Contrasting response of nitrification capacity in three agricultural soils to N addition during short-term incubation

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Abstract

Purpose Human disturbance is a major culprit driving imbalances in the biological transformation of nitrogen from the nonreactive to the reactive pool and is therefore one of the greatest concerns for nitrogen (N) cycling. The objective of this study was to compare potential nitrification rates and the abundance of ammonia oxidizers responsible for nitrification, with the amendment of external N in different agricultural soils.

Materials and methods Three typical Chinese agricultural soils, QiYang (QY) acid soil, ShenYang (SY) neutral soil, and FengQiu (FQ) alkaline soil, were amended with 0, 20, 150, and 300 $\mu\text{g NH}_4^+\text{-N g}^{-1}$ soil and incubated for 40 days. The abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA) at the end of incubation in the soil microcosms was determined using the real-time PCR.

Results and discussion There was a significant decrease in ammonium concentration in the QY soil from the highest to the lowest N-loading treatments, while no significant difference in ammonium concentrations was detected among the different N-loading treatments for the SY and FQ soils. A significantly higher potential nitrification rate (PNR) was observed in the FQ soil while lowest PNR was found in the QY soil. Quantitative PCR analysis of AOB *amoA* genes

demonstrated that AOB abundance was significantly higher in the high N-loading treatments than in the control for the QY soil only, while no significant difference among treatments in the SY and FQ soils. A significant positive correlation between PNR and AOB *amoA* abundance, however, was found for the SY and FQ soils, but not for the QY soil. Little difference in AOA *amoA* abundance between different N-loading treatments was observed for all the soils.

Conclusions This study suggested that ammonia oxidation capacity in the FQ and SY soils was higher than those in the QY soil with the addition of ammonium fertilizer for a short-term. These findings indicated that understanding the differential responses of biological nitrification to varying input levels of ammonium fertilizer is important for maximizing N use efficiency and thereby improving agricultural fertilization management.

Keywords Ammonia oxidizing bacteria · Ammonia oxidizing archaea · Ammonium fertilizer · Potential nitrification rate · Soil type

1 Introduction

Since the introduction of synthetic N fertilizer, agricultural ecosystems annually receive about 25 % of global nitrogen input predominately in the form of ammonium (NH_4^+) fertilizer (Gruber and Galloway 2008). Consequently, N use efficiency and environmental issues associated with large-scale fertilization are serious and legitimate concerns for fertilizer management and environmental sustainability. In particular, imbalances in the biological transformation of N from the nonreactive to the reactive pool by cause of human intervention, i.e. N fertilization, are of paramount significance. The negative consequences for ecosystems from external N additions are substantial, particularly for N-transformation processes such as

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nitrification (Singh et al. 2010). Biological nitrification is a two-step process, ammonia oxidation and nitrite oxidation. The conversion of ammonia to nitrite is catalyzed by a group of obligate chemolithoautotrophic prokaryotes collectively referred to as ammonia-oxidizing bacteria (AOB) (Kowalchuk and Stephen 2001) and the recently discovered ammonia-oxidizing archaea (AOA) affiliated with Thaumarchaeota phylum (Brochier-Armanet et al. 2008). Understanding the response of these microbial-mediated processes to N amendment, and how this biological response affects N turnover in the soil, is critical for knowledge-informed design and implementation of sustainable land management strategies.

