Effects of nitrogen deposition rates and frequencies on the abundance of soil nitrogen-related functional genes in temperate grassland of northern China

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Abstract

Purpose Microbial processes driving nitrogen (N) cycling are hot topics in terms of increasing N deposition. Abundances of N-related functional genes (NFG) can be most responsive to N deposition and commonly used to represent N transformation rates. However, empirically simulated N deposition has been exclusively conducted through large and infrequent N fertilization, which may have caused contrasting effects on NFGs.

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Therefore, experiments with small and frequent N additions closed to natural deposition are necessary.

Materials and methods Independently manipulated N addition rates (i.e., 0, 5, 10, 15, 20, and 50 g N m⁻² year⁻¹) and two frequencies (2 times per year addition as conventional large and infrequent N fertilization (2 N), and 12 times per year addition simulating small and frequent N deposition (12 N), respectively) were conducted in a long-term field experiment of a semiarid grassland in northern China. Quantification analysis using real-time PCR were carried out for NFGs, including *nifH* for N fixation, *chiA* for N mineralization, archaeal (AOA) and bacterial (AOB) *amoA* for nitrification, and *narG*, *nirS*, *nirK*, and *nosZ* for denitrification.

Results and discussion NFG abundances showed distinct sensitivities to N addition rates. The *nifH*, AOA-*amoA*, *nirS*, and *nosZ* gene abundances increased due to improved available N at low N rates, but suppressed by salt toxicity and acidification at high N rates. Large changes of *chiA* and AOB-*amoA* gene abundances highlighted their great sensitivities to the N enrichment. The abundance of AOB-*amoA* was more sensitive to N addition than AOA-*amoA*, but AOA-*amoA* dominated in absolute numbers and they predominated the ammonia-oxidation under different conditions. The N addition frequencies caused significant lower gene abundances of *nifH*, *nirS*, and *nosZ* under the 2-N frequency due to stronger suppression of acidification and salt toxicity and resulted in significant higher AOB-*amoA* gene abundances in response to higher N availability under the 2-N frequency.

Conclusions The NFGs abundances responded to N addition rates distinctly, highlighting that the driven processes involved in N cycling were altered by the N addition rates. The different effects of two N addition frequencies on NFG abundances demonstrated that conventional large and infrequent N fertilization cannot represent N deposition, and small and frequent

N addition should be employed to project the effects of N deposition on microbial functional groups as well as on N transformations.

Keywords Functional gene · Nitrogen addition frequency · Nitrogen addition rate · Nitrogen deposition · Nitrogen transformation · Temperate grassland

1 Introduction

Nitrogen (N) is considered to be the nutrient most likely to limit production in terrestrial ecosystems (Vitousek and Howarth 1991; Xu and Chen 2006; Xu et al. 2009). The rate of atmospheric N deposition has rapidly increased due to intensive anthropogenic activities and therefore results in the alteration of ecosystem processes and functions (Galloway et al. 2004, 2013; Sala et al. 2000; Xu and Chen 2006; Xu et al. 2009). There is increasing evidence that N enrichment can produce wide-ranging impacts on the microbial diversity and biomass (Sala et al. 2000; Treseder 2008) as well as the biogeochemical processes (Ramirez et al. 2012) including N transformation rates (Ochoa-Hueso et al. 2013) and the emissions of greenhouse gases (Treseder 2008). However, infrequent addition of simulated N deposition with infrequent N fertilization might cause contrasting evaluation of impacts on N cycling processes compared with continuous natural N deposition. It has been proven that the effects of N addition frequencies differed significantly on ecosystems, such as different available plant N concentration (Cheng et al. 2009), ammonia emissions (Zhang et al. 2014a), plant species loss (Zhang et al. 2014b), net ecosystem CO₂ exchange and radiation-use efficiency (Barton et al. 2008), and plant biomass (Bilbrough and Caldwell 1997). Consequently, experiments with wide range of rates and varying frequencies are imperative to understand the influences of N deposition on ecosystems (Zhang et al. 2014b), especially on microbial processes involving in N cycling.

