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Long-term nitrogen fertilization increases both potential denitrification activity and $N_2O/(N_2O+N_2)$ product ratio by altering activities and populations of denitrifying communities: a global meta-analysis

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Abstract

Excessive application of nitrogen (N) fertilizer has remarkably changed the global N cycle by doubling the input of reactive nitrogen (N) to the environment. Denitrification is one of the most important biological processes by which reactive N is removed in the biosphere, but the responses of potential denitrification activity (PDA) and associated gene abundances to long-term N fertilization are poorly understood. We compiled data from 1041 observations across 62 studies and quantified the effects of long-term N input on PDA, the denitrification $N_2O/(N_2O+N_2)$ product ratio, and abundances of denitrifying communities using meta-analysis. Long-term N fertilization significantly increased potential denitrification activity by 75.9% and increased the relative abundances of *nirK, nirS, and nosZ* gene copies by 60.4%, 77.0%, and 25.1%, respectively. Further, long-term N loading increased the denitrification $N_2O/(N_2O+N_2)$ ratio by 22.1% and *nir(K+S)/nosZ* ratios by 27.7%, respectively. The effect of long-term N fertilization on potential denitrification activity was positively correlated with soil organic carbon (SOC) (R^2=0.11; n=132; $P < 0.001$), while long-term N loading increased the SOC by 29.0% compared to the unfertilized control. The responses of *nirK*, *nirS*, and *nosZ* abundances to long-term N fertilization were positively correlated with soil pH, whereas N loading decreased the soil pH by 4.6%. Thus, we postulate that long-term N fertilization might have a double effect on the activities and populations of denitrifying communities due to the increased SOC and decreased soil pH. This stimulates denitrification-derived N_2O emissions by increasing both the denitrification activity and the denitrification end-product proportion of N_2O. Reducing excessive N

fertilization in intensive agricultural areas might cut N_2O emissions directly by reducing substrate for denitrification, and indirectly by increasing the reduction of N_2O to N_2 during denitrification.

Keywords: Potential denitrification activity; denitrifying; long-term N fertilization; N_2O/ (N_2O+N_2) ratio; soil properties.

1. Introduction

Denitrification is an anaerobic microbial process that includes all or part of the sequential reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) (Firestone, 1982; Bakken et al., 2012; Almaraz et al., 2020). It is catalyzed by several enzymes, including nitrate reductase, nitrite reductase, nitric oxide (NO) reductase, and N_2O reductase, which are encoded by the genes *narG*/*napA*, *nirK*/*nirS*, *norB*, and *nosZ*, respectively (Philippot et al., 2007). Denitrification is of great interest because it converts 30%–60% of reactive N (Nr) back into N_2 in terrestrial ecosystems (Davidson and Seitzinger, 2006). Though N_2 is the ultimate end product of denitrification, N_2O is often generated through an incomplete denitrifying pathway (Bouwman et al., 2002; Baggs, 2011; Butterbach-Bahl and Dannenmann, 2011). N_2O is a potent greenhouse gas that has the potential to destroy the ozone layer. Atmospheric N_2O concentrations have also increased by approximately 20% relative to pre-industrial levels (Montzka et al., 2011). Regulating the denitrification rate and its product stoichiometry (i.e., the $N_2O/(N_2O+N_2)$ ratio) has been proposed as a promising N_2O mitigation strategy (Bakken et al., 2012; Butterbach-Bahl et al., 2013). Nevertheless, due to extreme methodological difficulties in the analytical detection of N_2 emissions and the large spatial and temporal heterogeneity, the factors that control denitrification rates are currently poorly understood (Davidson and Seitzinger, 2006; Butterbach-Bahl et al., 2013; Almaraz et al., 2020). Several studies have reported that denitrification activity is correlated with denitrifying community diversity or abundance (Hallin et al., 2009). In contrast, many other studies have shown that the abundance or diversity of

denitrifiers is uncoupled with the variation in denitrification due to functional redundancy (Wertz et al., 2007), whereas denitrification activity and product stoichiometry are mainly affected by abiotic factors, such as the concentrations and availability of labile C, NO_3^-, and O_2, soil texture, soil organic carbon (SOC) concentrations and pH (Attard et al., 2011; Liu et al., 2014; Samad et al., 2016).

Globally, approximately 425 Tg of reactive N is fixed from unreactive atmospheric N_2 annually (IPCC, 2013). This is double that of the pre-industrial period, with the increase being driven by human activity. Specifically, this increase had largely been driven by the excessive application of N fertilizers in agricultural systems (Dimkpa et al., 2020; Scheer et al., 2020; Wang et al., 2020). As N is a limiting nutrient for plants in most terrestrial ecosystems (Vitousek et al., 2002), long-term reactive N fertilization typically enhances primary production and C storage in biomass (Tilman, 1987; Gough et al., 2011). However, N fertilization has multiple effects on microbially mediated belowground C and N dynamics, depending on the ecosystem and the length of exposure to reactive N (Dai et al., 2018; Zeng et al., 2016). Geisseler and Scow (2014) found that the long-term application of mineral fertilizers increases microbial biomass and SOC in cropping systems. However, several studies based on unmanaged ecosystems have shown that N fertilization strongly reduces soil microbial activity and microbial biomass (Treseder, 2008; Ramirez et al., 2012). Furthermore, studies focusing on the influence of long-term N loading on denitrification activity, denitrification stoichiometry, and denitrifier abundance remain fragmented. For instance, Hallin et al. (2009) showed that a 50-yearfertilization application period with

added mineral N significantly reduced potential denitrification rates and *nirK*, *nirS*, and *nosZ* gene abundance. Similarly, Liang et al. (2020) showed that more than 100 years of N fertilization reduced the temporal turnover of functional communities involved in denitrification. In contrast, several studies have found that N fertilization significantly increases *nirK*, *nirS*, and *nosZ* gene abundances (Ouyang et al., 2018). Consequently, there is currently no holistic understanding of how long-term excessive N exposure affects soil denitrification activity, soil denitrifier abundance, and community composition (Jia et al., 2020).

Understanding the response of denitrification functionality to long-term N loading is a basic premise for solving the "missing N" in N balance, and for the mitigation of the undesirable ''cascading effects'' of reactive N (Galloway et al., 2003). Our objective in this study was to answer the following questions: (1) how will denitrification potential, denitrification product stoichiometry, and the associated abundances of genes react to long-term N fertilization? (2) Which variables significantly affect the response? (3) What roles do altered SOC and pH play in this response?

2. Methods

2.1. Data extraction

We used several databases to investigate the effect of long-term N fertilizer application on soil potential denitrification activity (PDA), including the China Knowledge Resource Integrated Database, Web of Science, Google Scholar, CAB Abstracts (ISI), SCOPUS, and cross-referencing. We searched for relevant studies published before June 2020. Our key search words were: "denitrify AND long-term

fertilization" for the rate of soil denitrification or the potential denitrification activity and N_2O/N_2 ratio, and "denitrifier OR denitrifying OR denitrification enzyme AND long-term fertilization" OR "*narG* OR *nirK* OR *nirS* OR *nosZ* AND long-term fertilization" for other variables. To be included in our data set, studies had to meet the following criteria: 1) their researches were performed under field conditions with replicated experimental design that N fertilization treatments exceeded two years; 2) the plant species, soil type, and other management practices (e.g., planting pattern and irrigation) between the fertilization treatment and unfertilized treatment plots were identical; 3) the means and sample sizes of observations for these variables were either available for both treatments or could be calculated; 4) the start and end times, sample locations, and other basic information were clear. We collected data about author and year, experimental site, location, study type, land use, soil structure, soil text, cropping system, soil depth, pH, SOC, N fertilizer form, application rate, duration of fertilization and denitrifying communities (*nirK*, *nirS*, *nosZ* and *narG*), and extracted relevant data from graphs in the original publications using GetData Graph Digitizer software (version 2.22). In total, we obtained 1041 observations (201 observations of PDA, 228 observations of *nirK* gene copies, 251 observations of *nirS* gene copies, 230 observations of *nosZ* gene copies, and 92 observations of *narG* gene copies) from 62 studies across the globe. The ratios of both $N_2O/(N_2O+N_2)$ and *nir*(*K*+*S*)/*nosZ* were calculated from studies with available data.