A great number of molecular investigations have been carried out across the globe for the purpose of identifying and mapping key environmental factors and their complex interactions that influence soil nitrification. Two factors, ammonium and soil pH, have been identified as the most important ones. For example, many studies have addressed the dynamics of AOB populations in soils amended with N fertilizer, in particular ammonium sulfate, in soil microcosms or in situ (Phillips et al. 2000; Okano et al. 2004; Enwall et al. 2005). Additionally, the distribution patterns of AOB and AOA as a function of soil pH have been demonstrated at different land scales using molecular methods and bioinformatics analysis (Nicol et al. 2008; Gubry-Rangin et al. 2011; He et al. 2012; Hu et al. 2013). However, the effects of other factors, such as soil structure, on the dynamics of AOB and AOA as well as their nitrification activity are little understood (Wessen et al. 2011; Yao et al. 2013). Girvan et al. mentioned that soil type was the primary determinant of the composition of the total and active bacterial communities in arable soils (Girvan et al. 2003). Another study investigating three soil types, fluvo-aquic soil, permeable paddy soil, and red earth, showed that fluvo-aquic soil had the highest nitrification potential, based on quantitative analysis of AOB in soil, compared to the other two soil types (Yuan et al. 2005). Recently, surveys based on large-scale sampling clearly demonstrated a multifactorially driven pattern of distribution for both groups of ammonia oxidizers (Fernandez-Guerra and Casamayor 2012; Yao et al. 2013). Among these factors, soil type was identified as the key driver shaping ammonia oxidizer community structure and abundance in soils and sediments (Chen et al. 2010; Shen et al. 2014). Soil texture varying in clay or sand content could be one of the reasons explaining the fluctuation in the size and structure of AOB and AOA, as well as their function, measured in different soil types (Wessen et al. 2011; Silva et al. 2012). Comparative analysis for nitrification capacity of different agricultural soils is therefore both useful and necessary for assessing N use efficiency and by extension, N management practices.

Concerns about the ecological niche of ammonia oxidizers and their relative contribution to ammonia oxidation have been raised since the breakthrough discovery of AOA.

Results from soil microcosm experiments suggested that ammonia oxidation was mainly driven by AOA, rather than AOB, under conditions of low ammonia (Offre et al. 2009; Zhang et al. 2010, 2012), thus favoring the growth of AOA in these conditions (Verhamme et al. 2011; He et al. 2012). Other reports have demonstrated that nitrification is predominantly driven by AOB rather than AOA in habitats such as alkaline upland soils (Shen et al. 2008), paddy soils (Wu et al. 2011), and nitrogen-rich grassland soils (Di et al. 2009, 2010). The main explanation for these studies was that high amounts of available ammonia, preferentially favor nitrification by, and hence growth of AOB (Schauss et al. 2009; Hu et al. 2014). However, the link between ecological niche separation feature of ammonia oxidizers and their related function of nitrification is less understood. The objective of this study was therefore to determine the effect of ammonium fertilizer on potential nitrification rates and abundances of AOA and AOB in three agricultural soils with contrasting pH via microcosm experiments. This allowed us to determine the response of soil nitrification capacity to these important factors, knowledge of which is necessary to improve our understanding of the microbial mechanism underlying ammonia oxidation.

2 Materials and methods

2.1 Soil sites description and sampling

Soil samples were collected from sites at the ShenYang (SY), FengQiu (FQ), and QiYang (QY) stations, which are all long-term fertilization stations located from Northeast to Southern China, and which are characterized by different climatic properties and soil types. The detailed information of QY, FQ, and SY experimental sites has previously been described in He et al. (2007) and Shen et al. (2008, 2010), respectively. Briefly, all three sites have been managed for more than 15 years with different combinations of fertilizer nitrogen (N), phosphorus (P), potassium (K), and organic manure (M). Based on our previous results investigating the nitrification capacity in the agricultural soils (He et al. 2007; Shen et al. 2008), we chose three different soil types with variable pH ranging from acid (pH 4.04) to alkaline (pH 8.65) for the microcosm experiments. For each soil type, we selected two soils (i.e., CK (Control, no fertilizer) and NPK treatments from QY site, CK and NPK treatments from FQ site, and CK and N+M treatments from SY site) with contrasting fertilization backgrounds in order to compare the response of nitrification rates and AOA/AOB abundance with varying soil properties within the same soil type. The main soil characteristics are listed in Table 1. All the soils used in this study were collected from the upper 20 cm and stored at 4 °C until passing through a 2.0-mm sieve for the analysis of soil characteristics and microcosm construction.