Effects of N deposition have garnered considerable attention surrounding microorganisms (Stevens et al. 2010). In various ecosystems, microorganisms drive many fundamental N transformation processes (Hallin et al. 2009; Petersen et al. 2012; Veresoglou et al. 2012), such as N fixation, N mineralization, nitrification, and denitrification. N-related functional genes (NFG), such as *nifH* for N fixation, *chiA* for N mineralization, archaeal (AOA) and bacterial (AOB) *amoA* for nitrification, and *narG*, *nirS*, *nirK*, and *nosZ* genes for denitrification, encoding the key enzymes responsible for the N transformation processes and could be used as a proxy for measuring N transformation rates in soil (Colloff et al. 2008; Lindsay et al. 2010; Petersen et al. 2012; Zhang et al. 2013). N

deposition is promoting major alterations of N cycling processes (Ochoa-Hueso et al. 2013) and NFG abundances (Kandeler et al. 2009) in ecosystems. The form of N addition to soil and its effect on soil properties play important roles in the microbial community responses to N additions (Levy-Booth et al. 2014). For example, the research carried out by Jung et al. (2011) showed that the addition of N caused increased *nifH* gene abundance; Zhang et al. (2013) found decreased *chiA* gene abundance with N addition; AOA-*amoA* gene abundance might be important an factor in low-nutrient environments (Erguder et al. 2009), which was in contrast with AOB-*amoA* gene abundance (Di et al. 2010; Jia and Conrad 2009); and the size of the total nitrate reducer and denitrifier communities did not respond to the reduced N deposition (Kandeler et al. 2009). However, previous intensive research thus far investigates one single step, such as the initial nitrification step ammonia-oxidation (Leininger et al. 2006) or denitrification with loss of gaseous products (Attard et al. 2011; Kandeler et al. 2006), and the fact that N transformation is a network of closely interlinked processes and has been commonly neglected (Hai et al. 2009; Shen et al. 2014; Wang et al. 2014). Consequently, experiment of NFG quantification corresponding to all key steps should be conducted to better understand N transformation (Shen et al. 2014).

Therefore, we sought to investigate the effect of N deposition rates and frequencies on key N transformation processes including N fixation, N mineralization, nitrification, and denitrification. We hypothesized that different N frequencies induced different responsive patterns of NFG abundances, and further postulated that NFG abundances and corresponding N transformation processes were also influenced by different edaphic conditions resulting from N addition rates and frequencies. To test these hypotheses, we conducted a N deposition experiment in 2011, with independently manipulated N addition frequencies (2 N and 12 N) and rates (0, 5, 10, 15, 20, and 50 g N m⁻² year⁻¹), with quantifying NFG abundance, on a molecular basis through a real-time PCR method and linking the data to edaphic properties. This allowed us to detect the responses of NFGs to distinct N addition rates and frequencies, and to explore the possible underlying mechanisms.

2 Materials and methods

2.1 Experimental site

This study was carried out in a semiarid grassland ecosystem near the Inner Mongolia Grassland Ecosystem Research Station (IMGRES) of the Chinese Academy of Sciences (43°13' N, 116°14'E; 1250–1260 m.a.s.l). The experimental site had been fenced since 1999 to exclude large animal grazing. The average annual temperature is 0.9 °C and mean monthly temperature ranges from −21.4 °C in January to 19.7 °C in July.

Mean annual precipitation is 355.3 mm, with 60–80 % mainly occurring from May to August. The soil is classified as Haplic Calcisol and Calcic-orthic Aridisol by the FAO and US Soil Taxonomy classification system, respectively (Zhang et al. 2014b). The mean annual N deposition was less than $1.5 \text{ g N m}^{-2} \text{ year}^{-1}$ in this experimental area for the immediate past 20 years' period (Jia et al. 2014), and no fertilizer had been applied prior to the experiment (Zhang et al. 2014b). The N is limiting in the experiment field as Bai et al. (2010) found that the N saturate rate is approximately $10.5 \text{ g N m}^{-2} \text{ year}^{-1}$ at IMGERS. The vegetation is dominated by *Leymus chinensis*, *Stipa grandis*, *Cleistogenes squarrosa*, *Agropyron cristatum*, and *Achnatherum sibiricum*. The main dominant N-fixing bacteria are free-living diazotrophs as no legumes were present at this experimental site.

