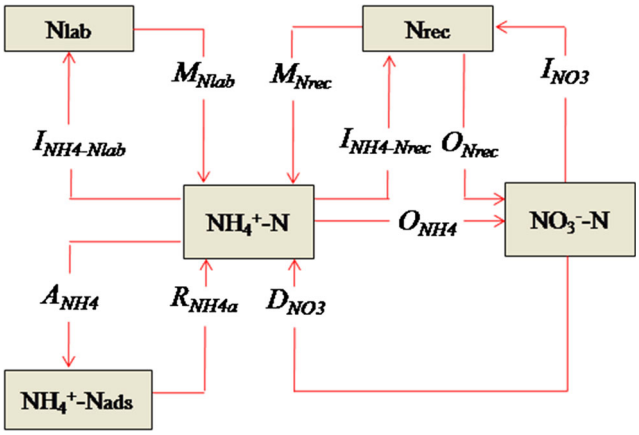

Fig. 1 Conceptual ^{15}N tracing model (Müller et al., 2007). N_{rec} recalcitrant organic N; N_{lab} labile organic N; NH_4^+ ammonium; NO_3^- nitrate; M_{Nrec} mineralization of recalcitrant organic N to NH_4^+; M_{Nlab} mineralization of labile organic N to NH_4^+; I_{NH4_Nrec} immobilization of exchangeable NH_4^+ to recalcitrant organic N; I_{NH4_Nlab} immobilization of exchangeable NH_4^+ to labile organic N; R_{NH4ads} release of adsorbed NH_4^+; A_{NH4} adsorption of NH_4^+ on cation exchange sites; O_{NH4} oxidation of exchangeable NH_4^+ to NO_3^- (autotrophic nitrification); O_{Nrec} oxidation of recalcitrant organic N to NO_3^- (heterotrophic nitrification); I_{NO3_Nrec} immobilization of exchangeable NO_3^- to recalcitrant organic N; and D_{NO3} dissimilatory NO_3^- reduction to NH_4^+

NH_4^+ immobilization ($I_{NH4} = I_{NH4\_Nrec} + I_{NH4\_Nlab}$), and gross NO_3^- immobilization ($I_{NO3} = I_{NO3\_Nrec}$) were calculated. The data supplied to the model were the concentrations (μmol N g^{-1} dry soil) and enrichments (at.% excess ^{15}N) of exchangeable NH_4^+ and NO_3^- in the form of mean ± standard deviations (SD) (Fig. S1). For each plot, the simulated concentrations and isotopic enrichments were generally within the range of the observed averages ± SD (see supporting information, Figs. S2–10).

The model calculated gross N transformation rates by simultaneously optimizing the kinetic parameters for each individual process via minimizing the misfit between modeled and observed concentrations of exchangeable NH_4^+ and NO_3^- and their respective ^{15}N enrichments. To identify the most suitable model that could best simulate the observed data and the measured soil N cycling, several model modifications, which varied in the number of N transformations, kinetic settings of individual processes (zero/first-order kinetics/Michaelise-Menten kinetics) and considered N pools, were tested. The final model (Fig. 1) was identified based on the lowest Aikaike's information criterion (AIC) (Cox et al. 2006). Initially, all parameters (N pools and N transformations) from the conceptual model (Fig. 1) were included in the optimization run and the kinetic settings adjusted to reach an AIC as low as possible. Parameters, which did not significantly improve the model fit, were excluded in the following step when they approached zero in the analysis. In general, zero-order kinetics are more appropriate to describe N transformations (e.g., M_{Nrec} and O_{Nrec}) originating from large pool sizes, while N transformations (M_{Nlab}, I_{NH4_Nrec}, I_{NH4_Nlab}, $I_{NO3\text{-}Nrec}$, and D_{NO3}) originating from small pool sizes are likely to follow first-order kinetics (Myrold and Tiedje 1986). During the incubation, the activity of nitrifying microorganisms may undergo a rapid change from non-NH_4^+ limiting conditions (zero-order kinetics) to NH_4^+ limiting conditions (first-order kinetics) (Müller et al. 2007). In this case, Michaelise-Menten kinetics, rather than zero- or first-order kinetics, could be more appropriate to describe NH_4^+ oxidation. A detailed description of stepwise modification of parameters and their kinetic settings to find the lowest resulting AIC have been reported (Inselsbacher et al. 2013; Müller et al. 2004, 2007; Rütting and Müller 2007). The model parameters were optimized with Markov Chain Monte Carlo Metropolis algorithm (MCMC-MA), which has been described in detail by Müller et al. (2007). To get a better resolution of soil processes, and in line with previous studies, soil organic N pool was conceptually divided into two fractions, a liable (active) and a recalcitrant (slow) pool (Inselsbacher et al. 2013; Müller et al. 2007; Zhang et al. 2013). The initial (i.e., $t = 0$) pool sizes of mineral N (^{14}N and ^{15}N) were estimated based on Müller et al. (2004)). The optimization procedure produced a probability density function (PDF) for each process, from which the average and standard deviation of each process was calculated. For N transformations following first-order kinetics, average gross rates were calculated by integrating the gross rates over the entire experimental period, divided by the incubation time (Inselsbacher et al. 2013; Müller et al. 2007). The gross N transformation rates were expressed in milligrams N per kilogram dry soil per day. The MCMC-MA routine was processed by the software Matlab (Version 7.2, The Math Works Inc.), which considers models separately.

