Effects of the nitrification inhibitor dicyandiamide (DCD) on N₂O emissions and the abundance of nitrifiers and denitrifiers in two contrasting agricultural soils

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Abstract

Purpose Nitrous oxide (N₂O) is a trace gas and is mainly produced through the microbial-mediated nitrification and denitrification processes in agricultural soils. Dicyandiamide (DCD) is a powerful nitrification inhibitor (NI) which can mitigate N₂O production from soil. However, it remains poorly understood how N₂O emission and associated microbes are affected by DCD application across different agricultural soils.

Materials and methods Here, we carried out a 21-day laboratory microcosm incubation experiment to examine the impact of ammonium sulfate and DCD applications on N₂O production and the abundance of nitrifiers and denitrifiers in two contrasting agricultural soils. The abundance of the functional genes was quantified by quantitative PCR to investigate the population changes in response to ammonium sulfate and DCD addition.

Results and discussion Higher N₂O production emitted followed the application of ammonium sulfate (287.9 ng N₂O–N g^{−1} soil) in the alluvial soil was obtained compared to that (84.9 ng N₂O–N g^{−1} soil) in the red soil. The average N₂O emission rate in the NH₄⁺ treatment from the alluvial soil

was significantly higher than that from the red soil, and DCD addition remarkably reduced N₂O emission rates in both soils during the 21-day incubation. We also observed that the abundance of ammonia-oxidizing bacteria (AOB) *amoA* gene and the *nirK* gene was positively correlated with total N₂O emissions in the alluvial soil, but only AOB *amoA* gene abundance was significantly related to the total N₂O emissions in the red soil.

Conclusions These results suggested that DCD was more effective in inhibiting N₂O production in the alluvial soil than in the red soil. DCD could effectively reduce N₂O emission by suppressing the growth of microorganisms containing AOB *amoA* gene and the *nirK* gene in the alluvial soil.

Keywords Agricultural soils · Denitrifiers · Nitrification inhibitor · Nitrifiers · Nitrous oxide

1 Introduction

Nitrous oxide (N₂O) is a potential greenhouse gas, with an approximately 300 times stronger global warming potential than that of carbon dioxide (CO₂) (IPCC 2007). Agricultural soils are believed to be the major source of N₂O emission for the atmosphere (Del Grosso et al. 2006), accounting for nearly 65 % of the total anthropogenic N₂O emissions (Davidson and Kanter 2014). The excessive application of mineral nitrogen (N) fertilizers considerably causes large amounts of N₂O production in the past decades (Galloway et al. 2008; Davidson 2009). Therefore, it is very important to develop mitigation strategies to enhance N fertilizer use efficiency and to reduce N₂O emissions from agricultural soil ecosystems.

The N₂O production from soil is mainly from nitrification-mediated processes (nitrifier nitrification and nitrifier

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denitrification) and denitrification (Hu et al. 2015). Nitrification is the oxidation of ammonium (NH_4^+) or ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-) and releases N_2O as a by-product (Wrage et al. 2001). The critical step of nitrification, the oxidation of NH_3 or NH_4^+ to hydroxylamine (NH_2OH), is carried out by ammonia-oxidizing bacteria (AOB) or archaea (AOA). The important enzyme of this process is ammonia monooxygenase (AMO) which is encoded by the *amoA* gene. AOB can generate N_2O directly through incomplete NH_2OH oxidation to nitric oxide (NO) and further to N_2O (ammonia oxidation pathway) or through the nitrifier denitrification pathway (sequential enzymatic reduction of NO_2^- to NO and N_2O , mainly under high soil moisture) (Arp and Stein 2003; Shaw et al. 2006). Unlike AOB, AOA are considered to produce N_2O during ammonia oxidation, from NO_2^- and an intermediate of ammonia oxidation through a new hybrid formation mechanism (Stieglmeier et al. 2014). Denitrification is the sequential reduction of NO_3^- to dinitrogen gas (N_2) through NO_2^- , NO, and N_2O ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) (Firestone and Davidson 1989; Di et al. 2014). Firstly, NO_3^- is reduced to NO_2^- by NO_3^- reducers. The second step ($\text{NO}_2^- \rightarrow \text{NO}$) is catalyzed by two different NO_2^- reductases encoded by the *nirS* or *nirK* genes. In the third step, NO reducers converted NO to N_2O , which is an end product under particular conditions. The final step ($\text{N}_2\text{O} \rightarrow \text{N}_2$) is carried out by N_2O reductase encoded by the *nosZ* gene in denitrifying microorganisms (Henry et al. 2006).

