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Is Nitrous Oxide Reduction Primarily Regulated by the Fungi-to-Bacteria Abundance Ratio in Fertilized Fields?

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ABSTRACT:

The production of nitrous oxide (N_2O) is a widespread trait in fungi and is of interest because denitrifying fungi lack the N_2O reductase gene (*nosZ*) that regulates N_2O reduction to nitrogen gas (N_2). The adaptive ability of soil fungi is better than that of bacteria in acidic soils. We investigated the N_2O reduction potential, described by the $N_2O/(N_2O+N_2)$ product ratio (R_{N2O}), in different soils. The abundance ratio of fungi to bacteria ($R_{F/B}$) was negatively correlated with the natural pH of the soil (P<0.01), but the high value of $R_{F/B}$ measured in vineyards was probably due to the large inputs of manure. When the denitrification potential was measured at natural pH values, R_{N2O} was negatively correlated with the natural pH of soils (P<0. 01). When the denitrification potential was measured after the short-term modifications of pH, however, no significant correlation was found between R_{N2O} and the modified pH. Based on stepwise multiple regression analysis, the soil pH and nitrate (NO_3^-) were the key factors regulating N_2O reduction in soils at natural pH values (R^2=0.88; P<0.001), whereas the key factor was the soil NO_3^- alone (R^2=0.83; P<0.001) when the soil pH was modified. When the effect of the soil chemical properties was weakened, a high $R_{F/B}$ value had the potential to affect the reduction of N_2O (P<0.01), but the role of fungi was offset by the presence of denitrifying bacteria. These results provide evidence that the direct effects of the chemical properties of the soil have a greater effect on the reduction of N_2O in fertilized soil than the indirect effects of $R_{F/B}$.

Key Words: fungi; acidification; vegetable fields; vineyards; manure.

INTRODUCTION

Urbanization has stimulated the development of suburban agriculture, especially vegetable production, in recent decades. The intensive management of suburban agriculture is characterized by high inputs of nitrogen fertilizer, which can lead to soil acidification and the accumulation of mineral nitrogen (Cao *et al.*, 2006; Guo *et al.*, 2010; Zhu *et al.*, 2011; Zeng *et al.*, 2017). Regions with a heavy nitrogen load inevitably become hotspots

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for the emission of nitrous oxide (N_2O). For example, vegetable fields contributed 20% of the total direct N_2O emissions from croplands in the 1990s (Zheng *et al.*, 2004), despite making up only 6% of the total area of cropland (National Bureau of Statistics of China, 2014). N_2O is an important greenhouse gas and is the primary destroyer of the ozone layer in the stratosphere (IPCC, 2007; Ravishankara *et al.*, 2009). Unfortunately, there is evidence to indicate a negative relationship between the flux of N_2O and pH in intensively managed vegetable fields (Zhu *et al.*, 2011), which suggests that the acidification of soils by high inputs of chemical nitrogen fertilizers may further intensify the emission of N_2O. The last step in denitrification (the reduction of N_2O to N_2) may be directly impaired by a low pH (Cuhel and Simek, 2011; Liu *et al.*, 2014; Qu *et al.*, 2014).

The fungi-to-bacteria abundance ratio ($R_{F/B}$) in soils may be another key factor regulating the reduction of N_2O in acidic soils. N_2O production is a widespread trait in fungi, but denitrifying fungi lack N_2O reductase (Shoun *et al.*, 1992; Jirout *et al.*, 2013; Maeda *et al.*, 2015). The adaptive ability of soil fungi is better than that of bacteria in acidic soils (Rousk *et al.*, 2010). A significant contribution of denitrification by fungi to N_2O emissions has been found across various ecosystems, especially in acidic environments (Laughlin *et al.*, 2009; Rütting *et al.*, 2013; Chen *et al.*, 2014; Huang *et al.*, 2017). It is reasonable to suggest that the reduction of N_2O is indirectly inhibited in acidic soils dominated by fungi. However, the soil pH can also influence the reduction of N_2O directly (Liu *et al.*, 2014; Qu *et al.*, 2014)—that is, the observed positive relationship between the emission rates of N_2O and the soil fungi-to-bacteria ratio may be a direct result of soil acidification (Rütting *et al.*, 2013; Chen *et al.*, 2014). The truncation of the denitrification process could lead to the emission of significant amounts of N_2O.