Data analysis

All the data were tested for normality (Shapiro–Wilk test) and homogeneity (Levene test) of variance before analysis. If preconditions of ANOVA were not met, we employed the Kruskal-Wallis H test with paired comparisons for testing differences among treatments. Data that showed normal distribution and homogeneity were tested by using the one-way ANOVA with least significant difference (LSD) test to compare the differences between treatments. Nitrogen dose was set as main effects. All analyses were conducted using SPSS version 20.0 (IBM Co., Armonk, NY, USA), and $\alpha < 0.10$ was considered as the threshold value for significance.

Results

Dynamics of N pool sizes and ^{15}N enrichments

The stimulated and observed concentrations and ^{15}N enrichments of exchangeable NH_4^+ and NO_3^- matched well. For each plot, the simulated concentrations and isotopic enrichments were generally within the range of the observed averages ± SD (Figs. S2–10). Concentrations of exchangeable NH_4^+ and NO_3^- increased with incubation time (Figs. S2–10a, b) The dilution of ^{15}N in the exchangeable NH_4^+ (or NO_3^-) pool when the N pool was labeled indicated an inflow of unlabeled exchangeable NH_4^+ (or NO_3^-) into the labeled N pool. ^{15}N of the exchangeable NH_4^+ pool under $NH_4{}^{15}NO_3$ labeling showed a very small change, suggesting that dissimilatory nitrate reduction to ammonium (DNRA) rates were negligible (Figs. S2–10c). The ^{15}N of the NO_3^- pool showed a slow increase under $^{15}NH_4NO_3$ labeling, indicating a low potential to oxidize exchangeable NH_4^+ to NO_3^- (autotrophic nitrification) (Figs. S2–10d).

Production and consumption of exchangeable NH_4^+

There were treatment effects of NO_3^- additions on gross N mineralization ($M_{tot} = M_{Nlab} + M_{Nrec}$, $p = 0.10$; Fig. 2). M_{tot} in the high N addition plots were 23.7 % lower than in the control plots ($p = 0.04$), while M_{tot} in the low N addition plots did not differ significantly from other plots at $p < 0.10$ (Fig. 2). Exchangeable NH_4^+ production via mineralization of recalcitrant organic N (M_{Nrec}) increased from 1.94 ± 0.12 mg N kg^{-1} soil day^{-1} in the control plots to 2.78 ± 0.24 mg N kg^{-1} soil day^{-1} in the low N addition plots ($p = 0.011$), and then decreased by 58.3 % to 1.16 ± 0.09 mg N kg^{-1} soil day^{-1} in the high N addition plots ($p < 0.001$) (Fig. 3). Exchangeable NH_4^+ production via mineralization of labile organic N (M_{Nlab}) showed the opposite in response to increasing NO_3^- additions (Fig. 3).