2.2 Experimental design

The experimental N deposition started in September 2008 and was laid out in a completely randomized block design as described in detail (Zhang et al. 2014b). Briefly, nine N addition rates (0, 1, 2, 3, 5, 10, 15, 20, and $50 \text{ g N m}^{-2} \text{ year}^{-1}$) with two N addition frequencies: 2 and 12 N additions per year were involved. In this study, we randomly selected 5 N addition rates (5, 10, 15, 20, and $50 \text{ g N m}^{-2} \text{ year}^{-1}$) with two N addition frequencies and control treatment (N addition rate $0 \text{ g N m}^{-2} \text{ year}^{-1}$) in three blocks, in brief, 33 plots were analyzed. Each replicate was designed as a block separated by 2-m buffer; the plot was randomly located within the block ($45 \text{ m} \times 70 \text{ m}$) and separated by 1-m buffer; size of each plot was $8 \text{ m} \times 8 \text{ m}$.

We constructed purified ammonium nitrate (NH_4NO_3) supplied as N resource. Firstly, experimental N deposition started on 1st September 2008 for the high frequency (12 N additions per year) and 1st November 2008 for the low frequency (2 N additions per year), and then the N addition continued on 1st day of every month for the high frequency and on the 1st of June and November for the low frequency. When the monthly N was added in August of each year, total annual loading N was equal between the 2-N and 12-N addition frequencies. The NH_4NO_3 was dissolved in distilled water and evenly sprayed to the plot (9.0 L for the 2 N in June and 1.5 L for the 12 N each month) during the growing season from May to October; from November to the next April, NH_4NO_3 was blended with washed fine sand and evenly applied by hand in November for the 2 N and on the 1st day of winter months from November to April for the 12 N.

2.3 Sampling

For each plot, we took six soil cores (3 cm in diameter) from surface to 10-cm depth at random and blended them as a composite sample after sieving through a 1-mm mesh

in August 2011. The composite sample was divided into four subsamples: one was stored at -80°C for nucleic acid extraction; the second was to determine soil water content after 24 h drying at 105°C ; the third was air-dried for measuring soil pH and total C, N, and P; and the fourth was used to measure ammonium and nitrate within 24 h after sampling.

2.4 Soil physical and chemical properties

Soil moisture was determined using the gravimetric method and soil pH (water/soil=2.5:1) was measured with a SevenEasy pH Meter (Mettler-Toledo; USA). Air-dried soil was ground with a ball mill (Retsch MM 400, Retsch GmbH & Co KG, Haan, Germany). Soil total C concentration was determined using $\text{H}_2\text{SO}_4\text{-K}_2\text{Cr}_2\text{O}_7$ oxidation method (Nelson and Sommers 1982). Total N concentration was determined using the Kjeldahl acid-digestion method with an Alpchem autoanalyzer (Kjektec System 1026 Distill Unit, Sweden). Total P concentration was determined colorimetrically at 880 nm after reaction with molybdenum blue. Inorganic N was extracted with 2 M KCl; extractable $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were analyzed colorimetrically using a continuous flow injection analyzer (FIASStar, Foss Tecator, Höganäs, Sweden).

2.5 Nucleic acid extraction

DNA was extracted from 0.5 g soil using the FastDNA[®] SPIN Kit (MP Biomedicals, Irvine, CA). Yield and purity of the extracted DNA were checked with a spectrophotometer (Nanodrop, PeqLab, Germany); the 350 μL DNA elution solution was used to elute extracted DNA in the tenth step. Afterwards, extracts were stored at -20°C until use.

2.6 Real-time PCR assays

Absolute quantification of all investigated genes was conducted on a ABI7500Fast Real-Time PCR System (Applied Biosystems, Germany) using SybrGreen as fluorescent dye. Quantitative real-time PCR was performed in 96-well plates for all eight target genes. To create a standard curve, plasmid DNA containing each target gene was serially diluted from 10^1 to 10^6 gene copies per microliter. All samples, standard series, and negative controls were run in triplicate. The 25- μL PCR amplification mixtures containing 12.5 μL SybrGreen qPCR Master Mix ($\times 2$) (Shanghai Ruian BirTechnologies Co., Ltd., Shanghai, China), 0.5 μL each primer (20 μM), 10 μL sterile and DNA-free water, and 1.5 μL standard plasmid. The bovine serum albumin (BSA) was included in the SybrGreen qPCR Master Mix to provide some resistance to inhibitors such as co-extracted polyphenolic compounds of the soil. The primers nifHF and nifHR for *nifH* gene (Rösch et al. 2002), chif2 and chir for *chiA* gene (Xiao et al. 2005),