Few studies have addressed whether the soil pH influences the reduction of N_2O directly, or indirectly via the microbial composition of the soil, which is itself influenced by the pH (Cuhel and Simek, 2011). A good understanding of these mechanisms will have important practical implications for the mitigation of N_2O emissions from fields treated with large amounts of nitrogen fertilizer. For example, the effect of the management of soil pH on N_2O mitigation was found to vary among large numbers of field experiments (Qu *et al.*, 2014), which may have been due to a lack of consideration of the role of fungi. A recent meta-analysis showed that the emission of N_2O did not decrease significantly when chemical nitrogen fertilizers were substituted by manure (Xia *et al.*, 2017), which may be due to fungi outcompeting bacteria in the use of complex organic substances (Zhang *et al.*, 2012; Gunina *et al.*, 2014; Wei *et al.*, 2014; Yamamoto *et al.*, 2017).

The main objectives of this study were to investigate the response of the soil $R_{F/B}$ to high fertilizer inputs and to explore the indirect role of fungi in the reduction of soil N_2O. Soils were sampled from two paddy fields, four vineyards, four intensively managed vegetable fields and an untilled soil (control treatment). The short-term soil N_2O reduction activity was evaluated using measurements of the denitrification potential, which can circumvent the effect of organic carbon and oxygen (Groffman *et al.*, 1999). Specifically, we wanted to answer the following questions: (1) does soil acidification enhance the soil $R_{F/B}$; (2) compared to untilled soils, does the application of manure increase the soil $R_{F/B}$; and (3) is the reduction of N_2O primarily regulated by the soil $R_{F/B}$?

MATERIALS AND METHODS

Soil sampling and site description

Samples of paddy soil and untilled soil were collected at depths of 0–10 cm from fields under long-term cultivation at the Changshu Agroecosystem Experimental Station (120° 65′ E, 31° 56′ N), Suzhou city, Jiangsu Province, China. Samples of soils from intensively managed vegetable fields and vineyards were collected at

depths of 0–10 cm from fields belonging to local farmers that were located near the experimental station. Based on the Food and Agriculture Organization World Reference Base for Soil Resources, the soils were classified as Anthrosols developed from lacustrine sediments. The soils were allowed to adjust to a moisture content of about 20% at room temperature 4 ℃--10 ℃ and were then sieved through a 2 mm mesh and stored at 4 ℃ until further use.

The vegetable fields had been converted from paddy fields at least 6 years previously and the rate of application of synthetic fertilizer was about 1044 N kg ha^{-1} yr^{-1}. The main crops in the vegetable fields were leafy vegetables (e.g. *Brassica chinensis* L. (pak choi), *Brassica rapa* subsp. *pekinensis* (Chinese cabbage) and *Brassica rapa* var. *glabra*) and the fields were cultivated three to four times each year. No fertilizer was applied to the bare untilled soil. The vineyards had been converted from paddy fields about 12 years previously and the rate of application of synthetic fertilizer was about 148 kg N ha^{-1} yr^{-1}. A large amount of organic fertilizer (~397 kg N ha^{-1} yr^{-1}) had also been applied to the vineyards to improve the flavor of the grapes. The organic fertilizers come from pig (or chicken) manure fermentation. The vegetable fields and vineyards were managed by local farmers. Urea was applied to the paddy fields at a rate of ~300 N kg ha^{-1} yr^{-1}; one of the paddy fields (paddy field 1) had also been used to grow rice seedlings before transplantation.

Determination and adjustment of pH

The pH of the soils was measured in deionized water at a soil to water ratio of 1:2.5. Three replicate samples were measured using a Sartorius pH meter. The soil samples were vigorously stirred for 2 minutes and then left to stand for >90 minutes before the measurements were taken.

Previous studies (Bergaust *et al.*, 2010; Liu *et al.*, 2014) have shown that there is a threshold pH for the reduction of N_2O by denitrifying bacteria. N_2O reduction is inhibited below the threshold value, but the inhibition is immediately eliminated when the pH is increased above the threshold pH. We used a pH value of 6.25 as the threshold pH. The $N_2O/(N_2O+N_2)$ ratio (R_{N2O}) in the soils was >0.5 in our preliminary experiment when the pH was <6.25. The pH of acidic soils (pH <6.25) was adjusted to above the threshold pH with 2 M potassium hydroxide (KOH) and the pH of alkaline soils (pH >6.25) was adjusted to below the threshold pH with 1 M sulfuric acid (H_2SO_4). The acid (or alkali) demand per gram of dry soil was recorded (Table Ⅰ).