The main consumption pathway for the produced exchangeable NH_4^+ was NH_4^+ immobilization rather than autotrophic nitrification (Fig. 2). Gross NH_4^+ immobilization rates (I_{NH4}) did not differ significantly among treatments ($p = 0.382$). Most of the produced exchangeable NH_4^+ was immobilized into labile organic N (59 to 71 %) rather than recalcitrant organic N (29 to 41 %) (Fig. 3), and the respective rate of NH_4^+ immobilization into labile (I_{NH4_Nlab}) and recalcitrant (I_{NH4_Nrec}) organic N did not differ significantly across treatments (Fig. 3).

Production and consumption of NO_3^-

Forty-one to 85 % of the NO_3^- production was derived from oxidation of N_{rec} (O_{Nrec}), and the rest was the result of NH_4^+ oxidation (O_{NH4}) (Fig. 2). Heterotrophic nitrification rates (O_{Nrec}) did not vary significantly among treatments (Fig. 2). Autotrophic nitrification rates (O_{NH4}) increased significantly with increasing NO_3^- additions (Fig. 2). However, gross rates of NO_3^- production ($O_{Nrec} + O_{NH4}$) were similar among treatments ($p = 0.313$). Nitrate immobilization (I_{NO3}) dominated

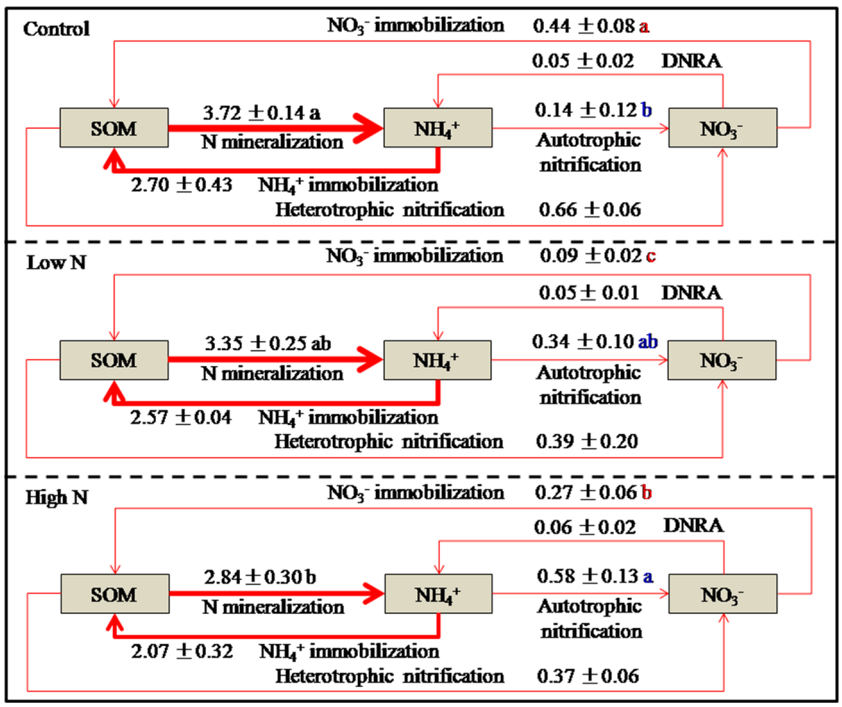

Fig. 2 Gross rates of N cycling in the surface soil (0–10 cm) in response to experimental NO_3^- additions. Rates are means with standard errors (mg N kg^{-1} dry soil day^{-1}; $n = 3$ plots), and see Fig. 1 for definition of abbreviations. There was no difference detected among treatments for NH_4^+ immobilization, heterotrophic nitrification, and DNRA. For N mineralization, autotrophic nitrification and NO_3^- immobilization, means followed by the same letter (*black*, *blue*, and *red* for N mineralization, autotrophic nitrification and NO_3^- immobilization, respectively) indicated no significance among treatments (one-way ANOVA, least significant difference test at $p < 0.10$)