2.2. Data statistical and meta-analysis

We grouped fertilizer type, fertilizer application rate, duration of N fertilizer use, land use type, and soil properties into several classes according to the data distribution. We used the following categories: (1) fertilizer type (chemical or manure/mixture); (2) N application rate (N $\leqslant$ 150, 150 < N $\leqslant$300, N > 300 kg N ha^{-1}), and (3) duration of fertilizer application (duration $\leqslant$ 10, 10 < duration $\leqslant$ 30, duration > 30 years), (4) land use type (cropland or forest), (5) soil texture (coarse, medium, or fine), (6) pH (pH $\leqslant$ 6.5, 6.5 < pH $\leqslant$ 7.5, pH > 7.5), and (7) SOC (SOC $\leqslant$ 10, 10 < SOC $\leqslant$ 15, SOC > 15 g kg^{-1}). Soil texture was determined using data from the United States Department of Agriculture (USDA; source: http://www.nrcs.usda.gov/). We used a conversion factor of 0.58 to convert soil organic matter (SOM) concentrations to SOC concentrations when only the former was reported (Xu et al., 2012).

We used a natural logarithm of the response ratio (lnRR) as the effect size in the meta-analysis to evaluate the effects of long-term N fertilizer application on the rate of soil denitrification, the $N_2O/(N_2O+N_2)$ ratio, and the abundances of denitrifying genes (Wang and Yan, 2016):

$$\ln RR = \ln(x_t) - \ln(x_c), \quad (1)$$

where X_t and X_c are the means of the N fertilizer addition and control groups, respectively.

We estimated the percentage changes in the denitrification rate or the soil denitrifying gene copies under the effect of long-term N fertilizer application as follows (Wang and Zou, 2020).

156 Percentage change=$\left(e^{\ln RR}-1\right)\times 100\%$……..……..……(2)

157 In our meta-analysis, many studies did not report variances. Moreover, extreme
158 weights may be generated by using a variance-based weighting function. We therefore
159 used replication-based weighting in this study, according to the following equation (Xia
160 et al., 2017):

161 $W=\frac{Q_t\times Q_c}{Q_t+Q_c}$ ……(3)

162 where Q_t and Q_c are the number of replicates of the treatment and control,
163 respectively. We analyzed the mean effect size and variance using a weighted random
164 effects approach. We used bootstrapping (4999 iterations) to generate the mean effect
165 sizes and 95% confidence intervals (CIs). If the 95% CIs did not overlap zero, we
166 considered the effects of long-term N fertilizer application to be significant. In addition,
167 we further analyzed the effects of long-term fertilization among different subgrouping
168 categories, from which we calculated the between-group heterogeneity (Q_b) (Table 1).
169 In addition to this meta-analysis procedure, we performed statistical analyses using
170 IBM SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). All differences discussed are
171 significant at a probability level of $P < 0.05$. The weighted effect sizes of this variable
172 were considered to differ among groups when the Q_b value was significant ($P < 0.05$)
173 (Ouyang et al., 2018). We also examined the relationships between the effect sizes and
174 continuous forcing factors using meta-regression analysis. We also conducted
175 publication bias using Orwin's method (negligible effect = 0.2) and Rosenthal's method
176 (α value= 0.05) (Dai et al., 2018). All of the meta-analysis procedures were conducted
177 using MetaWin 2.1 software (Sinauer Associates, Inc., Sunderland, MA, USA).

3. Results

3.1. Impact of long-term N fertilization on potential denitrification rate and gene abundances

Overall, long-term N fertilization significantly increased potential denitrification activity (PDA) by 76% (95% CI: 59%–94%, $P < 0.01$; Fig. 1) compared to the non-fertilized control. Long-term N fertilization significantly increased the abundances of *nirK* (60.45%, 95% CI: 41.31-82.07%; $P < 0.05$), *nirS* (77.02%, 95% CI: 56.39-101.74%; $P < 0.01$), and *nosZ* (25.09%, 95% CI: 4.96-48.75%; $P < 0.05$), but showed no significant effect on the abundance of *narG* (Fig. 1). Furthermore, long-term N fertilization significantly increased the $N_2O/(N_2O+N_2)$ ratio by 22.1% (95% CI: 3.13–47.18%, $P < 0.05$; Fig. 1) and increased the *nir*(*K*+*S*)/*nosZ* ratio by 27.7% (95% CI: 7.61–52.99%; $P < 0.05$; Fig.1) compared to the non-fertilized control. In addition, long-term N fertilization significantly increased the SOC by 29% (95% CI: 23–36%; Fig. 1) and decreased the soil pH by 4.6% (95% CI: -5.4 to 3.8%; Fig. 1) compared to that of the non-fertilized control.

3.2. Effects of categorical variables on potential denitrification rate and gene abundances

Between-group variability suggested that fertilizer type and land-use type were significant predictive variables that influenced the denitrification rate (Table 1). Long-term N fertilization significantly increased PDA in croplands (79%, 95% CI: 62–97%; Fig. 2, Table 1) but did not significantly affect PDA in forests (19%, 95% CI: -20–84%; Fig. 2, Table 1). The increase in soil denitrification was significantly higher ($P < 0.01$,

Table 1) in the organic/mixture fertilizer treatment (112%, 95% CI: 83–146%, $P < 0.01$) than in the inorganic treatment (48%, 95% CI: 31–66%, $P < 0.01$; Fig. 2). Similarly, the increases in *nirK*, *nirS*, and *nosZ* abundances following N fertilization were significantly higher in the organic/mixture N treatment than in the inorganic N treatment (Fig. 3). The N application rate and fertilization duration also greatly influenced the associated gene abundance regarding long-term N fertilization (Fig. 2; Table 1). The N application rate was positively correlated with the responses of *nirK* (n=228, $P < 0.001$, Fig. 5a) and *nosZ* abundances (n=230, $P < 0.05$; Fig. 5e). Notably, the response of *the nosZ* gene was significantly negatively correlated with the duration of fertilization (n=201, $P < 0.05$; Fig. 5f).

SOC and soil pH also greatly affected the responses of denitrification potential and associated gene abundance. The response of PDA to long-term N fertilization was positively correlated with SOC ($P < 0.001$, Fig. 4d). SOC was also significantly positively correlated with the response of *nosZ* abundance and was negatively correlated with the response of *nirS* abundance ($P < 0.001$, Fig. 6). Soil pH was positively correlated with the responses of *nirK* ($P < 0.001$), *nirS* ($P < 0.01$), and *nosZ* ($P < 0.05$) abundance under long-term N fertilization (Fig. 6).

4. Discussion

4.1 The response of potential denitrification rate, product stoichiometry and denitrifying microorganisms to long-term N fertilization

Long-term N fertilization simultaneously influences plants, soil N/C availability, and soil properties (McAndrew and Malhi, 1992; Raun et al., 1998; Carrara et al., 2018).