Measurement of denitrification potential at natural or modified values of pH

The soils were pre-incubated at 25 ℃ for 7 days and the bottle was sealed with parafilm (Bemis, the USA). The original mass water content of the soils was about 20%, which then decreased by less than 1% within 7 days. The microbial abundance of soils did not change significantly during preincubation according to our recent study (data not published). After pre-incubation, soil samples were divided into three portions. One portion was stored at -80 ℃ for the later extraction of microbial DNA, and the second portion was used to determine the amount of mineral nitrogen in the soil. The ammonium (NH_4^+) and nitrate (NO_3^-) present in the soil were extracted with a 2 M potassium chloride solution (KCl) at a soil-to-solution ratio of 1:5 and then determined using an Alliance Smartchem200 autoanalyzer (Table Ⅰ).

The third portion was used to measure the soil denitrification potential using the procedure outlined in a previous study (Groffman *et al.*, 1999). Fresh soil (5 g dry weight) was added to a 120-mL CNW serum bottle (Anpel, China) with 5 mL of a medium capable of supplying NO_3^- (100 mg kg^{-1} N dry weight soil) and glucose

(40 mg kg^{-1} dry weight soil). The bottle was sealed with the pre-assembled cap and septa (Anpel, China), evacuated for 5 minutes and then flushed with 99.99% helium (He) gas (20 minutes, four cycles). The soils were incubated under an atmosphere of He with or without the addition of 10% v/v acetone-free acetylene on a rotary shaker at 125 revs min^{-1} at 25 ℃. Gas samples were taken with syringes after 30 and 90 minutes and determined using an Agilent 7890A gas chromatograph with an electron-capture detector. Because the incubation was complete within 1.5 hours, it is reasonable to assume that there was no significant change in the microbial composition during the course of the experiment. Although the acetylene-based method has some drawbacks and could have underestimated the N_2O-deductase activity to some extent, there was good relationship in the results between the acetylene-based method and the direct-N_2 method in fertilized soils (Groffman *et al.*, 1999; Qin *et al.*, 2014)

The denitrification potential was determined at a modified pH. A KOH solution was added to increase the pH of the acid soils to ~6.50 (Table Ⅰ) before measurement. The pH of the neutral soils was adjusted to ~6.00 with H_2SO_4.

TABLE Ⅰ.

Chemical properties of soils and the acid or alkali demand for pH modification.

Field type	NO_3^--N	NH_4^+-N	NO_2^--N	Natural pH	Modified pH	Added OH^-	Added H^+
	(mg kg^{-1} dry soil)					(μmol g^{-1} dry soil)	
Vegetable field 1	800.61±2.03a[a)]	123.34±2.91b	0.04±0.02b	6.01±0.02j	6.53±0.04bc	7.67	Na[b)]
Vegetable field 2	348.04±6.03b	2.33±0.18c	0.04±0.01b	5.89±0.01h	6.57±0.04bc	8.00	Na
Vegetable field 3	16.30±0.22f	2.61±0.12c	0.05±0.01b	6.16±0.01f	6.58±0.00b	6.00	Na
Vegetable field 4	167.99±4.34c	155.07±10.83a	0.03±0.01b	4.91±0.01k	6.77±0.03a	38.00	Na
Vineyard 1	14.37±0.32fgh	1.95±0.13c	0.02±0.01b	5.86±0.01h	6.52±0.05	8.40	Na
Vineyard 2	11.43±0.2gh	2.39±0.10c	0.06±0.01ab	6.21±0.01e	6.52±0.00c	4.00	Na
Vineyard 3	15.14±0.14fg	1.55±0.01c	0.09±0.05ab	6.78±0.01b	6.00±0.03e	Na	8.59
Vineyard 4	9.94±0.29h	1.24±0.08c	0.03±0.01b	6.40±0.01d	5.82±0.02g	Na	4.66
Paddy field 1	47.03±0.30d	2.44±0.05c	0.08±0.02ab	6.69±0.01c	5.92±0.03f	Na	9.57
Paddy field 2	34.93±0.07e	2.11±0.04c	0.10±0.04a	6.79±0.01b	5.95±0.01ef	Na	9.57
Untilled soil	17.52±0.23f	2.65±0.06c	0.07±0.01ab	7.25±0.02a	6.10±0.04d	Na	11.04

a) Data are given as mean±standard error values (n=3). Significant differences in the mean values within each column are indicated by different letters; α= 0.05.

b) Na = no addition.