Arch-amoAF and Arch-amoAR for AOA-*amoA* gene (Francis et al. 2005), AmoA-1F and AmoA-2R for AOB-*amoA* gene (Rotthauwe et al. 1997), narGf and narGr for *narG* gene (De-lorme et al. 2003), cd3aF and R3cd for *nirS* gene (Michotey et al. 2000; Throbäck et al. 2004), nirK-1F and nirK-5R for *nirK* gene (Braker et al. 1998), and nosZ-F and nosZ-R for *nosZ* gene (Kloos et al. 2001) were used. The specificity of the amplification products was confirmed by melting-curve analysis, and the sizes of the amplified fragments were checked in a 2 % agarose gel. The thermal profile for genes of *nifH*, *chiA*, AOA-*amoA*, AOB-*amoA*, and *nirK* was as follow: 95 °C for 2 min and 40 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 1 min. The thermal profile for genes of *narG*, *nirS*, and *nosZ* was as follow: 95 °C for 2 min and 40 cycles of 95 °C for 10 s, and 60 °C for 1 min. The formula $\text{Eff}=[10^{(-1/\text{slope})}-1]$ was used to calculate the amplification efficiencies. Preceding tests with dilution series of the extracted DNA showed that the inhibitory effect was negligible.

2.7 Statistical analyses

Data normality distribution was checked by Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to test the variance of gene abundances and soil physicochemical properties in all plots. Two-way ANOVAs were used to test the interactive effect of N addition rates and frequencies on gene abundances and soil physiochemical properties. Gene abundances were determined by polynomial quadratic and linear regressions with N addition rates and physicochemical properties, and the best-fitting regression models were adopted and presented, statistical significance was set at $p<0.05$. Multiple regressions of NFG abundances with soil properties and N addition rates and frequencies were checked, statistical significance was set at $p<0.1$. All statistical analyses were performed using statistical software SAS V8 (SAS institute, USA) and Statistix 8.0 (Analytical Software, USA); all figures were created by graphing software SigmaPlot 11 (Systat software Inc, USA).

3 Results

3.1 Soil physical and chemical properties

Soil pH values in response to the two N addition frequencies revealed a statistically significant difference ($p<0.001$) (Fig. 1a, b), with N addition leading to more acidic conditions under the 2-N frequency. Soil pH with both frequencies negatively correlated with N addition rates. It was noteworthy that soil pH exerted a sharp plunge from 20 to 50 g N m⁻² year⁻¹, especially for the 2-N frequency. Higher NH₄⁺-N concentrations ($p<0.001$)

(Fig. 1c, d) and NO₃⁻-N concentrations ($p<0.001$) were detected under the 2-N frequency (Fig. 1e, f). Both NH₄⁺-N and NO₃⁻-N concentrations increased with the N addition rates for the two N addition frequencies.

There were no differences in soil total C, N, and P concentrations and their ratios between the two N addition frequencies. Total N concentration significantly positively correlated with N addition rates ($R^2=0.41$, $p=0.005$, 2 N; $R^2=0.28$, $p=0.025$, 12 N; Fig. 2b). Negative correlations were found between C/N ratios and N addition rates ($R^2=0.46$, $p=0.002$, 2 N; $R^2=0.37$, $p=0.008$, 12 N; Fig. 2d). The C/P ratios in the 12 N plots negatively ($R^2=0.26$, $p=0.029$; Fig. 2e) and N/P ratios in 2 N plots positively correlated with N addition rates ($R^2=0.40$, $p=0.005$; Fig. 2f), respectively.