222 It could therefore have a great influence on the abundance, diversity, and community
223 structure of microbes, and could thus affect biological denitrification activity
224 (Wallenstein et al., 2006; Liang et al., 2015). Furthermore, our results suggest that long-
225 term N loading significantly increased potential denitrification activity by 75.9%,
226 which is likely attributed to the positive responses of the activities and sizes of the
227 denitrifying communities to N fertilization (Fig. 1). Our results also show that long-
228 term N fertilization increased the abundances of the *nirK* and *nirS* genes (Fig. 1), which
229 is consistent with previous studies (Yu et al., 2018; Linton et al., 2020). As shown in
230 Fig. 3, the increases in *nirK* and *nirS* abundances following the long-term application
231 of organic fertilizer were higher than those under the application of inorganic fertilizer,
232 which supports the hypothesis that available C input contributes to the increase in soil
233 denitrification (Geisseler and Scow, 2014; Linton et al., 2020).

234 In line with previous studies (Geisseler and Scow, 2014; Ghosh et al., 2018), we
235 found that long-term N fertilization significantly increased the SOC concentration (n =
236 245, Fig. 1). N fertilization leads to greater primary productivity and increases the input
237 of plant-derived organic matter to the soil (Ghosh et al., 2018), while the higher labile
238 C inputs may be easily mineralized and increases heterotrophic respiration. This
239 temporarily creates an O_2 depletion zone that favors denitrification and N_2O emissions
240 (Senbayram et al., 2019). Furthermore, as the electron donor for denitrification, more
241 labile C increases the energy supply to denitrifiers and determines the overall
242 denitrification rate, especially in situations where the nitrate supply is sufficient (Surey
243 et al., 2020). The positive correlation between the response of PDA to N fertilization

244 and SOC indicates that SOC was likely the dominant factor contributing to the overall
245 increase in PDA ($P < 0.001$, Fig. 4d) (Čuhel et al., 2010; Carreira et al., 2020).

246 In line with previous studies (Barak et al., 1997; Guo et al., 2010), our results show
247 that long-term N fertilization led to a significant decline in soil pH (Fig. 1). Soil pH is
248 a fundamental factor regulating denitrification activity and product stoichiometry at
249 both proximal and distal scales (Čuhel and Šimek, 2011); it affects not only the
250 composition and abundance of the denitrifying community over the long term, but also
251 influence the kinetics of denitrification enzymes (Zhu et al., 2020). In our study, the
252 responses of *nirK*, *nirS*, and *nosZ* abundances to long-term N fertilization were
253 positively correlated with soil pH (Fig. 6). This indicates that soil acidification had a
254 negative effect on denitrifying microbes, which is consistent with the findings of study
255 by Hallin et al. (2009), who found a negative effect of 50-years ammonium sulfate input
256 on the abundances of *narG*, *nirS*, and *nosZ* with a soil pH < 4. The contrasting response
257 of *nirS* and *nosZ* to SOC concentration indicates that there might be some niche
258 differentiation among different denitrifiers. Therefore, we postulate that the lower pH
259 induced by long-term N loading could counteract part of the positive effects expected
260 by higher C inputs on denitrifiers. This might also explain the non-significant response
261 of *narG* abundance to N loading observed in our study (Fig. 1). As shown in Fig. 2, the
262 increase in PDA for cropland soils was higher than that of forest soils. This is likely
263 because denitrification rates in agricultural soils are generally known to be much higher
264 than those in forest soils (Barak et al., 1997). In addition, agroecosystems have been

265 subjected to larger N-related disturbances than other ecosystems (Chalk and Smith,
266 2020).

267 *4.2 The response of denitrification product stoichiometry to long-term N*
268 *fertilization*

269 Our results suggest that long-term N fertilizer significantly increased the
270 $N_2O/(N_2O+N_2)$ ratio, which likely occurred due to the combined effects of N input on
271 the soil pH, residual NO_3^- content, and denitrifying communities (Ma et al., 2019). It
272 has been frequently reported that the $N_2O/(N_2O+N_2)$ product ratio increases with
273 decreasing soil pH (Wijler and Delwiche, 1954; ŠImek and Cooper, 2002; Qu et al.,
274 2014; McMillan et al., 2016). The underlying mechanism is assumed to be that low pH
275 can interfere with the assembly of N_2O reductase in the periplasm, where the insertion
276 of Cu is possibly hindered by low pH (Liu et al., 2014; Schlüter et al., 2018). On the
277 other hand, a higher content of NO_3^- induced by N fertilization at denitrifying microsites
278 would likely increase the $N_2O/(N_2O+N_2)$ ratio, as NO_3^- is preferred over N_2O (Bremner
279 and Blackmer, 1978; Baggs et al., 2003; Ruser et al., 2006; Graham et al., 2010;
280 Senbayram et al., 2018).

281 The reduction of N_2O to N_2 by bacteria and archaea harboring N_2O reductase is
282 the only known N_2O sink in the biosphere (Jones et al., 2013). However, in terms of the
283 bioenergetics of denitrification, there is little disadvantage for denitrification microbial
284 communities in failing to perform the final step of N_2O reduction to N_2, especially under
285 conditions with a sufficient NO_3^- supply (Richardson et al., 2009; Kuypers et al., 2018).
286 In fact, most denitrifying fungi and about one-third of genomes that possess *nir* genes

encoding nitrite reductases are currently known to lack *nosZ*, resulting in N_2O as the end product of their denitrification (Shoun et al., 2012). We speculate that when NO_3^- is sufficiently supplied through consistent N loading, long-term N fertilization-driven selection might enhance denitrifying microorganisms encoding *nir*(*K*+*S*) rather than those encoding *nosZ* (Simon and Klotz, 2013). This could be supported by the significant increase in *the nir*(*K*+*S*)*/nosZ* ratio (27.71%) observed under N fertilization, and by the negative correlation between the response of *nosZ* and the duration of N loading ($P < 0.05$).

4.3 Implications for estimating N_2O emissions mitigation strategies

Human activities have remarkably changed the global N cycle during the last 50 years, creating a universal ecosystem with conditions that are "novel" to living organisms (Fowler et al., 2013; Galloway et al., 2004). Our conceptual diagram illustrates how long-term N fertilization influences N denitrification processes and related microbial community genes (see Graphical abstract). The uncovered simultaneous increase in the $N_2O/(N_2O+N_2)$ product and *nir*(*K*+*S*)*/nosZ* ratios under long-term N loading indicates the existence of an unfavorable feedback loop for soil N_2O emissions. Specifically, the application of more N leads to more N_2O being produced, but at the same time, less of this produced N_2O is being fully denitrified to N_2. This suggests that mitigation actions, such as reducing excessive N fertilization in intensive agricultural areas, may not only cut N_2O emissions directly, but also indirectly by increasing the N_2O reduction to N_2 process. A similar beneficial effect on soil N_2O emissions may occur in natural ecosystems when N deposition is reduced. We

recommend that future estimations of the N_2O reduction potential of reducing N inputs should include the additional quantification of the indirect effect of N loading on the $N_2O/(N_2O+N_2)$ product ratio. It should be noted that most of the included *nosZ* abundances in the current dataset were those of "typical" nitrous oxide reductase (*nosZ* I) (Jones et al., 2013; Orellana et al., 2014). It has been recently found that "atypical" N_2O reductase (*nosZ* II) gene diversity and abundance potentially play a significant role in N_2O consumption in soil (Xu et al., 2020; Zhao et al., 2020). Future studies are needed to investigate how long-term N fertilization affects the gene abundance of atypical *nosZ*.