Table 1 The basic properties of QY, SY, and FQ soils for microcosm construction

Site	Location	Soil type	Clay (%)	Silt	Sand	Soil name	pH (H ₂ O)	Organic matter g kg ⁻¹	Previous fertilization regime
QY	26° 45' N 111° 53' E	Red soil	45.0	6.3	8.7	QY1	5.26	13.6	CK
						QY2	4.04	16.8	NPK
SY	41° 49' N 123° 34' E	Brown soil	18.1	7.1	24.8	SY1	6.34	16.2	CK
						SY2	5.82	19.3	N+M
FQ	35° 00' N 114° 24' E	Sandy loam	11.0	75.0	17.0	FQ1	8.65	4.21	CK
						FQ2	8.37	5.71	NPK

2.2 Soil microcosm construction

Six soils as listed in Table 1 from QY (QY1 and QY2), SY (SY1 and SY2), and FQ (FQ1 and FQ2) stations were used for soil microcosm construction. Microcosms consisted of 50-g fresh soil in 100-ml centrifuge tubes that were incubated at 25 °C for 1 week before the followed treatment. The soils were kept at 60 % of field water capacity before and throughout the incubation by weighing tubes at 1–2 day intervals and drop-wise addition of sterile distilled water to replace moisture lost during incubation. Ammonium sulfate (NH₄)₂SO₄ fertilizer was added to the soils at a rate of 0, 20, 150, and 300 µg NH₄⁺-N g⁻¹ soil in four replicates termed as No, N1, N5, and N10, respectively. Soil samples were collected at day 40, subsamples from which were immediately stored at -80 °C for DNA extraction. The remaining soil was stored at 4 °C until analysis of soil characteristics.

2.3 Soil chemical analysis and potential nitrification rate (PNR)

Soil nitrate and ammonium were extracted with 2 M KCl and measured with a Continuous Flow Analyzer (SAN++, Skalar, Holland). Potential nitrification rates (PNR) were measured using the chlorate inhibition method as described previously (Kurola et al. 2005; He et al. 2007). Briefly, 20 ml phosphate buffer solution (PBS) (8.0 g l⁻¹ NaCl; 0.2 g l⁻¹ KCl; 0.2 g l⁻¹ Na₂HPO₄; 0.2 g l⁻¹ NaH₂PO₄; pH 7.4) with 1 mM (NH₄)₂SO₄ was used to culture 5.0 g fresh soil in a dark incubator at 25 °C for 24 h, while 10 mM potassium chlorate was added to the buffer solution to inhibit nitrite oxidation during the incubation. The nitrite concentration in the extracted solution after 24 h was expressed as the potential nitrification rate.

2.4 PCR amplification of *amoA* genes

Nucleic acids were extracted from frozen soil samples using reagents and protocol described previously with a MoBio UltraCleanTM soil DNA isolation kit (San Diego, CA) (He et al. 2007). DNA was eluted in 80 µl of solution S5 and stored at -20 °C.

Real-time PCR was performed on an iCycler iQ 5 thermocycler (Bio-Rad) using reagents and concentrations described previously (He et al. 2007; Shen et al. 2008) with sets of primers targeting *amoA* genes of either AOB or AOA from the extracted DNA templates (Table 2). Template DNA was diluted tenfold and bovine serum albumin (BSA, 0.4 mg ml⁻¹) was added to PCR reactions to reduce the interference of humic acid. For quantification of bacterial *amoA* genes using a 5'-nuclease assay, the primers A189/*amoA*-2R' and the probe A337 labeled at the 5' end with the reporter dye FAM (6-carboxy-fluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) were used with HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) in a 25 µl-reaction mixture containing 1× PCR buffer, 2.0 mM MgCl₂, 200 µM of each dNTP, and 2.5 U HotStarTaq DNA polymerase. A dilution series of the known amount of a plasmid (Promega) containing the *amoA* gene of *Nitrosomonas europaea* ATCC 19718 was used as a standard (He et al. 2007). For archaeal *amoA* gene analysis using SYBR Green I detection, the primers Arch-*amoA*F/Arch-*amoA*R (Francis et al. 2005) were used with SYBR^(R) Premix Ex TaqTM (TaKaRa) and a dilution series of known amount of correct insert of plasmid was used as the standard as described in our previously work (He et al. 2007). A melting curve analysis was performed to confirm archaeal *amoA* gene product specificity after amplification by measuring fluorescence continuously as the temperature increased from 55 to 95 °C. Triplicate samples for each treatment were used for real-time PCR. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

2.5 Statistical analysis

Copy numbers were log-transformed as needed to normalize the distributions prior to statistical analysis. All statistical analyses were performed using SPSS version 11.5, and one-way analysis of variance (ANOVA) followed by S-N-K-test was used to check for quantitative differences between treatments. *P*<0.05 was considered to be statistically significant.

