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Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: A meta-analysis of field studies

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ABSTRACT

Quantification of functional genes involved in nitrogen (N) transformation improves our understanding of N-cycling microbial population responses to environmental disturbance. Agricultural N fertilization affects N-cycling gene abundances in soil, but the general patterns and variability of N cycling gene abundances in response to N fertilization have yet to be synthesized. We conducted a meta-analysis comprising 47 field studies in agricultural ecosystems. We included five marker genes important to N-cycling: *nifH* (encoding nitrogenase; key enzyme for N fixation), *amoA* (encoding ammonia monooxygenase; key enzyme for nitrification), *nirK* and *nirS* (encoding nitrite reductase; key enzyme for denitrification), and *nosZ* (encoding nitrous oxide reductase; key enzyme for denitrification). We found that N fertilization had no effect on the abundance of *nifH*, but significantly increased archaeal *amoA* (31%), bacterial *amoA* (313%), *nirK* (53%), *nirS* (40%) and *nosZ* (75%), respectively. N fertilizer form (inorganic versus organic) strongly affected the response of most selected N-cycling genes to N fertilization; organic fertilizers often had a much stronger effect than inorganic fertilizers. N fertilization duration, crop rotation, and soil pH were also important factors regulating the response of most N-cycling genes to N fertilization. Genes involved in nitrification and denitrification were significantly correlated with each other. Improvement in understanding of the response of N-cycling gene abundance to enhanced N input will help develop quantitative models of N availability and N fluxes and improve strategies for reducing reactive N gas emissions and N management in agricultural ecosystems.

1. Introduction

Nitrogen (N) fertilization is a common agricultural practice for improving crop growth and productivity. Human input of N to cropping systems has increased rapidly over the past decades to meet food and biofuel production needs (Robertson and Vitousek, 2009). However, excessive and repeated input of N increases nitrate leaching and reactive N gas production, resulting in adverse environmental and human health impacts (Fowler et al., 2013; Robertson and Vitousek, 2009). Therefore, reduction of N loss and improvements in crop N use efficiency are essential for the sustainability of agricultural ecosystems (Tilman et al., 2002; Zhang et al., 2015).

The N cycle is a complex biogeochemical cycle with many N-transforming processes, including N-fixation, nitrification, and denitrification (Kuypers et al., 2018; Stein and Klotz, 2016). Although most N-fixation is carried out by symbiotic bacteria in root nodules of legumes, free-living N-fixation is a potential source for biological N inputs in non-leguminous crops in agricultural systems (Hsu and Buckley, 2009; Roper and Gupta, 2016), and its rate ranges from 0 to 60 kg N ha^{-1} $year^{-1}$ (Cleveland et al., 1999; Gupta et al., 2014). Nitrification and denitrification lead to considerable N loss through nitrate leaching and reactive N gas production in agricultural soils (Norton and Stark, 2011; Philippot et al., 2007). N-transforming processes are largely driven by microbes. Functional gene markers are often used to describe the abundance and diversity of microbial communities responsible for the specific N transformation processes. The most commonly studied N cycling marker genes include *nifH* (encoding nitrogenase reductase) (Gaby and Buckley, 2011; Zehr et al., 2003), *amoA* (encoding ammonia monooxygenase) (Leininger et al., 2006; Pester et al., 2012; Rotthauwe et al., 1997), *nirK* and *nirS* (encoding nitrite reductase) (Braker et al., 1998; Henry et al., 2004), and *nosZ* (encoding nitrous oxide reductase) (Henry et al., 2006). Quantification

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and characterization of functional genes involved in the N biogeochemical cycle help link N-cycling microbial groups directly to actual N processes and improve understanding of the ecological significance of N-cycling traits in soil (Levy-Booth et al., 2014).

Many individual studies have examined the response of N-cycling gene abundance to N fertilization in agricultural ecosystems, but the results of these studies vary widely in direction and magnitude of change with N fertilization (Ai et al., 2013; Baudoin et al., 2009; Ouyang et al., 2016; Reardon et al., 2014; Wang et al., 2016; Yin et al., 2014). Several factors attributed to the variation in the response of N-cycling gene abundances to N fertilization. For example, mineral and organic N fertilization often result in distinct changes in N-cycling gene abundances (Hai et al., 2009; Kelly et al., 2011; Sun et al., 2015; Yang et al., 2017). Soil organic carbon (SOC) and pH also exerts a strong influence on the abundance and diversity of N-cycling genes (Hallin et al., 2009; Prosser and Nicol, 2012; Wang et al., 2017). Comparisons of primer performance for N-cycling genes reveal differences in primer coverage and specificity (Gaby and Buckley, 2012; Meinhardt et al., 2015; Penton et al., 2013), which may further result in the inconsistent changes of N-cycling gene abundances in response to N fertilization when different primer sets are used to quantify the same gene across studies. A quantitative synthesis of a variety of studies may help detect meaningful patterns and elucidate the underlying mechanisms of N fertilization effects on N-cycling gene abundance. A recent meta-analysis examined the impact of N fertilization on the abundance of ammonia oxidizing archaea (AOA) and bacteria (AOB), and found that AOB are more responsive than AOA to N fertilization. However, this study only included 12 field studies from agricultural ecosystems (Carey et al., 2016). In addition, there is no quantitative synthesis on the response of genes involved in N-fixation and denitrification to N fertilization in agricultural ecosystems.