5. Conclusion

Our study highlighted that long-term N fertilization might have a double effect on the activities and populations of denitrifying communities due to simultaneously increased SOC and decreased soil pH. This mixed effect stimulates denitrification-derived N_2O emissions by increasing both the denitrification activity and the denitrification $N_2O/(N_2O+N_2)$ product ratio. Reducing excessive N fertilization in intensive agricultural areas might cut N_2O emissions directly by reducing substrate for denitrification, and indirectly by increasing the reduction of N_2O to N_2 during denitrification.

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563

Figure captions:

Fig. 1. Long-term N fertilization effects on PDA; $N_2O/(N_2O+N_2)$ ratio; *narG*, *nirK*, *nirS*, and *nosZ* abundances; the ratio of *nir*(*K*+*S*)/*nosZ*; soil pH; and SOC. Different color-filled circles indicate that the effect of long-term N fertilization is significant ($P < 0.05$) at 95% CIs. The sample size of each variable is presented beside each major tick label.

Fig. 2. Impacts of N fertilization type, rate, duration, soil texture, land use, soil pH, and SOC on the response of PDA to long-term N fertilization. Different color-filled circles indicate that the effect of long-term N fertilization is significant ($P < 0.05$) at 95% CIs. The sample size of each variable is presented beside each major tick label.

Fig. 3. Long-term N fertilization effects on *nirK*, *nirS*, and *nosZ* abundances under different N categories. Different color-filled circles indicate that the effect of long-term N fertilization is significant ($P < 0.05$) at 95% CIs. The sample size of each variable is presented beside each error bar.

Fig. 4. Bivariate relationships between the ln RR of PDA and N application rate, duration of fertilization, soil pH value, and SOC. R^2 is the coefficient of regression; when $P < 0.05$, it was considered to be statistically significant; The values (*n*) beside each dot without parentheses are the number of observations, and those in parentheses are the number of studies. The effect of long-term N fertilization on soil reflects the magnitude of the soil indicator, the ln RR of soil indicator, in the long-term N fertilization treatment and control.

Fig. 5. Bivariate relationships between the influence of N application rate and duration on ln RR for *nirK*, *nirS*, and *nosZ* abundances. R^2 is the coefficient of regression; when $P < 0.05$, it was considered to be statistically significant. The values (*n*) beside each dot without parentheses are the number of observations, and those in parentheses are the number of studies. The larger blue circles refer to the larger number of replicates (ranging from one to five). The number of observations is shown after N without parentheses, and the number of studies is shown in parentheses.

Fig. 6. Relationships between the influence of soil pH and SOC on the response ratio (ln RR) of nirK, nirS and nosZ abundance. R^2 is the coefficient of regression; when the $P < 0.05$ was considered to be statistically significant; The values (*n*) beside each dot without parentheses are the number of observations, and those in parentheses are the number of studies. The larger blue circles refer to the larger number of replicates, ranging from 1 to 5. The number of observations is after N without parentheses, and the number of studies is in parentheses.

Graphical abstract Conceptual diagram illustrating how long-term N fertilization influences N denitrification processes and related microbial community genes. The

608 symbols “+” and “−” in the circles associated with each arrow represent stimulatory
609 and inhibitory effects, respectively, on the N denitrification process.
610

Long-term nitrogen fertilization increases both potential denitrification activity and $N_2O/(N_2O+N_2)$ product ratio by altering activities and populations of denitrifying communities: a global meta-analysis

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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The Journal Office

Journal of Cleaner Production

Dear Editor:

I wish to submit an original article for publication in *Journal of Cleaner Production*, titled "**Long-term nitrogen fertilization increases both potential denitrification activity and $N_2O/(N_2O+N_2)$ product ratio by altering activities and populations of denitrifying communities: a global meta-analysis**".

Globally, approximately 425 Tg of reactive N is fixed from unreactive atmospheric N_2 annually. This is double that of the pre-industrial period, with the increase being driven by human activity. Specifically, this increase had largely been driven by the excessive application of N fertilizers in agricultural systems. Denitrification is one of the most important biological processes by which reactive N is removed in the biosphere. However, the responses of potential denitrification activity and associated gene abundances to long-term N fertilization are poorly understood. In addition, studies focusing on the influence of long-term N loading on denitrification activity, denitrification stoichiometry, and denitrifier abundance remain fragmented. To date, to the best of our knowledge, no meta-analysis study has been conducted to investigate the long-term effect of N loading on denitrification potential activity and $N_2O/(N_2O+N_2)$ product ratio. Understanding the response of denitrification functionality to long-term N loading is a basic premise for solving the "missing N" in N balance, and for the mitigation of the undesirable ''cascading effects'' of reactive N.

Here, we compiled data from 1041 observations across 62 studies and quantified the effects of long-term N input on potential denitrification activity, the denitrification $N_2O/(N_2O+N_2)$ product ratio, and abundances of denitrifying communities using meta-analysis. Our results showed long-term N fertilization significantly increased potential denitrification activity by 75.9%, and increased the relative abundances of *nirK*, *nirS*, and *nosZ* gene copies by 60.4%, 77.0%, and 25.1%, respectively. Further, long-term N loading increased the denitrification $N_2O/(N_2O+N_2)$ ratio by 22.1% and *nir*(*K*+*S*)/*nosZ* ratios by 27.7%, respectively. The effect of long-term N

fertilization on potential denitrification activity was positively correlated with soil organic carbon (SOC) (R^2=0.11; n=132; P < 0.001), while long-term N loading increased the SOC by 29.0% compared to the unfertilized control. The responses of *nirK*, *nirS*, and *nosZ* abundances to long-term N fertilization were positively correlated with soil pH, whereas N loading decreased the soil pH by 4.6%. This suggests that long-term N fertilization might have a double effect on the activities, populations, and compositions of denitrifying communities due to the increased SOC and decreased soil pH. The uncovered simultaneous increase in the $N_2O/(N_2O + N_2)$ product and *nir*(*K*+*S*)*/nosZ* ratios under long-term N loading indicates the existence of an unfavorable feedback loop for soil N_2O emissions. Reducing excessive N fertilization in intensive agricultural areas might cut N_2O emissions directly by reducing substrate for denitrification, and indirectly by increasing the reduction of N_2O to N_2 during denitrification. We believe that this paper will be of interest to the readership of your journal because the manuscript adheres to the aims and scope of *Journal of Cleaner Production*.