Nitrification inhibitors (NIs) are often applied together with N fertilizer in order to reduce N_2O emissions and NO_3^- leaching from soil. One of the most commonly used NIs is dicyandiamide (DCD, $\text{C}_2\text{H}_4\text{N}_4$), which has been studied for over 80 years (Lan et al. 2013). It has been reported that DCD can reduce the total N_2O production by 97 % in an alluvial soil from North China Plain (Huang et al. 2014a). DCD has been previously suggested to act as a Cu chelator and inhibits the activity of AMO and NirK (Kim et al. 2010; Subbarao et al. 2006). The oxidation of NH_4^+ or NH_3 to NO_2^- or NO_3^- is delayed by repressing the activities of AMO in ammonia-oxidizing microbes in soil (Cui et al. 2013). Thus, the NIs could directly decrease N_2O production by slowing down the nitrification rates or indirectly by reducing the concentration of NO_3^- for denitrification (Merino et al. 2001; Lan et al. 2013). Moreover, the effect of DCD in reducing N_2O emission in previous studies is simply ascribed to inhibition of nitrifiers, while it is unknown whether DCD also affects the growth of denitrifiers in agricultural soils.

The objective of this study was to investigate the effectiveness of ammonium and ammonium added with DCD on the abundance of nitrifiers and denitrifiers and N_2O emission in two contrasting upland soils under controlled incubation conditions. The abundance of nitrifiers and

denitrifiers was checked by quantifying the copy numbers of key functional genes involved in the nitrification and denitrification processes. We tested the hypothesis that the growth of nitrifiers and denitrifiers might be indirectly affected by DCD, probably due to the inhibitory effect on the nitrification process.

2 Materials and methods

2.1 Soil sample collection

Two arable soils were collected from Luancheng county ($40^\circ 7' 34'' \text{ N}$, $119^\circ 11' 27'' \text{ E}$), Hebei Province and Qiyang county ($26^\circ 24' 26'' \text{ N}$, $112^\circ 00' 45'' \text{ E}$), Hunan Province, China. The soil at the Luancheng site is classified as an upland alluvial soil according to US soil taxonomy (USDA 1994) and planted with a wheat-maize rotation under conventional agricultural management practices. The soil at the Qiyang site was collected from a maize-growing field in the Qiyang Agricultural Research Station and was classified as a red soil in the USDA Soil Taxonomy. Soil samples were collected from 0 to 20-cm layer, air-dried, and sieved ($<2 \text{ mm}$) prior to construction of microcosm incubation. Details of two soils properties were shown in Table 1.

2.2 Microcosm incubations

Twenty grams of soil (dry weight equivalent) from two types of soils was weighted in the 120-ml glass bottles. The soil samples were then adjusted to 40 % water hold content (WHC) and pre-incubated for 1 week at 30°C to activate the microbial population. After pre-incubation, soil was amended with deionized sterilized water (the control treatment without addition of N fertilizer or inhibitors) or a solution of $(\text{NH}_4)_2\text{SO}_4$ (150 mg N kg^{-1} soil) in the presence or absence of DCD (at a concentration of 10 % of the applied NH_4^+). The final soil moisture content was adjusted to 55 % WHC. In total, three treatments with three replicates were established as follows: control, NH_4^+ , and $\text{NH}_4^+ + \text{DCD}$. All the microcosms were incubated at 30°C for 21 days. The soil moisture content was maintained at 55 % WHC by adding distilled water every 2 or 3 days.

2.3 N_2O emission and mineral nitrogen measurement

Gas samples were collected at 1, 2, 3, 4, 7, 11, 14, and 21 days after pre-incubation. Before each gas sampling time point, bottles were closed for 24 h and then 10-ml gas samples were taken from the headspace of each bottle and transferred to pre-evacuated vials for N_2O analysis. The N_2O concentration was analyzed by a gas chromatograph

Table 1 Soil basic properties of the alluvial soil and the red soil. Soil pH, dissolved organic carbon (DOC), total nitrogen (TN), ammonium-N (NH_4^+ -N), and nitrate-N (NO_3^- -N) were tested

Soil tested	pH	DOC (mg kg ⁻¹)	TN (g kg ⁻¹)	NH_4^+ -N (mg kg ⁻¹)	NO_3^- -N (mg kg ⁻¹)
Alluvial soil	7.8a	46.0a	0.89b	2.4a	117.8a
Red soil	6.2b	33.0b	1.51a	3.0a	27.8b

Different letters denote significant difference ($P < 0.05$)

(HP7890A, Agilent Technologies, CA, USA). Three bottles per treatment were destructively sampled at 7, 14, and 21 days after incubation. Five grams of soil sample was extracted with 25 ml 0.01 M CaCl_2 solution, under shaking for 1 h, and then soil suspensions were filtered through filter papers (Whatman no. 42). Extracts were analyzed for NH_4^+ -N and NO_3^- -N contents on a flow injection analyzer (Skalar SAN++). The remaining soil samples were stored at -80°C for DNA extraction.