In this study, we synthesized 47 field studies with 157 observations to investigate the influence of N fertilization on the abundance of microbial N-cycling genes in agricultural ecosystems. We aimed to address the following questions: (1) Does N fertilization change the abundance of genes involved in N-cycling in agricultural ecosystems? (2) What factors control the response of N gene abundances to N fertilization? (3) Is there any relationship among different N-cycling genes or between gene abundance and process rate under N fertilization?

2. Methods

2.1. Data collection

We used ISI Web of Knowledge, Google Scholar and cross-referencing to search for relevant peer-reviewed studies published by March 2018. Studies were selected according to the following criteria: (1) real-time or quantitative PCR was used to measure the abundance of functional genes involved in the N cycle (e.g., *nifH*, *amoA*, and *nirK*); (2) N treatment and control plots were established under the same climate, soil and vegetation conditions to avoid confounding factors; (3) N fertilization rate was clearly reported; (4) the means, standard deviations and sample sizes of the target variables were directly reported or could be calculated; (5) only field experiments in agricultural ecosystems were included. Data were either collected from table and text, or from figures using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/). We aimed to collect all available N-cycling genes, but only *nifH*, archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, and *nosZ* had enough observations for this meta-analysis. A total of 157 observations from 47 peer-reviewed studies were collected for N-cycling gene abundances.

Meta-analysis requires that data sets are independent. Therefore, we only included values from the topsoil when data from several soil layers were reported. When studies measured N-cycling gene abundances repeatedly through time, the time points with reported soil properties (e.g., pH and SOC) and process rates were preferentially chosen, otherwise, datasets from the longest time point after fertilization were used for the meta-analysis to reduce the immediate effect of N fertilization. For studies with multiple agricultural management factors being manipulated (e.g., irrigation), we only extracted data from control plots and N fertilization plots.

For each study, we collected experimental information, including N fertilizer form, rate, and duration; crop type; cropping systems; tillage; and primer set for each gene. We also extracted SOC, total N, pH, N-fixation rate (NFR), nitrification potential (NP), denitrification enzyme activity (DEA), and location (latitude and longitude) when these data were reported along with the N-cycling gene abundances. In our dataset (Supporting data), P and K fertilizers were often added together with N; field plots were tilled with conventional practice in most experiments; most crops were non-leguminous grain crops. Since there were only three studies measured N-cycling gene abundances in the rhizosphere (Ai et al., 2013; Hai et al., 2009; Wang et al., 2017), our meta-analysis focused on the bulk soils, but the comparison between bulk soil and rhizosphere was also included in Table S1. Individual variables were further classified into categories. The following categorical groups were established. The N fertilizer form was grouped into inorganic N (IN; e.g., urea, NH_4^+, NO_3^-, NH_4NO_3), organic N (ON; e.g., compost and manure), and mixed use of inorganic N and organic N (IN & ON). N fertilization rate was grouped into $< 100\,kg\,N\ ha^{-1}\ yr^{-1}$, $100–200\,kg\,N\ ha^{-1}\ yr^{-1}$, and $> 200\,kg\,N\ ha^{-1}\ yr^{-1}$. N fertilization duration was divided into < 5 years, 5–20 years, and > 20 years. Cropping system was categorized into monoculture and rotation. Soil pH was divided into < 6, 6–7, 7–8, and > 8. If different primer sets were used to quantify the same genes, observations were grouped by primer set (Table S2). Categorical variable levels were sometimes combined into a new category if the sample size was small.

2.2. Data analysis

For each observation, the response ratio (RR) was used as effect size in the meta-analysis:

$$RR = In\left(\frac{\overline{X}_t}{\overline{X}_c}\right) \quad (1)$$

Where $\overline{X}_t$ and $\overline{X}_c$ are the means of the N fertilized treatment and control, respectively. The variance of effect size was calculated using Eq. (2):

$$v = \frac{s_t^2}{n_t \overline{X}_t^2} + \frac{s_c^2}{n_c \overline{X}_c^2} \quad (2)$$

Where s_t and s_c represent standard deviation of treatment and control groups, respectively; n_t and n_c were the sample sizes for the treatment and control groups, respectively.