3.2 Abundance of N functional genes

Except for *narG* gene, all the other NFGs were responsive to the N addition, and significant differences between the two N addition frequencies were detected in the abundances of *nifH*, AOB-*amoA*, *nirS*, and *nosZ* genes. For *nifH*, AOA-*amoA*, *nirK*, *nirS*, and *nosZ*, gene abundances first peaked then decreased. The N rate at 20 and 50 g N m⁻² year⁻¹ changed NFG abundances more largely than the other rates in both frequencies. Polynomial quadratic regression model showed *nifH* (2 N), *chiA* (12 N), AOA-*amoA* (2 N and 12 N), AOB-*amoA* (2 N and 12 N), *nirK* (2 N and 12 N), *nirS* (12 N), and *nosZ* (12 N) abundances had hump-shaped relationships with the N addition rates (see Fig. 3).

3.2.1 Abundances of *nifH* gene

The *nifH* gene abundance evidently increased in the 2 N (15 g N m⁻² year⁻¹) plots and 12 N (5, 10 and 15 g N m⁻² year⁻¹) plots (Fig. 3a), and the abundance was significantly higher under the 12-N frequency than that under the 2-N frequency ($p=0.040$). The *nifH* gene abundance negatively correlated with P concentrations ($r=-0.632$, $p=0.007$), and positively correlated with N/P ratios ($r=0.499$, $p=0.041$) under the 12-N frequency.

3.2.2 Abundance of *chiA* gene

The N addition reduced the *chiA* gene abundances in almost all rates of both frequencies excluding non-significant decreases in the 10 ($p=0.075$) and 20 ($p=0.075$) g N m⁻² year⁻¹ plots under 2 N frequency (Fig. 3b). Different N addition frequencies did not produce significant effects on *chiA* gene abundance ($p=0.871$).

Fig. 1 Soil pH, ammonium ($\text{NH}_4^+\text{-N}$), and nitrate ($\text{NO}_3^-\text{-N}$) concentration of 2 N (N addition 2 times/year) and 12 N (N addition 12 times/year) frequencies for control and N addition rates in August, 2011. Data are mean $\pm$ S.E. Bars with the same letter are not significantly different in Duncan's multiple range tests reported from one-way ANOVA. Regression parameters were calculated using log-linear models with N rates as a continuous predictor, i.e., $\text{pH} = \text{intercept} + \text{slope} \times \ln(\text{N})$. Significant regressions are reported as * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

3.2.3 Abundances of *AOA-amoA* and *AOB-amoA* genes

AOA-amoA gene abundances showed no significant difference between the two N addition frequencies (Fig. 3c); *AOB-amoA* gene abundances under the 2-N frequency were significantly higher than those under the 12-N frequency ($p = 0.030$, Fig. 3d). *AOA-amoA* abundance peaked in the 15 $\text{g N m}^{-2} \text{year}^{-1}$ plots of the 12 N. Strikingly, in both frequencies, *AOB-amoA* abundances showed substantial increases across the N addition rates. Independent of the N addition rates and frequencies, *AOA-amoA* gene abundances were clearly dominant over *AOB-amoA* abundances. Across the N addition rates, the ratio of *AOA/AOB* gene abundances ranged from 324 in the control plots to 6.04 under the 2 N and 7.85 under the 12-N frequency, respectively. *AOA-amoA* gene abundances had quadratic correlations with $\text{NH}_4^+\text{-N}$ concentrations ($R^2 = 0.43$, $p = 0.008$, 12 N; Fig. 4a) and $\text{NO}_3^-\text{-N}$ concentrations ($R^2 = 0.32$, $p = 0.026$, 12 N; Fig. 4b). *AOB-*

amoA gene abundances were found to have strong negative correlations with pH ($R = -0.816$, $p < 0.001$, 2 N; $R = -0.890$, $p < 0.001$, 12 N) and strong correlations with $\text{NH}_4^+\text{-N}$ concentrations (linear, $R^2 = 0.74$, $p < 0.001$, 2 N; linear, $R^2 = 0.53$, $p < 0.001$, 12 N; Fig. 4c) and $\text{NO}_3^-\text{-N}$ concentrations (linear, $R^2 = 0.70$, $p < 0.001$, 2 N; quadratic, $R^2 = 0.63$, $p < 0.001$, 12 N; Fig. 4d). In the 2-N frequency, *AOA-amoA* gene abundances positively correlated with soil P concentration, and *AOB-amoA* gene abundances also positively correlated with soil N concentrations and negatively correlated with soil C/N ratios and C/P ratios.