The manuscript has been edited to ensure that the language is clear and free of errors by professional editors. Please see attached the CERTIFICATE OF ENGLISH EDITING. This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. All authors have contributed to and read the current manuscript and agreed on submission to **Journal of Cleaner Production** in the present form.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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MANUSCRIPT TITLE

Effect of Long-term nitrogen fertilization on activities, populations, and compositions of denitrifying microbial communities and the consequent changes in potential denitrification activity and the denitrification product ratio

AUTHORS

Longcheng Li, Mengying Yang, Jincheng Li, Roland Bol, Zhangliu Du, Di Wu

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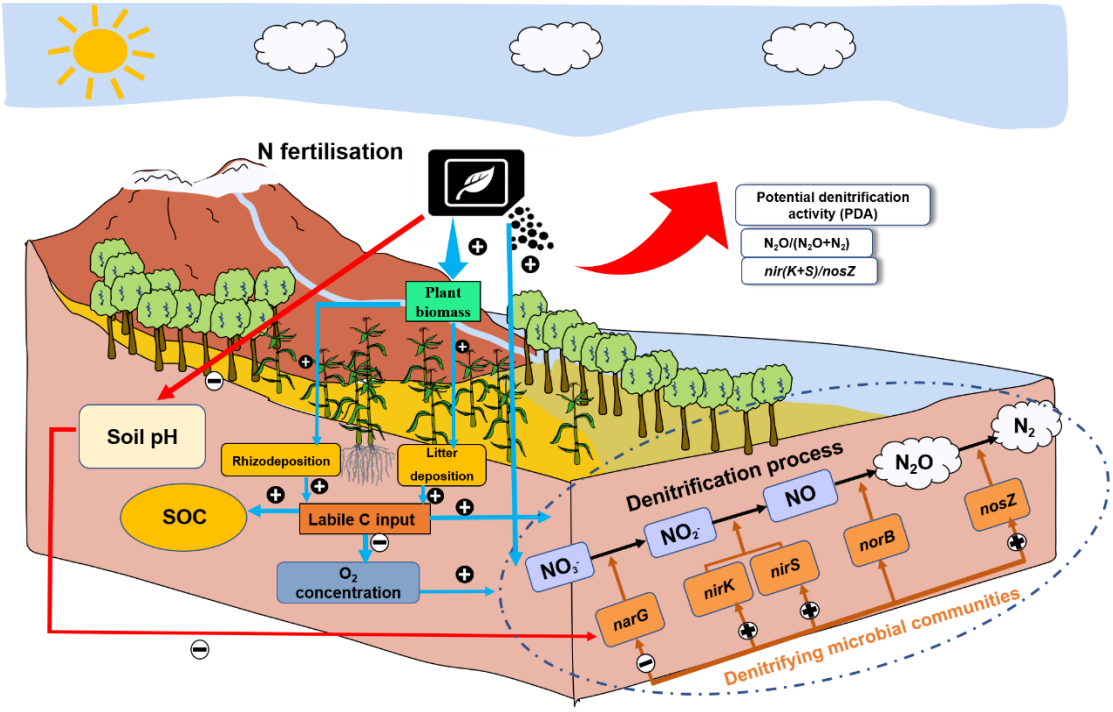
GRAPHICAL ABSTRACT



- Long-term N loading increased soil potential denitrification activity (PDA)
- Long-term N loading increased $N_2O/(N_2O+N_2)$ ratio and nir(K+S)/nosZ ratio
- The effect of increased SOC and decreased soil pH counteracts each other on PDA