The weighted response ratio (RR_{++}) and 95% bootstrap confidence interval (CI) were calculated using openMEE (Wallace et al., 2017) with the Hedges-Olkin random model. The RR_{++} was considered to be significant if the 95% CI did not overlap zero. We also transformed the RR_{++} to percentage change to evaluate the effect directly using Eq. (3):

$$\text{Percentage change (\%)} = (e^{RR++} - 1) \times 100\% \quad (3)$$

Subgroup analysis was also used to examine the effect of N fertilization on selected variables under different groups. Conventional heterogeneity statistics (Q-statistics) were used to test between-group heterogeneity (Q_M) (Wallace et al., 2017). The RR_{++} and 95% CI for each subgroup were calculated. Pearson correlation was used to explore the relationship among the RRs of soil properties, process rates, and genes.

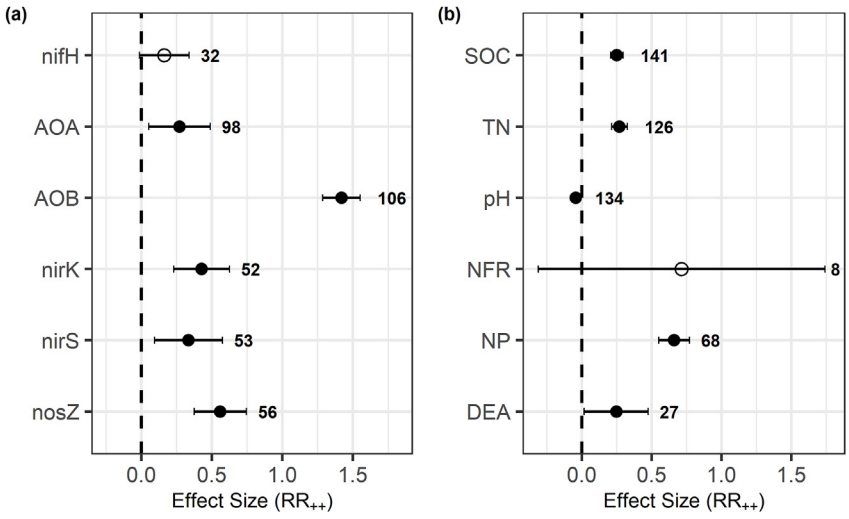

Fig. 1. Effect of N fertilization on (**a**) N cycling gene abundance and (**b**) soil properties and N process rates. Points are the effect size (weighted response ratio). Error bars represent 95% confidence intervals (CIs). The sample size of each variable is presented beside each bar. Filled circles indicate that the effect of N fertilization is significant ($p < 0.05$) at 95% CIs. Abbreviation: SOC, soil organic carbon; TN, total nitrogen; NFR, nitrogen fixation rate; NP, nitrification potential, DEA, denitrification enzyme activity.

3. Results

3.1. Overall effect of N fertilization on N-cycling gene abundances and soil properties

Across all studies, N fertilization had no effect on the abundance of *nifH*, but significantly increased AOA (31.1%), AOB (313%), *nirK* (53.3%), *nirS* (39.7%) and *nosZ* (75.1%) (Fig. 1a). As for the matched soil properties and N transformation rates, N fertilization significantly increased SOC and TN by 28.4% and 30.7%, respectively, but significantly decreased soil pH by 4.4% (Fig. 1b); N fertilization significantly increased NP and DEA by 93.7% and 27.9%, respectively, while NFR showed no significant change in response to N fertilization (Fig. 1b).

3.2. Impact of categorical variables on N-cycling gene abundances

N fertilizer form and soil pH significantly affected the response of *nifH* abundance to N fertilization (Fig. 2, Table 1). The application of organic N alone or mixed together with inorganic N significantly increased *nifH* abundance. The *nifH* abundance positively responded to N fertilization when N fertilization duration was less than five years, or when the soil pH was higher than 6. However, N fertilization rate, cropping system, and primer did not influence the response of *nifH* abundance to fertilization. The *nifH* abundance in the rhizosphere was significantly reduced by N fertilization (Table S1).

N fertilizer form and soil pH significantly affected the response of AOA to N fertilization (Fig. 3a; Table 1). The addition of organic N significantly increased AOA abundance while inorganic N had no effect on AOA abundance. N fertilizers significantly enhanced AOA abundance when soil pH was higher than 6. Interestingly, AOA abundance positively responded to N fertilization with the primer set arch-amoAF/R, while it showed no N fertilization effect with CrenamoA23F/616R and 19F/643R. For studies used the primer set arch-amoAF/R, the similar pattern of AOA abundance in response to N fertilization was observed (Fig. S1). The N fertilizer form, N fertilization rate, N fertilization duration, cropping system and soil pH significantly influenced the response of AOB abundance to N fertilization (Fig. 3b; Table 1). Overall, the greatest increase in AOB abundance in response to N fertilization was detected under the application of both inorganic N and organic N together, under crop rotation, and in the pH range of 7–8. Low N fertilization rate ($< 100\ N\ ha^{-1}\ yr^{-1}$) had no effect on AOB abundance.