Table 2 Primers, probes, and PCR conditions used for real-time PCR amplification

Target group	Primer and probe	Sequence (5'-3')	Length of amplicon (bp)	Concn (nM)	Thermal profile	Reference
AOB	Primer A189	GGHGACTGGGAYTTCTGG	670	100	15 min at 95 °C, followed by 40 cycles of 60 s at 94 °C, 45 s at 57 °C and 45 s at 72 °C	(Okano et al. 2004)
	Primer <i>amoA</i> -2R'	CCTCKGSAAAGCCTTCTTC		100		
	Probe A337	TTCTACTGGTGGTCRC ACTACCCCATCAACT		120		
AOA	Primer arch- <i>amoA</i> F	STAATGGTCTGGCTTAGACG	635	100	94 °C for 2 min followed by 40 cycles of 45 s at 94 °C, 1 min at 53 °C, 45 s at 68 °C, plate read at 83 °C.	(Francis et al. 2005)
	Primer arch- <i>amoA</i> R	GCGGCCATCCATCTGTATGT		100		

3 Results

3.1 Soil chemical properties and potential nitrification rates

We observed a significant influence of ammonium sulfate amendment on ammonia oxidation rates in these three types of soils. For the QY soil, after 40 days of incubation, both QY1 and QY2 soils showed a positive correlation between N-loading and final ammonium concentration (Fig. 1). In contrast, no significant difference in ammonium concentration as a function of N-loading was observed for SY and FQ soils. One exception was seen in SY1 soil, in which the ammonium content in the Nh treatment (the highest N-loading) was significantly higher than that of other N-loading treatments. The nitrate content (NO_3^- -N) after 40 days incubation was positively correlated with N-loading in all the soils, with the exception of QY2 (Fig. 2a). The soil nitrate content in the higher N-loading treatments (Nm and Nh) was significantly higher than other two treatments in SY and FQ soils. Similarly, the PNR significantly increased with the increasing ammonium loading in the SY and FQ soils, although this increase was not significant in the QY soil (Fig. 2b). Furthermore, PNR increased on average 95 and 436 % in the middle (Nm) and high (Nh) N-loading treatments in the FQ soil, respectively, compared to the control treatment (No), while increases in PNR of 9 % (Nm) and 10 % (Nh) were observed for the SY soil. The highest PNR was observed in FQ soil microcosms, while the lowest was observed in QY soil microcosms (Fig. 2b).

3.2 Abundance of AOB and AOA in the soils

Using real-time PCR, we quantified AOB and AOA *amoA* gene numbers as a proxy for AOB and AOA abundance, respectively. AOB *amoA* gene abundance in the FQ soil ranged from 2.07×10^6 to 1.56×10^7 copy numbers per grams of dry soil and from 1.75×10^6 to 7.74×10^6 copy numbers per

grams of dry soil in the SY soil. These abundances were found to be significantly higher than AOB *amoA* gene abundance in the QY soil, which ranged from 9.24×10^5 to 9.14×10^5 copy numbers per grams of dry soil (Fig. 3). In both QY1 and QY2 soils, AOB *amoA* gene abundance was significantly higher in the Nh and Nm treatments than in the No control. In SY and FQ soils, no significant differences were observed in AOB *amoA* gene abundance among treatments, except in the FQ1 soil, which had a significantly higher AOB *amoA* gene abundance in the middle N-loading treatment (Nm).

The archaeal *amoA* gene copy numbers in FQ1 and QY2 soils, ranging from 7.11×10^5 to 4.49×10^6 copy numbers per grams of dry soil, were significantly lower than copy numbers detected in other soils, where the range was from 4.40×10^6 to 2.56×10^7 copy numbers per grams of dry soil (Fig. 3). There was no consistent trend in AOA *amoA* abundance along the treatment gradient of N addition for any soil. Neither was a significant difference in AOA *amoA* abundance detected among treatments for each soil with the exceptions of SY2 and FQ2 (Fig. 3). The abundance of AOA *amoA* was higher than AOB *amoA* except in the treatments of N1 and Nm for the FQ1 soil, in which the AOA:AOB ratio ranged from 58.77 to 1.03. The ratio of AOA to AOB decreased on average 51, 49,