Microbial abundance

Four replicate subsamples of fresh soil were used for DNA extraction using an MP Biomedicals FastDNA SPIN kit for soil according to the manufacturer's instructions. The quality of the DNA solutions was determined using a Thermo Scientific NanoDrop 2000 spectrophotometer.

After DNA extraction, copies of the 18S rRNA and 16S rRNA genes were determined by quantitative polymerase chain reaction (qPCR) amplification according to the method of Rousk *et al.* (2010) to assess the abundance of fungi and bacteria. Although the number of ribosomal gene copies per microbial genome can vary between species, the qPCR results largely reflect the microbial abundance in diverse ecosystems (Fierer *et al.*, 2005; Rousk *et al.*, 2010). The abundance of the N_2O reductase gene, *nosZ*, was measured according to the method of Wang *et al.* (2013). The qPCR experiments were performed on an Applied Biosystems QuantStudio 3 Real-Time PCR system. The 20 µL reaction system contained 10 µL of SYBR Premix Ex Taq (Takara, Japan), 1 µL (5 µM) of each primer, 0.08 µL of ROX reference solution, 7.02 µL of DNA-free water and 2 µL (~0.8 ng) of DNA template.

The standards are plasmids containing the 16S rRNA gene, 18S rRNA gene or *nosZ* gene, which can represent bacteria, fungi, and denitrifying bacteria, respectively. Standard plasmids were obtained through the following steps. Briefly, targeted genes were PCR amplified from extracted DNA solutions using the method described above, and the amplicons were then cloned into the plasmids called pMD®19T Vector (Takara, China). The standard plasmids were verified by pyrosequencing. The standard curves were generated using a 10-fold serial dilution of a plasmid-containing target gene with between 10^2 and 10^8 gene copies μL^{-1}. The efficiencies of the amplification of the gene copies and the R^2 values of the standard curves were 91%--94% and 0.998--0.999, respectively. Melting curves were used to check the specificities of the amplification product at the end of the real-time PCR run.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 16.0 software. The differences in the chemical properties among the soils were examined by one-way ANOVA (analysis of variance). Unitary regression was used to elucidate the relationships between the biochemical properties and the R_{N2O}. Stepwise multiple analyses were used to explore the key factors affecting this ratio.

RESULTS

Relative ratios of microbial abundance in soils

The $R_{\mathrm{F/B}}$ in the soils, represented by the ratio of rRNA copies, was negatively correlated with the pH (P<0.01) (Fig. 1). This ratio was significantly higher in the vegetable fields and vineyards than in the paddy fields and the control site (P<0.001). The highest value of $R_{\mathrm{F/B}}$ was found in vegetable field 5, which had the lowest pH (4.91). There was no significant difference in $R_{\mathrm{F/B}}$ among the four vineyards, although the original soil pH values varied from 5.86 to 6.78.

Fig. 1. Correlation of $R_{\mathrm{F/B}}$ with natural soil pH values across four types of field: vegetable fields (blue), vineyards

(green), paddy fields (red) and a control field (black, untilled soil). Error bars represent the standard error (n=4); $R_{F/B}$ = soil fungi-to-bacteria abundance ratio.

The ratios between the copy numbers of *nosZ* and the 16S rRNA gene could represent the relative abundance of bacterial denitrifiers with the capacity to reduce N_2O in the soil bacterial community. Figure 2 shows that the relative abundance of *nosZ* was significantly higher in the vineyards than in the other sample sites.

Fig. 2. Relative number of N_2O reductase genes (*nosZ*/16S, the relative abundance of bacterial denitrifiers) across four types of field: VF, vegetable fields; VY, vineyards; P, paddy fields; and CK, control treatment (untilled soils). Error bars represent the standard error (n= 4).