Figure

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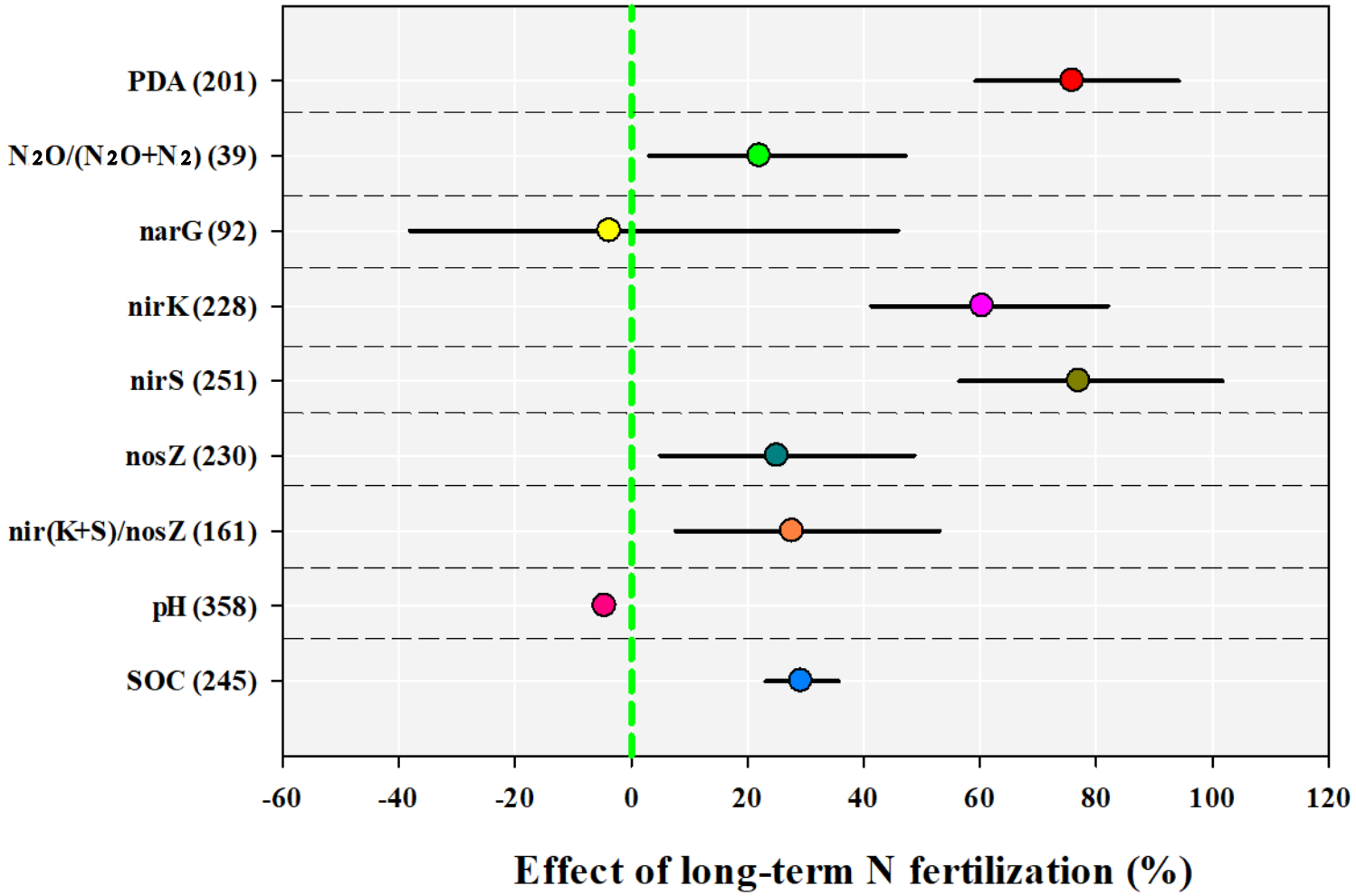
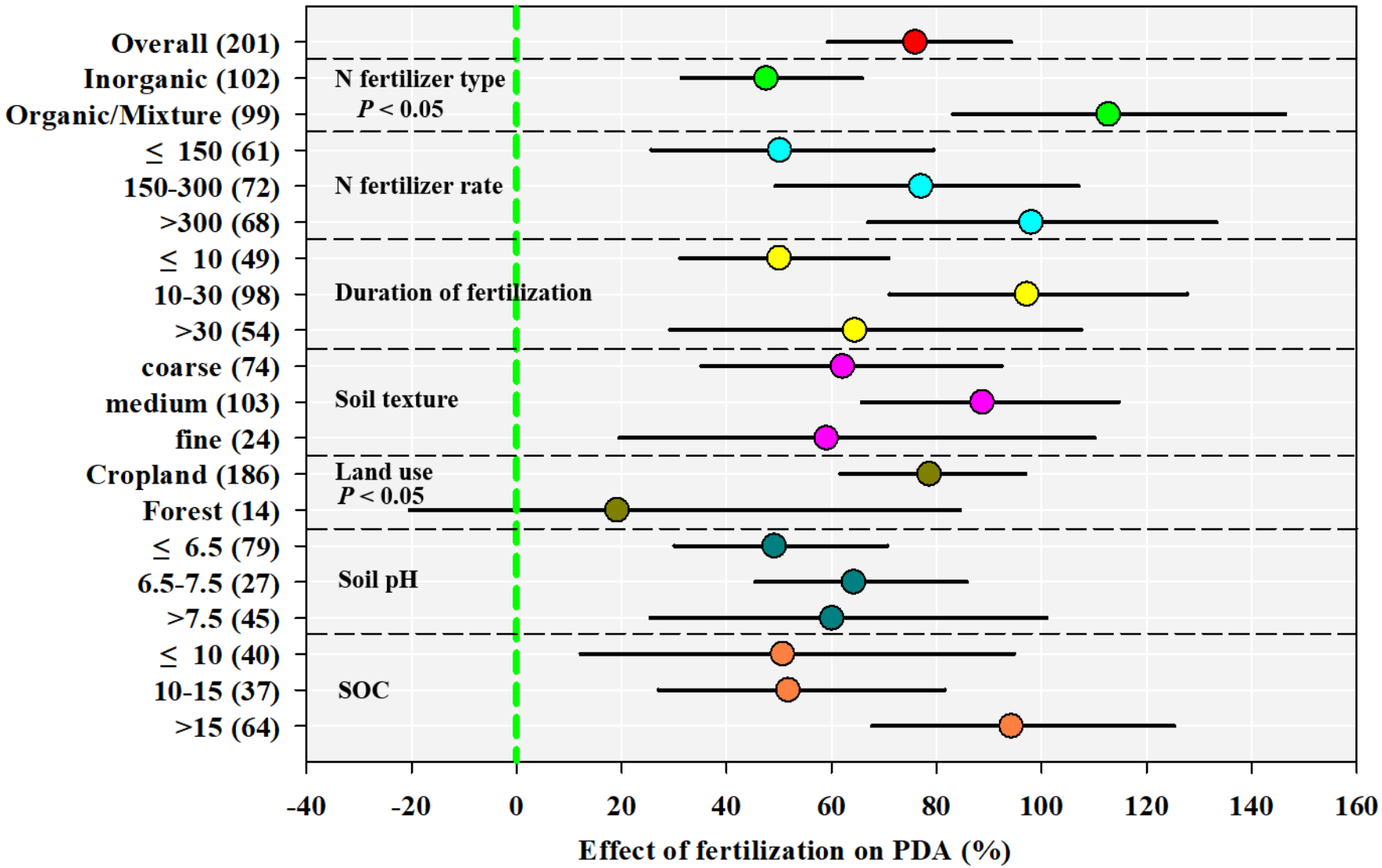