Fig. 1 Soil ammonium concentration after 40 days incubation in three different soils amended with different amounts of ammonium sulfate. Treatments consisted of 0 (No), 20 (N1), 150 (Nm), and 300 (Nh) $\mu\text{g NH}_4^+$ -N g^{-1} soil. Letters above bars indicate a significant difference ($P < 0.05$) between treatments for each soil

Fig. 2 Soil nitrate concentration (a) and PNR (b) after 40 days incubation in three different soils amended with different amounts of ammonium sulfate. Treatments are the same as in Fig. 1. Letters above bars indicate a significant difference ($P < 0.05$) between treatments for each soil

and 17 % in the highest N-loading treatment (Nh) for the QY, SY, and FQ soils, respectively, compared to the control treatments (No) (Fig. 4).

3.3 Correlation analysis for soil properties and the abundance of ammonia oxidizers

In order to get a comparative analysis for nitrification capacity between soil types, correlation analysis was carried out based on each soil type. Both soil PNR and AOB *amoA* abundance were significantly positively correlated with nitrate content for all soil types (Table 3). A significant correlation between PNR and AOB *amoA* abundance, however, was only observed in the SY and FQ soils, while a significant correlation between NH_4^+ -N concentration and AOB *amoA* abundance was found only in the QY and SY soils. There was no significant relationship detected between AOA *amoA* abundance and other soil properties, with the exception of a negative correlation between AOA *amoA* abundance and nitrate content in the SY soil.

4 Discussion

In this study, the highest and lowest nitrification potential rates were found in the FQ and QY soils, respectively. Soil ammonia oxidation activity varied greatly among soils, partly due to

Fig. 3 Soil bacterial (AOB) and archaeal (AOA) *amoA* gene copy numbers after 40 days incubation in three different soils amended with different amounts of ammonium sulfate. Treatments are the same as in Fig. 1. Letters above bars indicate a significant difference ($P < 0.05$) between treatments for each soil

their different chemical and physical properties (Ste-Marie and Pare 1999). Soil pH affects the chemical form and availability of substrate for ammonia oxidation, thereby influencing microbial growth and activity (Kowalchuk and Stephen 2001). Previous studies have found that nitrification decreased or even ceased at pH below 7 and 4.5, respectively (Haynes 1986). Our previous findings from acid red soils have indicated a significant correlation between PNR and soil pH in a long-term field study (He et al. 2007). As demonstrated by a

Fig. 4 The ratio of AOA:AOB *amoA* abundance between treatments in three soil types. Treatments are the same as in Fig. 1. Letters above bars indicate a significant difference ($P < 0.05$) between treatments for each soil

Table 3 Spearman's correlation analysis between soil N content, PNR, and the abundance of *amoA* copy numbers

	QY			SY			FQ		
	PNR	NH ₄ ⁺	NO ₃ ⁻	PNR	NH ₄ ⁺	NO ₃ ⁻	PNR	NH ₄ ⁺	NO ₃ ⁻
PNR		—	*		*	**		—	**
AOB	—	**	*	**	*	*	*	—	**
AOA	—	—	—	—	—	*	—	—	—

Triplicates were used for correlation analysis for each treatment and there were 24 samples totally for each soil type

*Correlation is significant at the 0.05 level, **Correlation is significant at the 0.01 level, the *in bold means the correlation is negative correlated. PNR potential nitrification rate

systematic analysis for 23 typical Chinese soils, it is clear that the distribution of AOA communities is mainly governed by pH (Shen et al. 2012). Although no data of soil pH for all the soils after incubation was available, FQ and SY with higher pH showed higher PNR compared to QY acid red soil soils, indicating the differential response of PNR to soil pH. Additionally, nitrification activity is also strongly dependent on ammonia availability, which can be highly variable with soil type (Yao et al. 2013). It has been recognized that ammonia, but not the ammonium ion, is the direct substrate for autotrophic ammonia oxidation (Suzuki et al. 1974). Previous work has demonstrated that inorganic N fertilizer, such as NH₄Cl, has little effect on nitrification activity (Levicnik-Hofferle et al. 2012). Therefore, for the QY soil in this study, the availability of the ammonia may be limiting in low pH habitats due to the ionization of ammonia to ammonium.