N fertilizer form and cropping system significantly affected the response of *nirK*, *nirS* and *nosZ* abundances to N fertilization (Fig. 4, Table 1). The application of organic N both alone and mixed with inorganic N often significantly increased all three genes. Compared with monoculture, crop rotation had a stronger N fertilization effect on the abundance of these three genes. Primer set also significantly influenced the response of *nirK* to N fertilization. There was no N fertilization effect

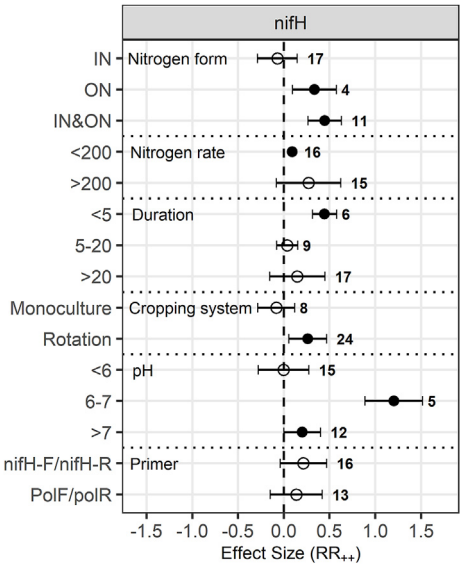

Fig. 2. N fertilization effects on *nifH* abundance under different categories. Points are the effect size (weighted response ratio). Error bars represent 95% confidence intervals (CIs). The sample size of each variable is presented beside each bar. Filled circles indicate that the effect of N fertilization is significant ($p < 0.05$) at 95% CIs. The unit of N application rate and duration are kg ha^{-1} $year^{-1}$ and year, respectively.

Table 1
Between group variability (Q_M) indicating the effect on N fertilization on N cycling gene abundance across categorical variables. Asterisks highlight significant p values (**p < 0.001,*p < 0.05).

Categorical variables	*nifH*	AOA	AOB	*nirK*	*nirS*	*nosZ*
N form	14.7**	6.09*	42.8**	18.3**	7.61*	17.7**
N amount	0.97	0.26	103**	4.65	1.36	8.87*
Duration	3.34	0.26	19.5**	1.96	8.66*	14.3*
Cropping system	3.13	0.28	256**	12.4**	10.6**	15.4**
pH	50.5**	21.3**	57.9**	0.38	33.1**	14.4*
Primer	0.24	3.91	1.15	11.6**	N/A	1.75

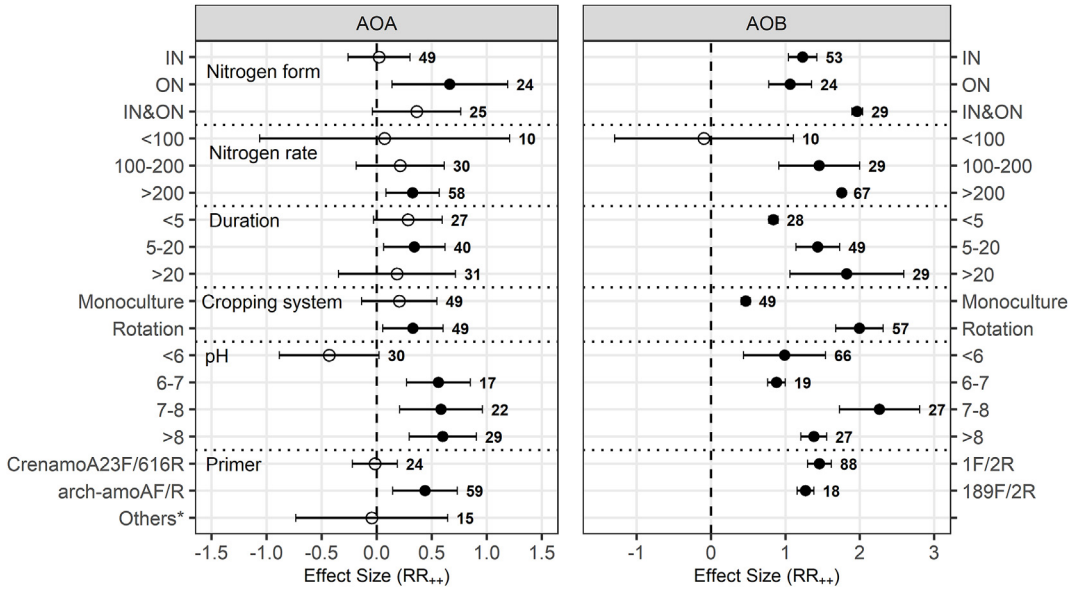

Fig. 3. N fertilization effects on AOA and AOB abundance under different categories. Points are the effect size (weighted response ratio). Error bars represent 95% confidence intervals (CIs). The sample size of each variable is presented beside each bar. Filled circles indicate that the effect of N fertilization is significant ($p < 0.05$) at 95% CIs. The unit of N application rate and duration are kg ha^{-1} $year^{-1}$ and year, respectively. "Others*" indicates the combined primer sets for AOA, including 19F/616R and16F/643R.