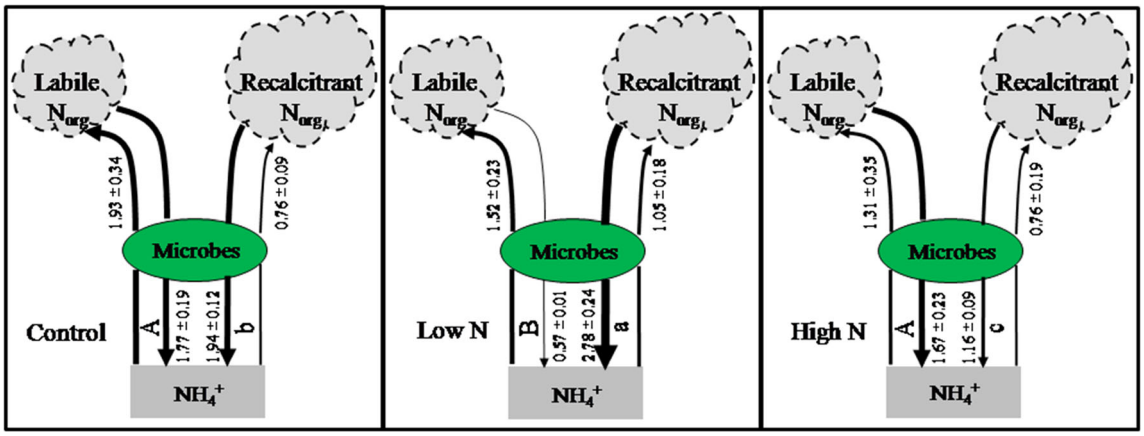

Fig. 3 Mineralization–immobilization turnover in the surface soil (0–10 cm) and its response to experimental NO_3^- additions. Rates are means with standard errors (mg N kg^{-1} dry soil day^{-1}; $n = 3$ plots), and see Fig. 1 for definition of abbreviations. There was no difference detected among treatments for exchangeable NH_4^+ immobilization into labile and recalcitrant organic N. For mineralization of labile and recalcitrant organic N to NH_4^+, means followed by the different letter (*upper case* for mineralization of labile organic N to NH_4^+, and *lower case* for mineralization of recalcitrant organic N to NH_4^+) indicated significance among treatments (one-way ANOVA, least significant difference test at $p < 0.10$)

over DNRA in NO_3^- consumption, and I_{NO3} rates differed significantly among treatments ($p = 0.016$; Fig. 2). I_{NO3} rates decreased from 0.44 ± 0.08 mg N kg^{-1} soil day^{-1} in the control plots to 0.09 ± 0.02 mg N kg^{-1} soil day^{-1} in the low N addition plots ($p = 0.006$), and then increased by 200 % to 0.27 ± 0.06 mg N kg^{-1} soil day^{-1} in the high N addition plots ($p = 0.074$) which were still lower than the controls ($p = 0.088$) (Fig. 2). DNRA retained 6.2 to 6.8 % of the NO_3^- produced ($O_{Nrec} + O_{NH4}$), and DNRA rates did not change significantly with treatments (Fig. 2).