Aside from pH, soil composition has been recognized as the primary determinant of bacterial community in arable soils (Girvan et al. 2003). In this study, the QY soil with higher relative clay content had lower PNR than the SY and FQ soils. A meta-analysis of over 700 samples from Scotland covering seven different soil types has demonstrated that factors such as land use and soil type had strong effects on the abundance and community composition of AOB and AOA by the creation of specific niches for these two functional guilds (Yao et al. 2013). It has been reported that soil clay content was negatively correlated to AOA abundance in samples across a 44-ha farm in Sweden (Wessen et al. 2011), while in the Netherlands was observed a positive correlation between soil clay content and AOB and AOA abundance for eight different soils (Silva et al. 2012). It appears therefore that soil type influences ammonia oxidation through variable rates of PNR irrespective of the magnitude of nitrogen input.

The higher PNR in the FQ and SY soils compared to QY soils may also due to the differential response of ammonia oxidizing microorganisms to N fertilizer addition. Comparative studies on the relative contribution of AOB and AOA to ammonia oxidation demonstrated that AOA have

a predominant role in acid soils, while AOB is responsible for the majority of ammonia oxidation in alkaline and N-rich neutral soils (Shen et al. 2012; Hu et al. 2014). Additionally, accumulated evidence from molecular investigations and physiological analyses of pure cultures has indicated low and high ammonia preferences of AOA and AOB, respectively (Martens-Habbena et al. 2009; Offre et al. 2009; Zhang et al. 2012). As such, large amounts of ammonium fertilizer provide a favorable substrate-rich habitat for AOB-dominated ammonia oxidation in neutral and alkaline soils (SY and FQ in this study), while caused an unfavorable niche for AOA-dominated acid soils (QY soil in this study). AOA has been shown to be sensitive to high ammonium concentrations (Martens-Habbena et al. 2009; Schleper 2010; Verhamme et al. 2011; He et al. 2012). Additionally, several microcosm studies provided strong evidence for AOA preference for mineralized ammonia instead of ammonium fertilizer (Zhang et al. 2010; Levicnik-Hofferle et al. 2012), which favors the growth of AOB (Di et al. 2009, 2010). These findings were corroborated by the observed small difference in AOA *amoA* abundance between different N-loading treatments in this study.

We observed a general trend of higher AOA than AOB abundance in soils irrespective of N input. This trend is clearly noticeable in QY soils, which is consistent with the previous observations of AOA dominance in a range of soils (He et al. 2007; Taylor et al. 2012). Moreover, the higher ratio of AOA to AOB in acid QY soils as compared to other non-acidic soils further confirmed the dominant role of AOA in acid soil habitats (He et al. 2007). However, our results showed a decreasing trend in the ratio of AOA: AOB with increasing levels of N input, indicating that growth rates of AOB in soils usually greatly outcompete the AOA growth rates when provided with larger amount of ammonium fertilizer. As PNR represents the potential ammonia oxidation activity, the significant relationship between PNR and AOB, but not AOA abundance for the SY and FQ soils, indicates the predominant role of AOB nitrification activity in neutral and alkaline soils.

5 Conclusions

Understanding the mechanisms underlying nitrification-associated loss of N fertilizer is increasingly driven by the desire to mitigate nitrous oxide emissions and reduce nitrate leaching. The niche differentiation of AOB and AOA, for which pH and ammonia concentration plays essential roles, could therefore provide important clues for agricultural fertilization management practices aimed at maximizing N use efficiency and minimizing nitrogen loss due to leaching. The results of this study indicated the responses of AOB and AOA to inorganic ammonium fertilizer addition in the FQ and SY soils were different from those in the QY soil. N addition increased nitrification activity in the FQ and SY soils via stimulating the growth of AOB, while little influence on the abundance of ammonia oxidizer in the QY soil. Future work about the effects of different fertilizer types and management regimes on nitrification capacity and associated microbial groups is necessary for a comprehensive understanding of the efficiency of applied N fertilizers in agroecosystems (Shen et al. 2013). Our investigation of PNR variability in different agricultural soils contributes to a greater understanding of the effects of N fertilizer management with regard to soil microbial community dynamics and biological nitrification.

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