on *nirK* when nirk876/1040R was used to quantify *nirK* abundance, but a significant N fertilization effect was detected when other *nirK* primers (e.g., F1aCu/R3Cu) were used. N fertilization duration and soil pH also significantly affected the response of *nirS* and *nosZ* to N fertilization; effect sizes of *nirS* and *nosZ* were greatest when N was added for 5–20 years or when pH was higher than 8.

3.3. Correlation analysis

SOC was significantly correlated with *nifH*, *nirK*, *nirS*, and *nosZ* abundances (Table 2). The soil pH was also significantly correlated with AOB, AOA, *nirS*, and *nosZ* abundances. Genes involved in nitrification and denitrification were significantly correlated with each other (Table 2). Except for *nifH* and *nirK*, other genes were positively and significantly correlated with their corresponding processes (Table 3).

4. Discussion

Quantification of functional genes involved in N transformation provides useful information on the dynamic of N-cycling microbial populations, especially uncultured organisms, in response to N fertilization. Uncertainties still remain across studies regarding the effect of N fertilization on the abundance of genes involved in N-fixation (Reardon et al., 2014; Wang et al., 2016), nitrification (Carey et al., 2016) and denitrification (Baudoin et al., 2009; Yin et al., 2014). Our meta-analysis across 47 field studies showed that N fertilization had no effect on

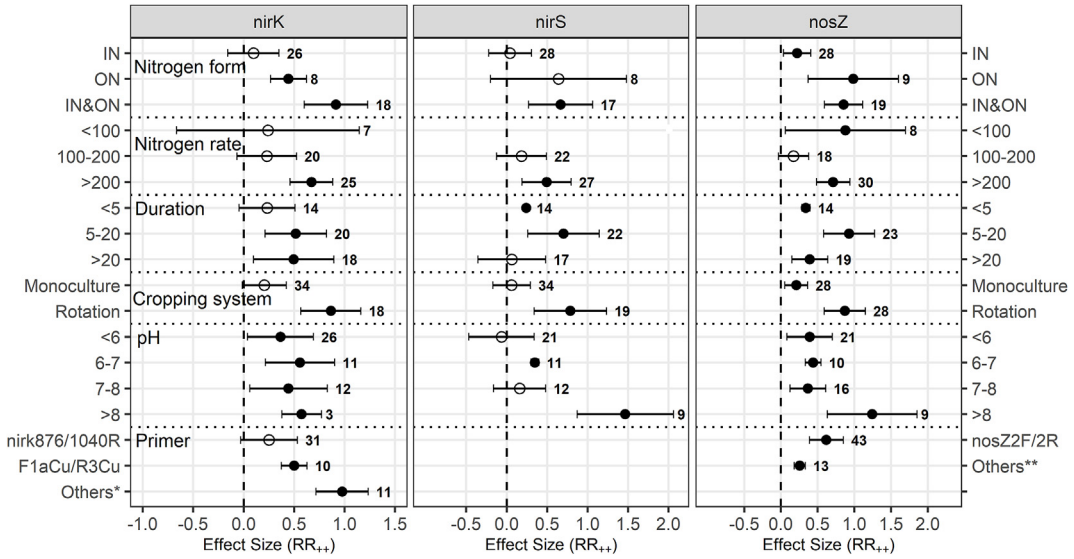

Fig. 4. N fertilization effects on *nirK, nirS* and *nosZ* abundance under different categories. Points are the effect size (weighted response ratio). Error bars represent 95% confidence intervals (CIs). The sample size of each variable is presented beside each bar. Filled circles indicate that the effect of N fertilization is significant ($p < 0.05$) at 95% CIs. The unit of N application rate and duration are kg ha^{-1} $year^{-1}$ and year, respectively. "Others*" indicates the combined primer sets for *nirK*, including nirK1F/5R and nirK876/5R. "Others**" indicates the combined primer sets for *nosZ*, including 1126qF/1318R, nosZ-F/1662R, nosZ1F/1R, and nosZ-F/nosZ-R.