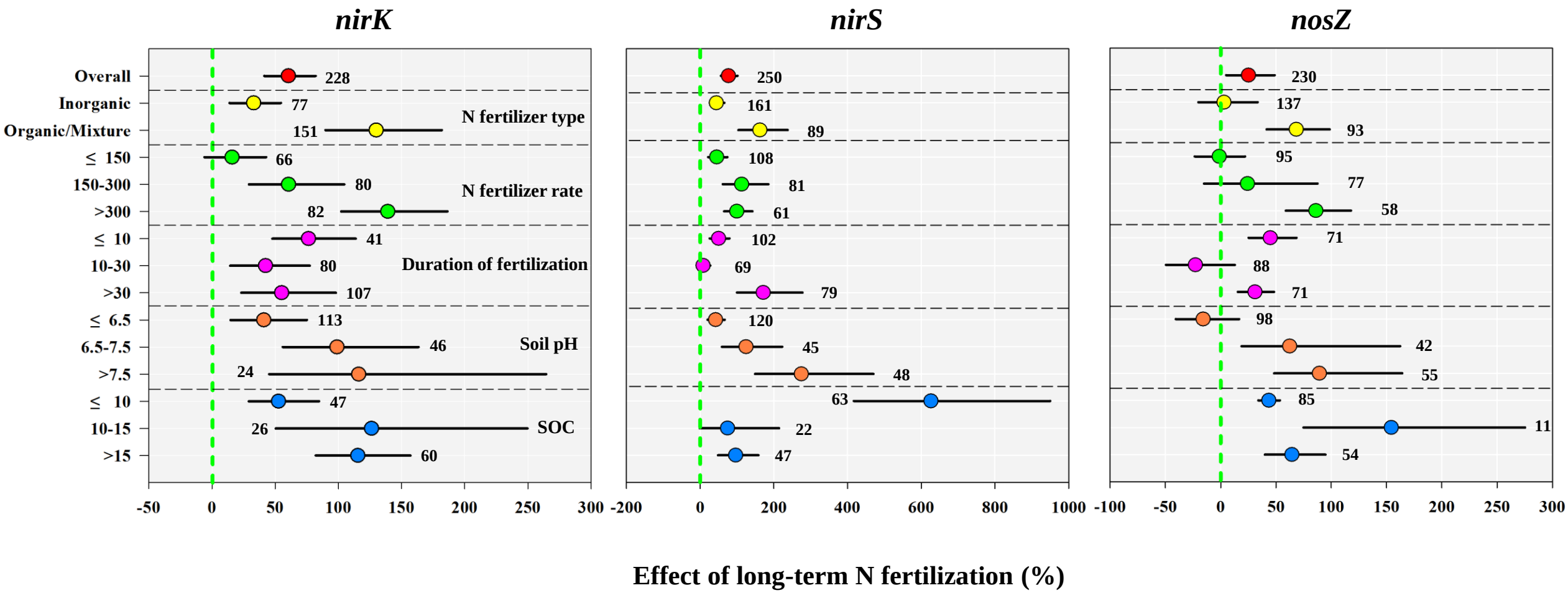
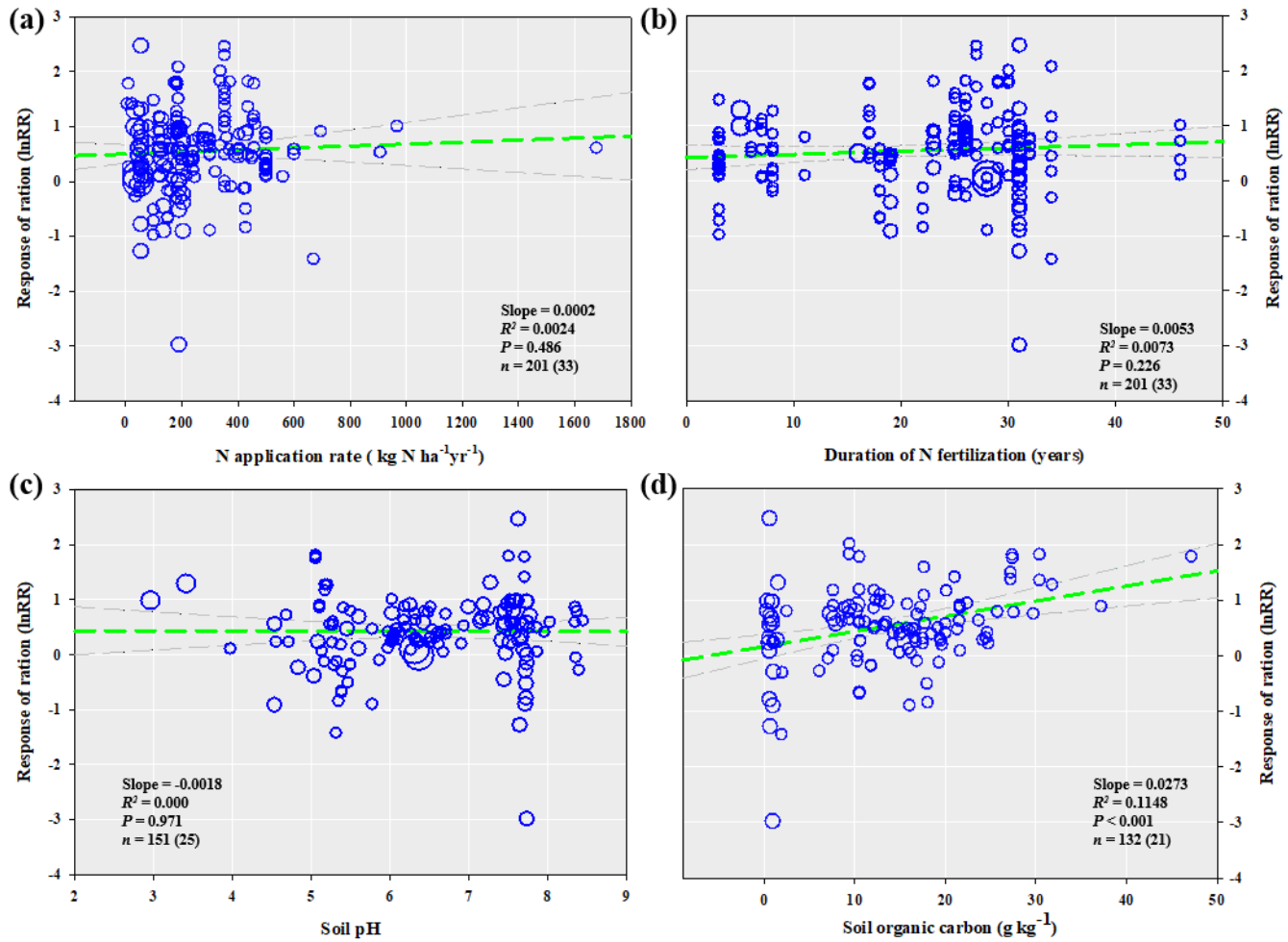
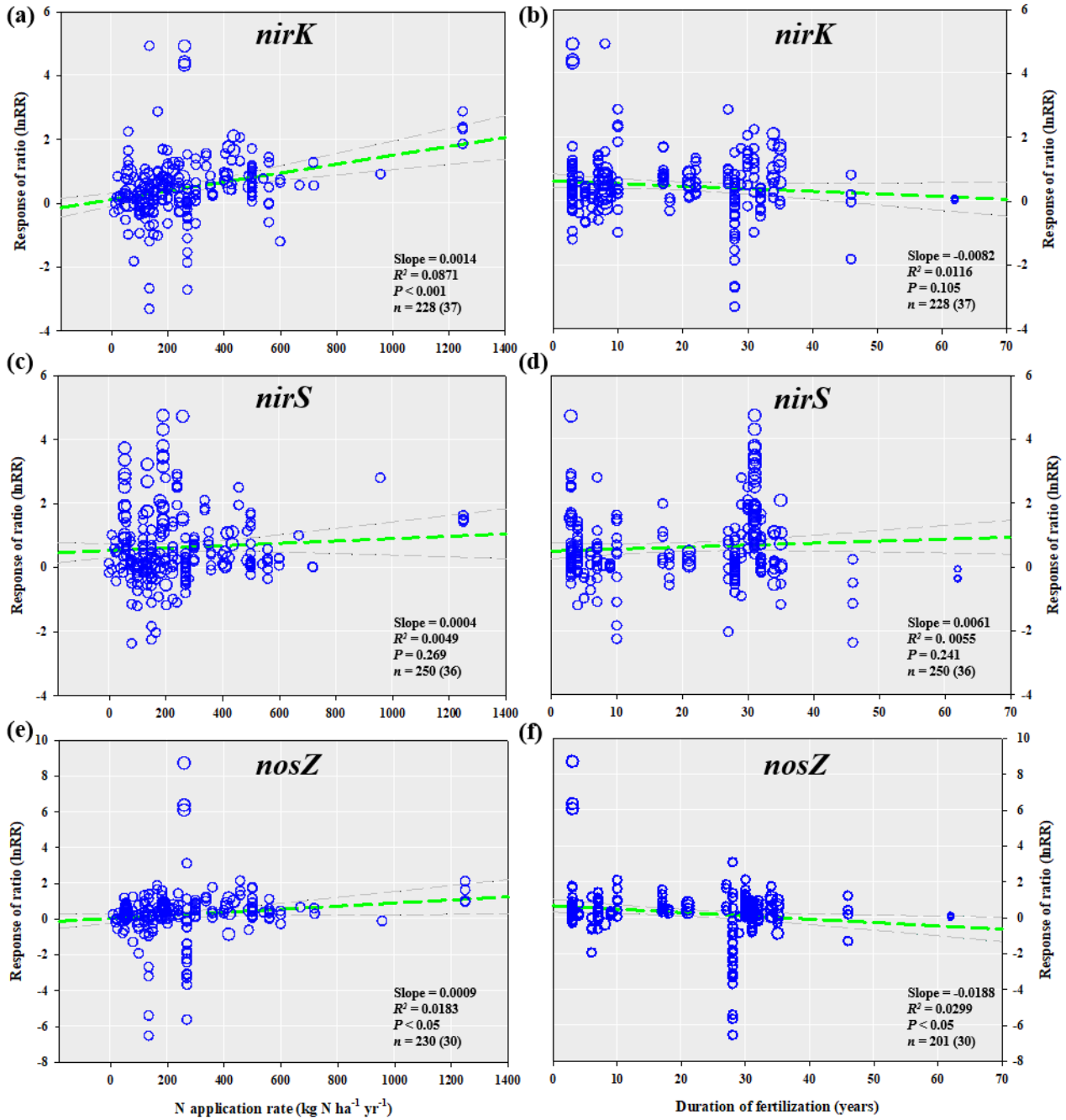
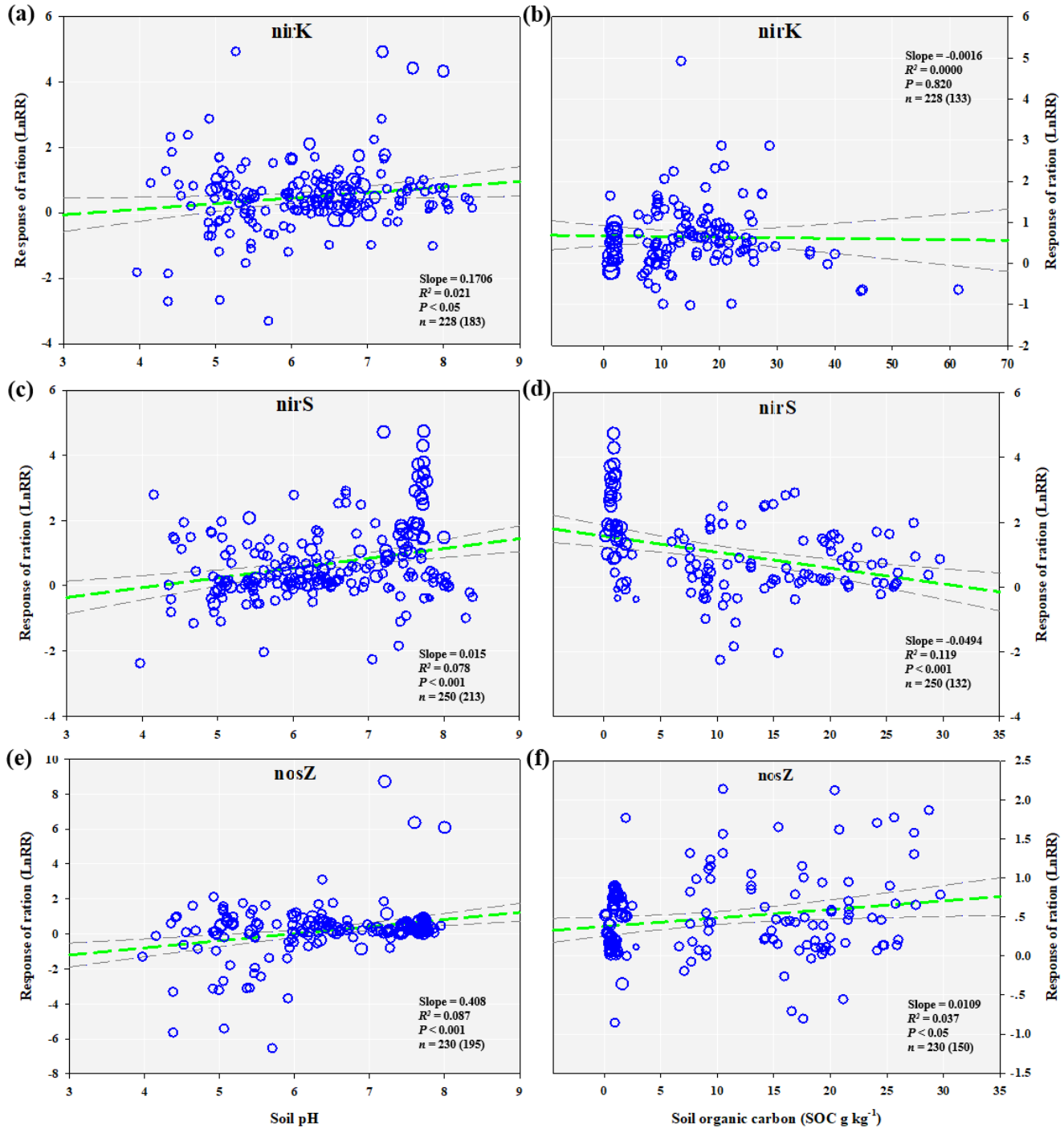