Discussion

The effects of NO_3^- addition on microbial NH_4^+ cycling

Previous studies have indicated that soil organic matter fractions, characterized by different size, quality, and turnover time, and linked to physiologically and functionally different soil microbes, respond differently to N deposition over time (Cusack et al. 2011; Frey et al. 2014; Janssens et al. 2010; Koranda et al. 2014; Maaroufi et al. 2015; Swanston et al. 2004). Thus, in the current study, we quantified two specific gross N mineralization rates, related to either a rapid (N_{lab}) or a slower (N_{rec}) turnover of organic N pool (Fig. 1). Obviously, the reduced gross N mineralization in the high N addition plots was primarily a result of decreased mineralization of N_{rec} (M_{Nrec}). The reduction in M_{Nrec} rates under high NO_3^- additions is not surprising. Unlike mineralization of N_{lab}, mineralization of N_{rec} requires a depolymerization step, which is generally carried out by exoenzymes that are often produced by fungi (Schimel and Bennett 2004). There is growing evidence of N enrichment-induced reductions in fungal biomass and activity in forest soils (Demoling et al. 2008; Frey et al. 2014; Maaroufi et al. 2015). Moreover, the observed inhibition of several fungal lignolytic enzyme production and activity, e.g., fungal phenol oxidases and peroxidases of white-rot basidiomycetes, by excessive additions of NO_3^- further supports our results (Ekberg et al. 2007; Kaiser et al. 2010; Waldrop and Zak 2006). It has been suggested that fungal decline under excessive N additions might be a result of enhanced stabilization of organic matter into recalcitrant compounds, as which are resistant to microbial decay or impairs fungal metabolism (Janssens et al. 2010; Maaroufi et al. 2015).

Low NO_3^- addition treatment had a contrasting effect on mineralization of N_{rec} relative to high NO_3^- addition treatment. Low NO_3^- additions stimulated mineralization from N_{rec}. This finding is in line with studies of inorganic N addition to a beech forest soil (Koranda et al. 2014) or an arable soil (Zhang et al. 2012). These occur partly because under low NO_3^- addition treatment, (1) microbial N limitation (especially fungal N limitation) was alleviated, resulting in increased respiratorily responsive biomass and growth-active biomass (especially fungi) to stimulate decomposition of complex C/N substrates (Koranda et al. 2014); (2) decreased C/N ratio of N_{rec} (Aber et al. 1998); and (3) a combination of (1) and (2).

In support of our first hypothesis, the directions of changes in M_{Nrec} and M_{Nlab} rates were opposite under increased NO_3^- additions (Fig. 3). Our results suggested that microbial mineralization of N_{rec} (M_{Nrec}) would be upregulated when microbial mineralization of N_{lab} (M_{Nlab}) was downregulated under low NO_3^- additions, and the possible mechanisms have been presented above. Two, mutually non-exclusive, mechanisms proposed by Janssens et al. (2010) might contribute to the contrasting responses of M_{Nrec} and M_{Nlab} under high NO_3^- addition treatment: enhanced stabilization of organic matter into recalcitrant compounds; shifts in synthesis and activity

of microbial extracellular enzymes towards preferential decomposition of liable, energy-rich compounds.

The change in N mineralization (gross N mineralization, mineralization of N_{rec}, and mineralization of N_{lab}) did not induce a corresponding change in exchangeable NH_4^+ immobilization (gross NH_4^+ immobilization, exchangeable NH_4^+ immobilization into labile organic N, and exchangeable NH_4^+ immobilization into recalcitrant organic N). Such a phenomenon might be explained by the high variance of the observed exchangeable NH_4^+ immobilization rates (Fig. 3). Thus, our results contrast with other findings. A number of researchers have reported decreased NH_4^+ immobilization with either increased or decrease N mineralization in forests receiving high levels of N deposition (Corre et al. 2003, 2007, 2010; Venterea et al. 2004). However, NH_4^+ immobilization might not necessarily change with the change in N mineralization if there is ample NH_4^+ availability, and microbes are lack of N limitation in the soil, as we observed in this study site. Neither an increase nor a decrease in the rates of exchangeable NH_4^+ immobilization into recalcitrant organic N occurred, suggesting that microbial N demand did not change under increased NO_3^- additions (Rütting et al. 2010). High microbial demand for exchangeable NH_4^+ (including autotrophic nitrifies), together with reduced exchangeable NH_4^+ production from recalcitrant organic N, indicates that plant–microbe competition for available NH_4^+ might increase in the high NO_3^- addition plots.