Relationship between the N_2O product ratio and soil factors

Figure 3 shows the influence of the soil pH and the modified pH on the reduction of N_2O. The natural soil pH was negatively correlated with the N_2O product ratio (R_{N2O}) (P<0.001) (Fig. 3a). After the soil pH had undergone short-term modification, R_{N2O} decreased in the acidic soils (pH <6.25) amended with KOH solution and increased in the alkaline soils (pH >6.25) amended with H_2SO_4 solution (Fig. 3). However, R_{N2O} did not change in proportion with the soil pH; R_{N2O} in the soils from the vegetable fields remained at high levels when the soil pH was increased. These results show that other factors may affect N_2O reduction in the short term.

Fig. 3. Relationship between R_{N2O} and the (a) natural or (b) modified pH of soils. The pH of acidic soils (pH <6.25) was modified to ~6.50 with KOH solution and the pH of alkaline soils (pH >6.25) was adjusted to ~6.00 with H_2SO_4. R_{N2O} = $N_2O/(N_2O+N_2)$, the N_2O product ratio.

Other factors (e.g. the soil residual NO_3^-, NH_4^+, $R_{F/B}$ and N_2O reductase gene copies (*nosZ*/16S)) may also affect N_2O reduction in soils. According to the stepwise multiple regression analysis, the soil pH and NO_3^- content were the key factors regulating N_2O reduction in soils at natural pH values (R^2 = 0.88; P<0.001), and the key factor was soil NO_3^- alone (R^2 = 0.83; P<0.001) when the soil pH was modified.

Effect of $R_{F/B}$ on the reduction of N_2O

Soil acidification and a high NO_3^- concentration significantly affected the reduction of N_2O. To investigate the effect of fungi in the reduction of N_2O, soils with a high pH and low residual NO_3^- concentration (pH >6.25, NO_3-N <168 mg kg^{-1}) were analyzed further. The pH threshold was selected according to our preliminary experiments. The threshold for the concentration of residual NO_3^- in the soils was selected according to Figure 4, which shows that R_{N2O} changed as the pH was modified when the soil residual NO_3^--N was <168 mg kg^{-1}.

Fig. 4 Effect of the modfication of pH on R_{N2O} at different soil residual NO_3^- concentrations. $\Delta R_{N2O} = R_{N2O}$(natural pH) − R_{N2O}(modified pH); ΔpH = natural pH − modified pH.

When the effect of pH and soil mineral nitrogen was weakened, further analysis showed that the soil R_{N2O}

was influenced significantly (P<0.01) by $R_{F/B}$ (Fig. 5a). However, the relationship between $R_{F/B}$ and R_{N2O} was similar to a parabolic curve. The N_2O reduction potential first increased and then decreased with the increasing relative abundance of fungi. The relative abundance of denitrifying bacteria was also significantly influenced by the relative abundance of fungi (p<0.01), but in the opposite direction (Fig. 5b).

Fig. 5. Effect of $R_{F/B}$ on (a) R_{N2O} and (b) the copy ratio (*nosZ*/16S) in vegetable fields and vineyards. Only the soils with a high pH and low residual NO_3^- (pH >6.25, NO_3-N <168 mg kg^{-1}) were analyzed.

DISCUSSION

Effect of fertilization on the soil $R_{F/B}$

The soil pH in this study decreased significantly (P<0.01) after the input of high nitrogen fertilizers, especially in the vegetable fields (Table Ⅰ), in agreement with previous studies (Guo *et al.*, 2010; Zhu *et al.*, 2011). Some studies have investigated the soil fungi-to-bacteria ratio at different pH values based on several methods, including phospholipid fatty acids, the substrate-induced respiration–inhibition method, and qPCR (Rousk *et al.*, 2010; Rütting *et al.*, 2013; Chen *et al.*, 2014; Zhou *et al.*, 2016). The gradients in pH were created by the application of chalk (Rousk *et al.*, 2010), nitrogen fertilizers (Zhou *et al.*, 2016) or natural processes (Rütting *et al.*, 2013; Chen *et al.*, 2014). These previous studies showed that fungi are likely to be more tolerant of soil acidification than bacteria. In this study, the vegetable fields and vineyards showed higher soil $R_{F/B}$ values than the other sample sites (Fig. 1). There was an overall negative relationship between the abundance ratios of fungi to bacteria and the soil pH.