Overall (201)
Inorganic (102)
Organic/Mixture (99)
N fertilizer type
P < 0.05
≤ 150 (61)
150-300 (72)
>300 (68)
N fertilizer rate
≤ 10 (49)
10-30 (98)
>30 (54)
Duration of fertilization
coarse (74)
medium (103)
fine (24)
Soil texture
Cropland (186)
Forest (14)
Land use
P < 0.05
≤ 6.5 (79)
6.5-7.5 (27)
>7.5 (45)
Soil pH
≤ 10 (40)
10-15 (37)
>15 (64)
SOC
-40
-20
0
20
40
60
80
100
120
140
160
Effect of fertilization on PDA (%)


nirK
nirS
nosZ
Overall
Inorganic
Organic/Mixture
≤ 150
150-300
>300
≤ 10
10-30
>30
≤ 6.5
6.5-7.5
>7.5
≤ 10
10-15
>15
N fertilizer type
N fertilizer rate
Duration of fertilization
Soil pH
SOC
228
77
151
66
80
82
41
80
107
113
46
24
47
26
60
250
161
89
108
81
61
102
69
79
120
45
48
63
22
47
230
137
93
95
77
58
71
88
71
98
42
55
85
11
54
-50 0 50 100 150 200 250 300
-200 0 200 400 600 800 1000
-100 -50 0 50 100 150 200 250 300
Effect of long-term N fertilization (%)


(a)
Response of ration (lnRR)
N application rate (kg N ha-1yr-1)
Slope = 0.0002
R2 = 0.0024
P = 0.486
n = 201 (33)
(b)
Response of ration (lnRR)
Duration of N fertilization (years)
Slope = 0.0053
R2 = 0.0073
P = 0.226
n = 201 (33)
(c)
Response of ration (lnRR)
Soil pH
Slope = -0.0018
R2 = 0.000
P = 0.971
n = 151 (25)
(d)
Response of ration (lnRR)
Soil organic carbon (g kg-1)
Slope = 0.0273
R2 = 0.1148
P < 0.001
n = 132 (21)


(a)
nirK
Response of ratio (lnRR)
Slope = 0.0014
R^2 = 0.0871
P < 0.001
n = 228 (37)
(b)
nirK
Response of ratio (lnRR)
Slope = -0.0082
R^2 = 0.0116
P = 0.105
n = 228 (37)
(c)
nirS
Response of ratio (lnRR)
Slope = 0.0004
R^2 = 0.0049
P = 0.269
n = 250 (36)
(d)
nirS
Response of ratio (lnRR)
Slope = 0.0061
R^2 = 0. 0055
P = 0.241
n = 250 (36)
(e)
nosZ
Response of ratio (lnRR)
Slope = 0.0009
R^2 = 0.0183
P < 0.05
n = 230 (30)
N application rate (kg N ha^{-1} yr^{-1})
(f)
nosZ
Response of ratio (lnRR)
Slope = -0.0188
R^2 = 0.0299
P < 0.05
n = 201 (30)
Duration of fertilization (years)


(a)
nirK
Response of ration (LnRR)
Slope = 0.1706
R^2 = 0.021
P < 0.05
n = 228 (183)
(b)
nirK
Slope = -0.0016
R^2 = 0.0000
P = 0.820
n = 228 (133)
Response of ration (LnRR)
(c)
nirS
Response of ration (LnRR)
Slope = 0.015
R^2 = 0.078
P < 0.001
n = 250 (213)
(d)
nirS
Slope = -0.0494
R^2 = 0.119
P < 0.001
n = 250 (132)
Response of ration (LnRR)
(e)
nosZ
Response of ration (LnRR)
Slope = 0.408
R^2 = 0.087
P < 0.001
n = 230 (195)
Soil pH
(f)
nosZ
Slope = 0.0109
R^2 = 0.037
P < 0.05
n = 230 (150)
Response of ration (LnRR)
Soil organic carbon (SOC g kg^{-1})

Tables：

Table 1

Between-group variability (Q_b) among observations (n) suggesting their potential as predictive variables influencing the denitrification rate, and *nirK*, *nirS*, and *nosZ* gene copy responses to long-term fertilization.

Variables	Denitrification rate		*nirK*		*nirS*		*nosZ*	
	n	Q_b	n	Q_b	n	Q_b	n	Q_b
All studies	201	N/A	228	N/A	250	N/A	230	N/A
N fertilizer type	201	4.17**	228	9.82**	250	12.98**	230	8.43**
N application rate	201	1.51	228	12.32**	250	5.06*	230	9.27*
Duration	201	1.75	228	1.37	250	7.56**	230	12.52**
Soil texture	201	0.77	228	5.45*	250	32.31**	230	10.86**
Land use	200	1.22*	228	4.76*	250	3.88	230	0.09
Soil pH	151	0.15	183	2.60	213	13.25**	195	14.52**
SOC	131	1.10	133	2.05	132	6.80*	150	1.14

Asterisks indicate significant *P* values (*$P < 0.05$, **$P < 0.01$).