The effects of NO_3^- addition on microbial NO_3^- cycling

Nitrate additions did not stimulate gross nitrification in the subtropical forest soil. This result does not agree with previous findings of increased gross nitrification in tropical forests under enhanced N deposition (Baldos et al. 2015; Corre et al. 2010; Silver et al. 2005). Contrary to our second hypothesis, heterotrophic nitrification did not change significantly with increasing NO_3^- additions. Previous studies have suggested that in acidic subtropical/tropical forests, soil C/N, fungal biomass, and soil pH were the key controls of heterotrophic nitrification, and their reductions could with a decline in the heterotrophic nitrification rates under enhanced N deposition (De Boer and Kowalchuk 2001; Zhang et al. 2015; Zhu et al. 2013). Thus, the lack of significant response from heterotrophic nitrification might be related to the lack of changes in soil C/N and soil pH (Table 1). Zhu et al. (2013) found that oxidation of recalcitrant organic N (heterotrophic nitrification) in acid forest soils of subtropical China decreased as gross N mineralization increased (or when mineralization of organic N was relatively high). This suggests that microbial mineralization of organic N to NO_3^- and NH_4^+ might be mutually exclusive or competitive. However, this is not confirmed by our data as heterotrophic nitrification in the high N addition plots showed a decreasing trend with decreased N mineralization (Fig. 2).

However, autotrophic nitrification rates were observed to increase with increasing NO_3^- additions, possibly as a result of NO_3^- addition-induced reductions in microbial assimilation of NH_4^+ (although not statistically significant; Fig. 3) and the associated increase in NH_4^+ availability (Booth et al. 2005; Corre et al. 2010). Specifically, autotrophic nitrification dominated over heterotrophic nitrification in NO_3^- production under high NO_3^- addition treatment, indicating an increase in the risk of N losses.

Again, inconsistent with our third hypothesis, NO_3^- immobilization first decreased, then increased with increasing NO_3^- additions. However, NO_3^- immobilization in the high N addition plots was still lower than the control plots (Fig. 3). Previous studies have suggested that NO_3^- immobilization depends directly on heterotrophic nitrification for substrate generation, and both pathways are functionally linked in subtropical/tropical acidic forest soils (Gao et al. 2016). At our site, NO_3^- immobilization rates were higher in the control plots with higher heterotrophic nitrification rates, and were lower in the N addition plots with lower heterotrophic nitrification rates (Fig. 2). In other subtropical/tropical acidic forest soils with a high rate of heterotrophic nitrification, NO_3^- immobilization rates were also higher, but this was not the case for forest soils with high autotrophic nitrification rates (Huygens et al. 2007, 2008; Zhang et al. 2013; Zhu et al. 2013). Also, autotrophic nitrification rates in grassland (Müller et al. 2011; Rütting et al. 2010) and arable (Zhang et al. 2012) soils were generally high; however, NO_3^- immobilization rates in these soils were extremely low, possibly due to the effectiveness of low NH_4^+ concentrations in inhibiting NO_3^- assimilation (Rice and Tiedje 1989). Thus, the reduced NO_3^- immobilization under NO_3^- additions could be related to decreased heterotrophic nitrification and increased autotrophic nitrification.

Low NO_3^- addition treatment had a greater inhibition on NO_3^- immobilization than high NO_3^- addition treatment (Fig. 2). This may be due to NH_4^+ being energetically favorable for microbes, and the low microbial NO_3^- demand under high NH_4^+ immobilization (Jansson et al. 1955; Puri and Ashman 1999). Indeed, rates of NH_4^+ immobilization into recalcitrant organic N in the low NO_3^- addition plots were obviously greater than in the high NO_3^- addition plots (Fig. 3). Thus, changes in rates of NH_4^+ immobilization into recalcitrant organic N, heterotrophic nitrification, and autotrophic nitrification could explain the nonlinear changes in NO_3^- immobilization rates under increased NO_3^- additions.