3.2.4 Abundances of *narG*, *nirK*, *nirS*, and *nosZ* genes

The *narG* gene abundance was not affected by the N addition rates and frequencies (Fig. 3e). Abundances of *nirK* gene (Fig. 3f) were found to be suppressed at 50 $\text{g N m}^{-2} \text{year}^{-1}$ of both N addition frequencies ($p = 0.024$, 2 N; $p = 0.011$,

Fig. 2 Soil C, N, and P concentration and the ratio of C/N, C/P, and N/P of 2 N (N addition 2 times/year) and 12 N (N addition 12 times/year) frequencies for control and N addition rates. Significant linear regression coefficients of the best-fitting models are reported as * $p<0.05$; $p<0.01$ **;
*** $p<0.001$. NS indicates non-significant ($p>0.05$)

12 N). The abundance of *nirS* (Fig. 3g) gene predominated over *nirK* gene abundance independent of all treatments, even though their functions were the same. The *nirK* gene abundances were found to have strong quadratic correlations with NH_4^+ -N concentrations ($R^2=0.56$, $p=0.001$, 2 N; $R^2=0.51$, $p=0.003$, 12 N; Fig. 4e) and NO_3^- -N concentrations ($R^2=0.47$, $p=0.005$, 2 N; $R^2=0.55$, $p=0.002$, 12 N; Fig. 4f), and positive correlation with pH ($R=0.538$, $p=0.026$, 12 N). The *nirS* gene abundances were significantly enhanced by the N rates at 5 (2 N), 10, and 15 (2 N and 12 N) g N m⁻² year⁻¹. The 12-N frequencies produced higher gene abundances of *nirS* ($p=0.0457$). Concerning *nosZ* gene (Fig. 3h), abundances showed increases at the 10 ($p=0.010$), 15 ($p=0.059$), and 20 ($p=0.015$) g N m⁻² year⁻¹ under the 12-N frequency. Additionally, *nosZ* gene numbers under the 12-N frequency were significantly higher than those under the 2-N frequency ($p=0.0189$). The *nosZ* gene abundances were found to have negative correlations with P concentrations ($R=-0.651$, $p=0.005$) under the 2-N frequency and negatively correlated with NH_4 -

N concentrations ($R=-0.500$, $p=0.041$) under the 12-N frequency.

4 Discussion

4.1 Soil available N and pH are main controlling factors for the NFG abundances

Soil available N is a crucial ecological factor for growth, metabolism, and community of microorganisms (Cheever et al. 2013; Ramirez et al. 2012), especially in N-limited soils. The N limiting can be mitigated by fertilization and deposition (Levy-Booth et al. 2014), supplying nutrient and energy for basic metabolism and growth of microorganisms, which contributed to the significant increases of *nifH* (2 N and 12 N), AOA-*amoA* (12 N), AOB-*amoA* (2 N and 12 N), *nirS* (2 N and 12 N), and *nosZ* (12 N) gene abundances at the low N

Fig. 3 Gene copy numbers of functional genes involved in major steps of the nitrogen cycle (*nifH*, *chiA*, AOA-*amoA*, AOB-*amoA*, *narG*, *nirK*, *nirS*, and *nosZ*) under different N addition rates and frequencies. Significant polynomial quadratic regression coefficients of the best-fitting models are reported as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS indicates non-significant ($p > 0.05$)

addition rates. The gene abundances of *nifH*, AOA-*amoA*, *nirS*, and *nosZ* largely peaked at relative low N rate 10–15 g N m⁻² year⁻¹ under the two frequencies. Previous research also found that the improved available N stimulated the abundances of NFG genes (Chon et al. 2009; Erguder et al. 2009; Hai et al. 2009; Hayden et al. 2010; Hofferle et al. 2010; Kowalchuk and Stephen 2001; Okano et al. 2004; Zhang et al. 2013). Oxidation of ammonia to nitrite catalyzed by ammonia-oxidizing bacteria and archaea could be an important pathway of nitrite (Huang et al. 2014), which might reduce the dependence of nitrate reduction on *narG* gene and resulted in no abundance changes.