Table 2
Pearson correlation coefficients between effect size (RR) of soil properties and N cycling gene abundance. Numbers in bold font indicate significant correlation coefficients at $p < 0.05$. The number of observations is given in parentheses.

	nifH	AOB	AOA	*nirK*	*nirS*	*nosZ*
SOC	**0.57 (32)**	0.14 (93)	0.01 (87)	**0.40 (44)**	**0.32 (45)**	**0.45 (48)**
TN	**0.40 (32)**	0.05 (78)	−0.17 (72)	**0.33 (44)**	−0.13 (45)	0.01 (48)
pH	0.27 (32)	**0.24 (87)**	**0.53 (81)**	0.19 (43)	**0.42 (42)**	**0.30 (41)**
nifH		−0.25 (14)	0.01 (19)	**0.94 (5)**	**0.95 (5)**	**0.88 (5)**
AOB			**0.49 (93)**	**0.72 (33)**	**0.57 (36)**	**0.58 (36)**
AOA				**0.53 (27)**	**0.86 (33)**	**0.75 (33)**
nirK					**0.78 (45)**	**0.89 (43)**
nirS						**0.88 (45)**

Table 3
Pearson correlation coefficients between effect size (RR) of N cycling gene abundance and corresponding process rates. Numbers in bold font highlight significant p values ($p < 0.05$).

gene	process	r	n	p
nifH	NFR	0.33	8	0.43
AOA	NP	**0.36**	60	0.004
AOB	NP	**0.32**	63	0.01
nirK	DEA	0.36	27	0.08
nirS	DEA	**0.68**	24	< 0.001
nosZ	DEA	**0.59**	18	0.01

AbbreviationNFR, nitrogen fixation rate; NP, nitrification potential, DEA, denitrification enzyme activity.

the abundance of genes involved in N-fixation, while it significantly and positively increased the abundance of genes involved in nitrification and denitrification. We also identified several factors regulating the direction and magnitude of N-cycling gene abundances in response to N fertilization, including N fertilization form and duration, cropping system, soil pH, and primer set.

4.1. Impact of N fertilization on N-cycling gene abundances

In our meta-analysis, N fertilization did not affect either *nifH* abundance or NFR in bulk soils in agricultural ecosystems. However, a recent quantitative synthesis reported that free-living NFR was significantly suppressed by N fertilization in natural terrestrial ecosystems (Dynarski and Houlton, 2018). The lack of significant effects of N fertilization on NFR in our study could be due to the small sample number (n = 8), but there was also notably large variability among studies, suggesting not that NFR responds little to N fertilization, but that the response is contingent on other factors. The response of *nifH* abundance and NFR to N fertilization may also be different between agricultural and natural ecosystems. Legumes are often rotated in agricultural ecosystems to increase crop yields (Smith et al., 2008) and primers for *nifH* do not differentiate between symbiotic and free-living diazotrophs (Gaby and Buckley, 2012). Thus, our *nifH* abundance data likely includes the abundance of symbiotic N-fixers, which live in plant-controlled environments and therefore are less likely to respond to environmental change (Smercina et al., unpublished data). It is also important to note that we include both organic and inorganic N fertilizers in our meta-analysis and that inorganic N fertilizers are often applied together with phosphorus (P) in agricultural ecosystems, while organic fertilizers include readily accessible C for microbes. The inclusion of P and micronutrients as well as C with IN&ON fertilization may help explain the observed positive effects on *nifH* abundance as N-fixers are often C and P limited (Dynarski and Houlton, 2018; Reed et al., 2011).

N fertilization significantly increased AOA and AOB abundances in agricultural soils. Furthermore, AOB were much more responsive than AOA to N fertilization; effect size of N fertilization on AOB was 9 times higher than that of AOA. These findings are in line with a previous meta-analysis of AOA and AOB (Carey et al., 2016). AOB have larger cell sizes than AOA (Lehtovirta-Morley et al., 2016; Prosser and Nicol, 2012). AOB and AOA have distinct ammonia oxidation pathways (Könneke et al., 2014; Kozlowski et al., 2016). These may affect their physiological responses to ammonium availability (Martens-Habbena et al., 2009; Ouyang et al., 2017). Ouyang et al. (2017) found that the maximum activity (V_{max}) and half saturation constant (K_m) of AOB were 10–20 times and 15–40 times higher, respectively, than those of AOA in an agricultural soil treated with ammonium fertilizers. This difference in ammonia oxidation kinetics may explain the much greater response of AOB than AOA to N fertilization in our meta-analysis. The implication of our finding is that AOB is a more important target group for N management to reduce N loss and improve N use efficiency. Physicochemical approaches such as urea and nitrification inhibitors and plant-based approaches such as breeding plants to secrete nitrification inhibiting root exudates may be essential management strategies for controlling the growth of AOB after N fertilization in the field (Hu and He, 2018).