The importance of DNRA in N retention for tropical/subtropical forests and its response to N deposition vary with site conditions, and DNRA rates in situ may be underestimated by laboratory incubation (Baldos et al. 2015; Huygens et al. 2008; Silver et al. 2005; Templer et al. 2008;

Zhang et al. 2013). Although DNRA rates (0.05 to 0.06 mg N kg^{-1} soil day^{-1}), quantified by our laboratory incubation, were low, they were still equal to or higher than NO_3^- leaching (0.04 ± 0.02 mg N kg^{-1} soil day^{-1}) and denitrification activity (<0.015 mg N kg^{-1} soil day^{-1}) measured in other tropical forests (Silver et al. 2005; Templer et al. 2008), highlighting the importance of DNRA in N retention in subtropical/tropical forest ecosystems. DNRA rates were similar among treatments, suggesting that increased NO_3^- additions did not affect the rate of N retention via this pathway, even though NH_4^+ production declined significantly in the high NO_3^- addition plots.

The effects of laboratory incubations of mixed, cold-stored soil on microbial N cycling

In the current study, gross N transformation rates were estimated by laboratory incubations of disturbed/mixed, cold-stored soil. To ensure uniform labeling, soil samples were sieved and homogenized. However, soil sieving could result in reductions in fungi-mediated transformations, e.g., heterotrophic nitrification and NO_3^- immobilization because of its disruption of established mycelium networks (Huygens et al. 2007; Johnson et al. 2005; Zhu et al. 2013). However, mixing the soil might have increased exchangeable NH_4^+ production (Schimel et al. 1989). As suggested by Arnold et al. (2008), the cold storage and laboratory incubation of soil samples might have led to a reduction in gross N mineralization and NH_4^+ consumption, and an increase in gross nitrification and NO_3^- immobilization. This occurred because microorganisms in subtropical/tropical soils are typically accustomed to relatively high temperatures with small fluctuations, and easily mineralizable organic N decreases during storage (Arnold et al. 2008). Zhang et al. (2013) found that DNRA rates in situ might be also underestimated by laboratory measurements. In brief, although laboratory measurement of gross rates of N transformations might allow identifying changes in N transformations between treatments (Paterson 2003), it did not reflect in situ N cycling rates (Arnold et al. 2008). Thus, ^{15}N injection into intact soil cores, in situ incubation, and mineral N extraction in the field to estimate gross N transformations of forest soils are recommended.

Conclusions

Our work revealed that microbial mineralization of fast and slow turnover organic N pool respond differently to NO_3^- additions, and high NO_3^- addition treatment had a contrasting effect relative to low NO_3^- addition treatment. After 2.5 years of treatments, mineralization of labile organic N first decreased, then increased, while mineralization of recalcitrant organic N changed oppositely in response to increasing NO_3^- additions, showing support for our first hypothesis. Our results also identified the suggested mechanisms mediating microbial cycling of N in forest soils under enhanced N deposition: when NH_4^+ production via mineralization of fast turnover organic N was blocked, microbial mineralization of slow turnover organic N be upregulated, and vice versa. Exchangeable NH_4^+ immobilization (exchangeable NH_4^+ immobilization into labile and recalcitrant organic N, and gross NH_4^+ immobilization), however, were not changed significantly by increasing additions of NO_3^-. These results suggests that in N-rich subtropical forests, enhanced NO_3^- deposition might have more of an effect on NH_4^+ production than NH_4^+ consumption in the soil. Contrary to our second hypothesis, heterotrophic and gross nitrification did not change with increasing additions of NO_3^-. However, it remains to be seen whether enhanced NO_3^- deposition will affect heterotrophic nitrification in this acidic subtropical soil by decreasing soil C/N ratio and/or fungal biomass in the long term. Again, inconsistent with our third hypothesis, immobilization of NO_3^- into recalcitrant organic N (NO_3^- immobilization) decreased under increased NO_3^- additions, with a greater reduction under low NO_3^- addition treatment than high NO_3^- addition treatment. Unexpectedly, autotrophic nitrification increased with increasing additions of NO_3^-. Thus, we conclude that enhanced NO_3^- deposition will increase the risk of N loss from this subtropical forest ecosystem by stimulating autotrophic nitrification, inhibiting NO_3^- immobilization, or both. Microbial mechanisms controlling the changes in NO_3^- production and soil retention of NO_3^- under enhanced NO_3^- deposition need to be explored.

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