When considering the vineyards only, the soil $R_{F/B}$ did not change significantly with the natural pH, although the pH values were significantly different (P<0.001) among the soils. One possible explanation for this is that fungi are more likely than bacteria to use the complex organic compounds present in soil (Zhang *et al.*, 2012; Gunina *et al.*, 2014; Sherman *et al.*, 2014). The vineyards in this study have received a large amount of organic fertilizer every year. The results of a previous study showed a similar pattern in an area of cropland where the surface of organic fertilizer was covered by fungal mycelia, which led to large emissions of N_2O from the soil (Wei *et al.*, 2014). The application of manure may therefore be an important cause of the increase in the relative abundance of fungi in the vineyards in our study.

Key factors affecting the reduction of N_2O in soils

The reduction of N_2O in soils was likely to have been caused by soil acidification (Fig. 3a, P<0.001). It is generally recognized that N_2O reductase activity decreases with decreasing soil pH (Cuhel and Simek, 2011; Liu *et al.*, 2014; Qu *et al.*, 2014). A plausible mechanism for this is that the assembly of functional N_2O reductase is hindered at low pH (Bergaust *et al.*, 2010; Liu *et al.*, 2014). According to this explanation, this inhibitory effect is relieved when the ambient pH is increased in the short term; and this has been verified in previous studies (Bergaust *et al.*, 2010; Cuhel and Simek, 2011). However, the results from this study indicate that this mechanism is questionable in acidic soils amended with large amounts of nitrogen fertilizer. Figure 3b shows that the observed relationship between the modified pH and R_{N2O} was completely different to the expected relationship: although the pH of the acidic soil (pH <6.25) increased, the R_{N2O} value did not decrease in proportion. These results suggest that other important factors may also affect the reduction of N_2O.

The stepwise multiple regression showed that high concentrations of residual NO_3^- decreased the reduction

of N_2O. The effect of residual NO_3^- may be due to the competition for electrons among different nitrogen oxide reductases (Pan *et al.*, 2012; Wang *et al.*, 2013) or the inhibitory influence of nitrite on nitrous oxide reductase (Gaskell *et al.*, 1981). These factors, whether pH or NO_3^-, could directly affect the reduction of N_2O in the bacterial denitrification process. If the soil N_2O emissions were dominated by soil fungi, the reduction of N_2O were more strongly affected by the ratio of fungi to bacteria than by the soil NO_3^- content and pH. The evidence points to the probability that the contribution of bacteria to N_2O production was more important than that of fungi in most instances. In addition, the emission of N_2O from soils through fungal pathways across various ecosystems may have been overestimated in previous studies when the cycloheximide was used to inhibit bacterial process (Crenshaw *et al.*, 2008; Laughlin *et al.*, 2009; Chen *et al.*, 2014).

Role of fungi in the reduction of N_2O in soils

Although the soil pH and NO_3^- content are the key factors regulating N_2O reduction in intensively managed fields, it is unwise to neglect the role of fungi in the reduction of N_2O in soils when the effects of pH and mineral nitrogen are reduced. For example, a high soil $R_{F/B}$ in vegetable fields has the potential to inhibit N_2O reduction (Fig 5a) and R_{N2O} did not obviously change with short-term modifications of the pH (Fig. 4), which suggested that the soil denitrification process was more affected by the composition of the denitrifying community than by the soil pH. This is because fungi lack the gene (*nosZ*) that facilitates N_2O reduction and fungal denitrification is therefore not regulated directly by the soil pH. These results show that fungi may dominate the emission of N_2O from soil in vegetable fields, in agreement with our previous study in which we showed that 99% of the N_2O emitted from such soils results from soil fungi (Ma *et al.*, 2017).

Fortunately, this undesirable role of fungi may be offset by the presence of denitrifying bacteria in vineyards (Fig. 5b). Our results show that fungi may indirectly affect the communities of bacteria, which is similar to the results reported by Burke *et al.* (2012). In their study, based on profiles of the terminal restriction fragment length, they found that the fungal biomass may be a more important factor than plants in structuring the distribution of communities of denitrifying bacteria in forests. The vineyards in our study had received a large amount of organic fertilizer. Bacteria may be able to utilize the easily degradable organic molecules released during the fungal degradation of complex organic matter (de Boer *et al.*, 2005; Romani *et al.*, 2006), suggesting that the joint effect of fungi and denitrifying bacteria on the emission of N_2O should be considered in organic agricultural systems.