Similar to AOA and AOB, the abundance of genes involved in denitrification was also significantly increased by N fertilization. Genes involved in nitrification and denitrification were significantly correlated with each other, largely because a similar N fertilization effect was observed for these genes. Many studies often see no relationship between nitrifier and denitrifier gene abundances, which is generally attributed to these groups having very different life strategies (e.g. autotrophic versus heterotrophic; aerobic versus anaerobic) and therefore different mechanisms controlling changes in population dynamics (Jin et al., 2014; Kastl et al., 2015; Ruiz-Rueda et al., 2009; Szukics et al., 2010). For example, as soil water-filled pore space increases, nitrifier abundances generally decreases (AOB and AOA) while denitrifier abundance (*nirK* and *nosZ*) increases (Gleeson et al., 2010; Ligi et al., 2014). The simultaneous increase in both nitrifier and denitrifier gene abundances and process rates with N fertilization could help explain relatively high rates of N loss and inefficient use of N fertilizer by crops (Liang and MacKenzie, 1994; Robertson and Vitousek, 2009).

4.2. Factors controlling the response of N-cycling gene abundances to N fertilization

N fertilization form is a very important factor influencing the response of N-cycling gene abundances to N fertilization. The application of organic N fertilizers often had a much stronger effect than inorganic N fertilizers. A meta-analysis found that organic amendments increased soil microbial biomass C and N by 36% and 27%, respectively, across 414 observations (Kallenbach and Grandy, 2011). Organic fertilizers provide SOC and nutrients for soil, which supports the growth of microbial populations. Soil N-cycling microbial populations will also likely increase with overall increases in soil microbial biomass under organic N fertilization, especially for heterotrophs, such as denitrifiers. This is also supported in our study by the significant correlation between SOC and genes involved in N-fixation and denitrification.

Another meta-analysis reported that repeated application of inorganic N fertilizers also increased soil microbial biomass C by 15% across 107 observations in cropping systems (Geisseler and Scow, 2014). However, in our study, we found that inorganic N fertilizers only increased AOB and *nosZ* abundances, but had no effect on other genes.

In our meta-analysis, organic fertilizers significantly increased both AOA and AOB abundances; organic and inorganic N fertilizers had a similar effect on AOB abundance. However, another meta-analysis showed that N fertilization form affected the response of AOB rather than AOA to N fertilization (Carey et al., 2016). It also found that organic N fertilizers had no effect on AOA, and had a smaller effect on AOB than inorganic N fertilizers. Such inconsistencies may be due to the differences between agricultural and natural ecosystems. Their meta-analysis also reported that AOA and AOB respond more strongly in unmanaged wildland soils than agricultural soils (Carey et al., 2016). Our result implies that organic fertilizer may not be as desirable and effective a practice to control nitrate production in cropping systems as was previously thought (Bhattacharyya et al., 2007; Sun et al., 2015), since it stimulated the growth of both AOA and AOB.

Soil pH also significantly influenced the response of N-cycling genes to N fertilization. Previous studies reported that soil pH strongly affects overall microbial community composition (Lauber et al., 2009; Rousk et al., 2009). Soil pH also exerts a strong influence on the abundance and diversity of N-cycling genes (Hallin et al., 2009; Hu et al., 2013; Liu et al., 2010; Prosser and Nicol, 2012). In our study, we found that soil pH often significantly correlated with N-cycling gene abundances. N fertilization had no effect on *nifH*, AOA, and *nirS* when pH was below 6, while it significantly increased gene abundance at higher pH values. Geisseler and Scow, 2014 also concluded that N fertilization strongly increased microbial biomass with a soil pH above 5. Interestingly, the soil pH range corresponding to the greatest response of N-cycling genes to N fertilization varies among different genes. For example, responses of *nifH* to N fertilization were greatest with a pH between 6 and 7; AOB were between 7 and 8; and *nirS* and *nosZ* were with a pH higher than 8. This indicates that different N-cycling populations may have different optimum soil pH affecting the response of growth to N fertilization.

The response of N-cycling gene abundances to N fertilization often varied with fertilization duration and under cropping systems. *nifH* and *nosZ* showed the greatest positive change when fertilization duration was less than 5 years and between 5 and 20 years, respectively, while AOB showed a stronger change when fertilization duration was higher than 20 years. This demonstrates the different sensitivities of N-cycling functional groups in response to N fertilization duration. Interestingly, AOB and denitrifiers' responses to N fertilization under crop rotation were stronger than those under monoculture. Because rotations increases SOC, soil total N, and microbial biomass C and N (McDaniel et al., 2014), the large C and N input to soils may explain the stronger response of AOB and denitrification genes to N fertilization under crop rotation.

In our study, PCR primer set strongly affected the response of AOA and *nirK* to N fertilization, largely because multiple forward and reverse primer sets were used to quantify these two genes. We also found that only *nirS* was quantified with the same primer set across various studies. Most of the current primers for N-cycling functional genes were designed based on relatively few references and therefore often had very low overall coverage and preferentially targeted certain clusters of functional populations (Helen et al., 2016; Penton et al., 2013). Therefore, it is important to note that qPCR data obtained from the current primers may largely underestimate N-cycling gene abundances and only capture the responses of certain sub-groups of microbial populations to N fertilization. The primer effect observed in our meta-analysis may imply that different clusters of AOA and denitrifiers had different sensitivity to N fertilization.