However, the microbes are not always limited by N, the continual increase of N addition will cause negative effects (Treseder 2008), including soil acidification (Shen et al. 2008), toxic osmotic potential of soil solution (Broadbent 1965), and altered nutrient status imbalance (Vitousek 1997; Waldrop and Zak 2006), on soil microbial gene abundances. The long-term and high amount N addition definitely induced the soil acidification (Enwall et al. 2005; Kowalchuk and Stephen 2001; Shen et al. 2008), which resulted in the significant decrease of *nifH*, AOA-*amoA*, *nirS*, and *nosZ* gene abundances at the highest N addition rate, compared with their peak abundances which were improved by the N addition

Fig. 4 Relationships between *AOA-amoA*, *AOB-amoA*, and *nirK* gene abundance, with ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) concentration of 2 N (N addition 2 times/year) and 12 N (N addition 12 times/year) frequencies. The inorganic N concentration is log natural transformed. No regression is fitted for relationship between *AOA-amoA* abundance and inorganic N (ammonium and nitrate, respectively) concentration in 2-N frequency. Significant linear regressions are best fitted for the relationships between *AOB-amoA* abundances and ammonium concentration (2 N and 12 N) and nitrate concentration (2 N). Significant quadratic regressions are best fitted for all the other relationships. Coefficients of the best-fitting models are reported as * $p<0.05$; ** $p<0.01$; *** $p<0.001$

under the two frequencies. Soil pH appeared to be an important factor influencing NFG abundances and microbial functions (Lindsay et al. 2010; Wakelin et al. 2009; Wallenstein et al. 2006). Microorganisms prefer certain pH levels in the substance or environment in which they grow (Rousk et al. 2010). The low pH may also cause strong absorption of molybdenum, which is a regulator of the N fixers (Jarrell and Dawson 1978). Zhang et al. (2013) found that soil pH contributed to the decrease of *chiA* gene abundance. The *nirK* gene abundances were significantly decreased at rate $50 \text{ g N m}^{-2} \text{ year}^{-1}$ under the two N addition frequencies compared with abundances in the control plots, which can be explained by the suppression of soil pH (Lindsay et al. 2010); positive relationship with soil pH was only found under the 12-N frequency. For *AOA-amoA* gene, compared to abundances in the control plots, there was a slight reduction ($p=0.064$) at rate $50 \text{ g N m}^{-2} \text{ year}^{-1}$ of the 12-N frequency with pH 5.5. The generally accepted explanation is the exponential reduction in ammonium availability with decreasing

pH (Frijlink et al. 1992), and the low soil pH caused by the N addition was a more important factor than the added N itself as substrates supply (He et al. 2007).

The hump-shaped relationships between *AOA-amoA* gene abundances and nitrate concentrations, and between *nirK* gene abundances and inorganic N concentrations, reveal that there should be optimum inorganic N concentrations for their livings. Additionally, accumulated N (ammonia, nitrate, and nitrite) at relatively high N addition rates resulted in high salt concentrations and toxicity which also inhibited the abundance of *nifH*, *chiA*, *AOA-amoA*, *nirK*, *nirS*, and *nosZ* genes (Hayden et al. 2010; Lindsay et al. 2010; Töwe et al. 2010; Wallenstein et al. 2006). The gene abundances of *nifH* (12 N) and *nosZ* (2 N) were negatively correlated with P concentrations, and *AOB-amoA* (2 N) were negatively correlated with C/N and C/P ratios indicating that the enrichment of N resource in the soil may have shifted microbes from being limited by the N resource to the C and P limitation (Zhang et al. 2013), which were also regulators of gene abundances.

Small changes in the N cycling are hypothesized to induce disproportionately large shifts in the dynamic of ecosystems (Yergeau et al. 2007). The increase of gene abundances would improve soil N transformation; in contrast, the decrease would suppress the N transformation. Therefore, it is of central importance to elucidate the extent to which the gene abundance would be improved or suppressed by the N addition.