Meta-analyses have shown that N_2O emissions do not significantly decrease or increase when chemical nitrogen fertilizers are replaced by manure (Xia *et al.*, 2017; Zhou *et al.*, 2017). The large amount of N_2O emitted from soils may be associated with an imbalance between denitrifying fungi and bacteria. Detailed studies are required to explore the effect of the composition of the communities of denitrifying bacteria and fungi on N_2O emissions in organic agricultural systems, particularly because the current rapid rates of urbanization may lead to the use of large amounts of organic waste in suburban agriculture. Notably, $R_{F/B}$ may not reveal the actual denitrifying fungi-to-denitrifying bacteria ratios in soils, and previous studies have only considered the $R_{F/B}$ when discussing the role fungi (Laughlin et al., 2009; Rütting et al., 2013; Chen et al., 2014; Huang et al., 2017). In future work, the reporting of some environmental denitrifying microbial sequences will be helpful to refine PCR tools and to comprehensively assess the abundance and activity of microbes involving N_2O flux, such as the *p450nor* gene in fungi and atypical *nosZ* genes in bacteria (Orellana *et al.*, 2014; Chen and Shi, 2017). Studies involving the management of manure could be an important component of future efforts to reduce the anthropogenic sources of N_2O emissions (Davidson, 2009; Chadwick *et al.*, 2015).

CONCLUSIONS

This study shows that not only soil acidification, but also the application of manure promotes the relative abundance of fungi in the microbial composition of soils. The reduction of N_2O was more strongly affected by the soil NO_3^- content and pH than by the ratio of fungi to bacteria, although fungi lack the N_2O reductase gene (*nosZ*). It is possible that the expression of *nosZ* and the function of N_2O reductase could be inhibited by soil acidification and a high residual content of mineral nitrogen, respectively. However, a high $R_{F/B}$ still has the potential to inhibit the reduction of N_2O in vegetable fields, but the undesirable role of fungi may lessen with an increase in the relative abundance of denitrifying bacteria in vineyards treated with manure.

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Figure

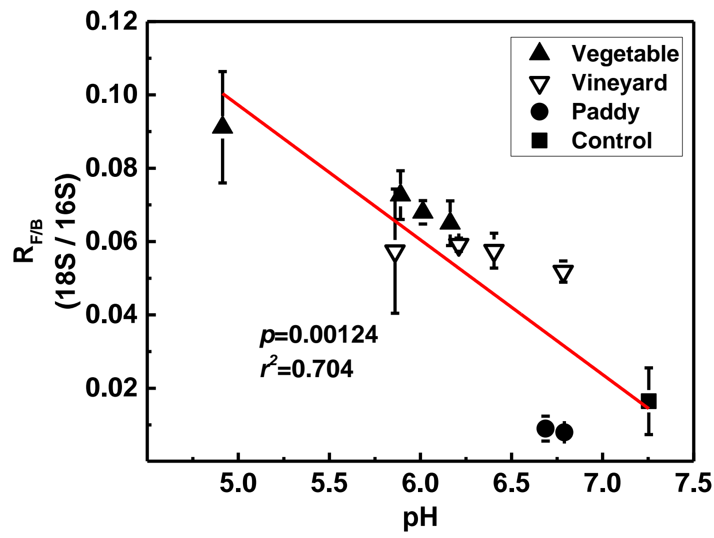

Fig. 1.