Several past studies have compared the performance of different primer sets for N-cycling genes (Bonilla-Rosso et al., 2016; Gaby and Buckley, 2012; Helen et al., 2016; Meinhardt et al., 2015; Penton et al., 2013). For example, Gaby and Buckley, 2012 reported that the nifHF/nifHR and PolF/PolR primer set only covered 26% and 25% of *nifH* diversity in their curated database. Soil AOA abundance showed differences in the order of magnitude among certain primer sets (Meinhardt et al., 2015). The most commonly used *nirK* and *nirS* primers preferentially targeted the *Proteobacteria* and that their primer coverages were very low (Bonilla-Rosso et al., 2016; Helen et al., 2016; Penton et al., 2013). It is unrealistic to comprehensively cover the diversity of N-cycling genes, such as *nifH* and *nirK*, with a single primer pair (Penton et al., 2013). A desirable solution is to develop environment-specific and clade-specific primers (Bonilla-Rosso et al., 2016; Helen et al., 2016). Together, these findings demonstrate that greater efforts are needed to improve the coverage and specificity of primers for N-cycling gene amplification.

Rhizosphere represents an important hotspot for microbial biomass and activity due to the continuous input of root exudates and rhizodeposits from plants (Kuzyakov and Blagodatskaya, 2015). Surprisingly, there were only three studies quantified the abundance of N-cycling genes in the rhizosphere under N fertilization (Ai et al., 2013; Hai et al., 2009; Wang et al., 2017). Direct comparison between the bulk soil and rhizosphere showed that abundances of N-cycling genes, such as *nifH* and AOA, in the rhizosphere were significantly higher than those in the bulk soil under N fertilization (Ai et al., 2013; Wang et al., 2017). Interestingly, our meta-analysis showed that N fertilization significantly suppressed the *nifH* abundance in the rhizosphere (Table S1), while N fertilization had no effect on the *nifH* abundance in the bulk soil. This suggests that N-fixers are more sensitive to N addition in the rhizosphere and the application of N fertilization may reduce the potential of biological N input from free-living N-fixation. In addition, N fertilization stimulated the growth of nitrifiers and denitirifers in the rhizosphere relative to the bulk soil (Table S1). Again, these patterns were obtained from less than ten observations. More studies should be conducted to examine how N fertilization influences N-cycling gene abundances in the rhizosphere.

4.3. Linkages between N-cycling gene abundances and process rates

We found that AOA, AOB, *nirS* and *nosZ* were significantly correlated with corresponding process rates. This is consistent with a meta-analysis examining relationships between gene abundance and corresponding process (Rocca et al., 2015) that found that narrow processes such as nitrification and denitrification have a stronger correlation between gene abundance and process rate than broad processes (Rocca et al., 2015). However, in their meta-analysis Carey et al. (2016) reported that AOB rather than AOA were significantly correlated with NP. This inconsistency could be due to the limited number of agricultural field studies (n = 12) included in their meta-analysis, and which therefore underestimates the contribution of AOA to NP under organic fertilizers. We also found that *nifH* and *nirK* were not correlated with corresponding process rates. In addition, even though the other four genes had a significant relationship with process rates, correlation coefficients were relatively low. Low coverage of current primers mentioned above may be an important contributing factor. An ongoing project in our group is developing high-throughput qPCR (Zhu et al., 2013) for N-cycling functional genes to capture a more comprehensive coverage of N-cycling gene abundances. The improved diversity of N-cycling genes may be able to more robustly predict soil N processes.

5. Conclusions

We conducted a meta-analysis to determine the response of N-cycling gene abundances to N fertilization in agricultural ecosystems. We found that N fertilization had no impact on the abundance of *nifH*, but significantly increased AOA, AOB, *nirK*, *nirS* and *nosZ*. N fertilization form and duration, crop rotation, and soil pH were important factors regulating the response of N-cycling genes to N fertilization. AOA, AOB,

nirS and *nosZ* were significantly correlated with their corresponding process rates. However, our analysis included only most studied N-cycling genes involved in N-fixation, nitrification and denitrification. More studies are needed to assess other involved genes such as comammox (Pjevac et al., 2017) and fungal denitrification (Chen and Shi, 2017). In addition, genes involved in other N-cycling transformations are needed for further examination, such as N mineralization (Ouyang et al., 2018) and anammox (Shen et al., 2013). Comprehensive quantification of N-cycling gene responses to N fertilization will help develop more accurate models of N availability and N flux and improve strategies for reactive N gas emissions and N management in agricultural ecosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.08.024.

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