4.2 Roles of microbial functional genes with high sensitivity in N deposition

All the N addition rates reduced *chiA* gene abundances by the same extent even at the lowest N addition rates, and we suggested that a very small N dose ($0\text{--}5\text{ g N m}^{-2}\text{ year}^{-1}$) can greatly influence *chiA* gene abundances, and then the lower N addition levels ranged and related abiotic factors should be studied exclusively to locate the point at which the *chiA* gene abundance would begin to decrease, which is necessary to prove the contribution of *chiA* group in decomposition processes. The AOB-*amoA* gene abundance presented sharp growth with increasing N addition rates and inorganic N concentrations, including absolute growth at lowest N rate, indicating the sensitive response to the N addition and a stimulating effect of N addition on AOB-*amoA* population size (Di et al. 2010; Hallin et al. 2009; Weng et al. 2013; Zhang et al. 2013). In consideration of high sensitivity of *chiA* and AOB-*amoA* gene abundances to the N addition, and their functions in regulating N cycling, N mineralization and nitrification processes can certainly be perturbed by the N deposition even at a small dose. Therefore, *chiA* and AOB-*amoA* gene can be used as sentinel organisms to project and monitor future N deposition by using ambient gradients of N addition (Stevens et al. 2004; Zhang et al. 2014b).

4.3 Frequencies of N addition prompted distinct responsive patterns of the NFG abundances

Abundances of *nifH*, AOB-*amoA*, *nirS*, and *nosZ* genes were diverged by the two N addition frequencies, indicating that large and infrequent N fertilization would likely cause biased assessment of the effects on NFGs as well as on N transformation processes, compared with small and frequent N deposition. There should be mechanisms responsible for these differences within the soil–microbe system. The N addition frequencies lead to distinct accumulated mineral N concentrations and the associated soil acidification, which principally regulated the responsive patterns of different gene abundances. At the same site, the low N addition frequency may result in a weaker potential than the high N addition frequency to emit gas (in the form of ammonia) (Zhang et al. 2013), and then more mineral N was accumulated under the 2-N frequency; consequently, the toxicity solution and acidification of soil inhibited the gene abundances of *nifH*, *nirS*, and *nosZ* under

the 2-N frequency. In contrast, the 2-N frequency induced significantly higher AOB-*amoA* gene abundances compared with the 12-N frequency, mainly due to higher N availability under the 2-N frequency. According to the negative correlation between N availability and soil acidification, we suggested that in the pH range 5.5–7.5 in this study, the promoting effect of N availability outstripped the inhibiting effect of soil acidification, and then N availability was the main driver for AOB-*amoA* gene abundance rather than the soil acidification.

An additional reason for the difference was that, when the same total N loading in a singular pulse rather than frequent deposition, the toxicity of mineral N and soil acidification tend to be aggravating, and then the peak effect of infrequent N addition prompted distinct responsive patterns of the NFG abundances. Then, the accumulative toxicity and acidification resulted in the lower abundances of *nifH*, *nirS*, and *nosZ* under the 2-N frequency, and peak enriched available N resulted in higher abundances of AOB-*amoA* gene under the 2-N frequency, respectively. Boyle-Yarwood et al. (2008) found that the plant species were linked to the differences in AOA-*amoA* and AOB-*amoA* community. The rapid plant species loss under the 2-N frequency (Zhang et al. 2014b) might lead to different rhizospheric conditions, such as soil organic C, which also influence response patterns of NFG abundances. The plant–microbe mechanism involved in this experiment should further studied, to reveal how the plant species changes regulate the abundance and function of NFGs.

Overall, according to the abundance response of NFGs, the large and infrequent fertilization in previous N enrichment experiments might potentially underestimate the effects of N deposition on N fixation and denitrification processes and overestimate the effects of N deposition on nitrification process. We should not simply simulate N deposition by large and infrequent N addition or confound their influences.

5 Conclusions

Our results indicated that N addition rates largely altered almost all the gene abundances measured in this study and consequently altered the driven processes. The *chiA* and AOB-*amoA* groups with great sensitive responses to rates of N addition can be used as indicators for future aerial N deposition. It is crucial to track the rate at which the gene abundances having reverse changes, to reveal the changes of processes of N cycling. The large and infrequent N addition caused diverged responsive patterns of *nifH*, *nirS*, *nosZ*, and AOB-*amoA* gene abundances, compared with small and frequent N addition; the corresponding functions and processes were also deduced to be influenced. Thus, the continuous, long-term field experiments with independently manipulated rates and

frequencies are required to accurately elucidate the influences of N deposition on N cycling processes.

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