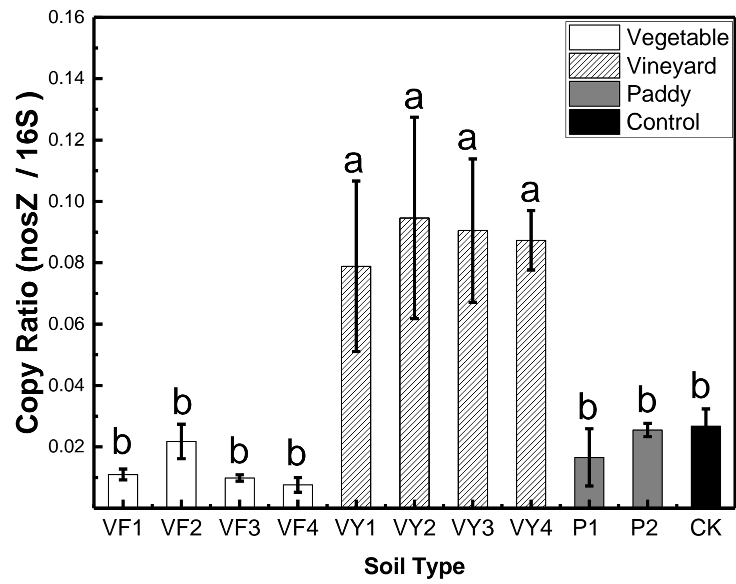

Fig. 2

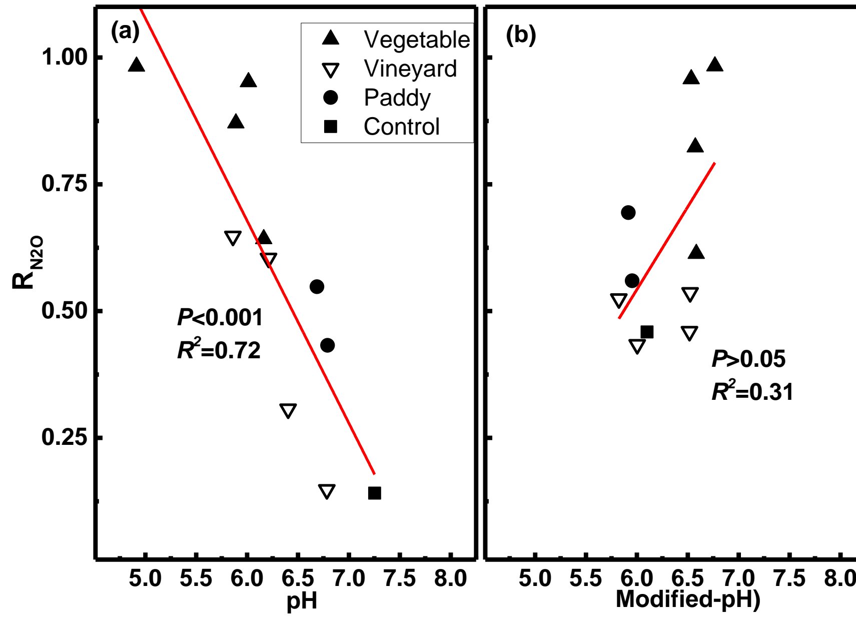

Fig. 3

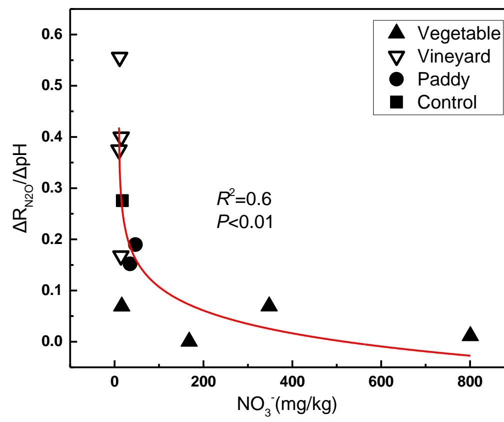

Fig. 4

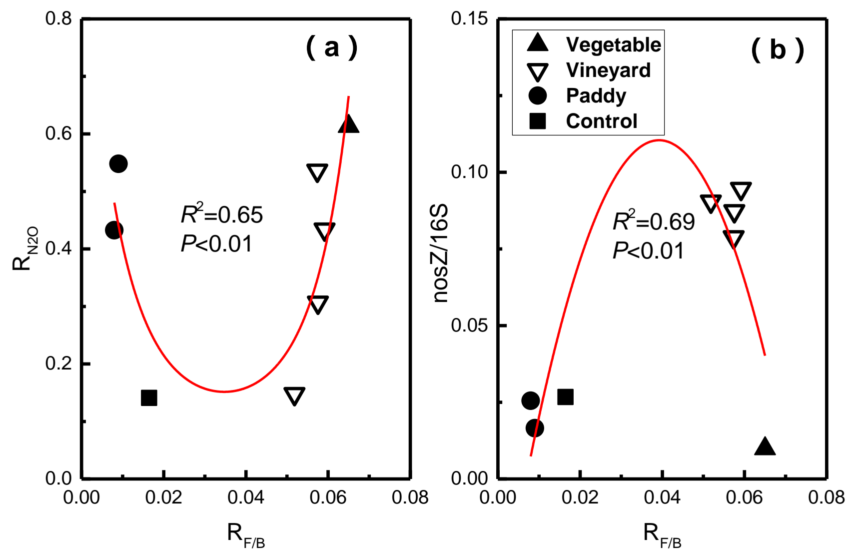

Fig. 5.