2.4 DNA extraction and quantification by real-time PCR

Soil DNA was extracted from 0.4 g soil with Powersoil™ DNA isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. Total soil DNA was quantified by a Nanodrop-1000 (Thermo Fisher Scientific). Soil DNA extracts were stored at -80°C until use.

AmoA gene was used to explore the shifts in the abundance of AOB and AOA. The *nirK* and *nosZ*-type denitrification genes, which encoded NO_2^- reductase (NirK) and NO reductase (NosZ) were also estimated. The PCR primers used to amplify functional genes from nitrifiers and denitrifiers were listed in Table 2. PCR reactions were carried out on a CFX96 (Bio-rad, USA). The 25 μl reaction mixture included 12.5 μl of 2 $\times$ Supermix (Bio-rad), 0.5 μl of BSA, 0.5 μl of each primer (10 mM), and 2 μl of template DNA (1–10 ng) and 9 μl of sterilized deionized water. The thermal-cycling profiles for every gene were listed in Table 2. The specificity of amplicons was checked by agarose gel electrophoresis and melting curve analysis. Standard curves were obtained using a tenfold serial dilution of plasmids harboring AOB *amoA*, AOA *amoA*, *nirK*, and *nosZ* genes.

2.5 Statistical analysis

The statistical analysis was performed using SPSS 17.0 software (IBM Co., Armonk, NY, USA). ANOVA was performed to assess the significance of different treatments on N_2O production; the *amoA*, *nirK*, and *nosZ* genes abundance; and NH_4^+ -N and NO_3^- -N concentrations. Correlations between the functional genes abundance and

total N_2O emissions were assessed by Pearson's correlation procedure.

3 Results

3.1 Soil pH

As for the alluvial soil, soil pH was around 7.8 after 21-day incubation across all the treatments. As for the red soil, soil pH in the control treatment was slightly reduced at the end of incubation (Fig. 1). The addition of NH_4^+ caused an obvious decline in soil pH from 6.2 to 4.6 ($P < 0.05$) after 21-day incubation in the red soil. When DCD was applied with NH_4^+ , soil pH was much higher than that in the NH_4^+ treatment but lower than that in the control treatment in the red soil ($P < 0.05$).

3.2 N_2O emission rates

In the control treatment, N_2O emission rates were very low during the 21-day incubation in both soils (Fig. 2). NH_4^+ addition significantly increased N_2O emission rates, reaching a peak of 92.4 ng N_2O -N g⁻¹ soil day⁻¹ at day 2 in the alluvial soil and then decreased quickly. In the red soil, N_2O emission rates ranged from 0.5 to 7.0 ng N_2O -N g⁻¹ soil day⁻¹. The application of DCD with NH_4^+ significantly reduced the average N_2O emission rates to 0.90 ng N_2O -N g⁻¹ soil day⁻¹ in the alluvial soil and 0.51 ng N_2O -N g⁻¹ soil day⁻¹ in the red soil, respectively.

3.3 Total N_2O emissions

In the two tested soils, the total N_2O production was very low in the control treatment, (Fig. 3). In the alluvial soil, NH_4^+ addition significantly increased N_2O emission to 287.9 ng N_2O -N g⁻¹ soil, compared to that in the control treatment ($P < 0.05$). The application of DCD remarkably ($P < 0.05$) reduced N_2O emission by 94.3 %. Likewise, NH_4^+ addition significantly increased N_2O emission from the red soil, but to a less extent, reaching 84.9 ng N_2O -N g⁻¹ soil ($P < 0.05$). N_2O emission in the NH_4^+ + DCD treatment was reduced by 87.4 % in the red soil, compared to the NH_4^+ treatment.

Table 2 Primer sequences, thermal cycling conditions and efficiencies of the quantitative PCR reactions

Target group	Primer name	Sequence (5′–3′)	Amplicon length (bp)	Thermal profile	Amplification efficiency (%)	Reference
AOB	amoA1F	GGGGTTTCTACTGGTGGT	491 bp	95 °C for 3 min × 1 cycle; 95 °C for 30 s, 57 °C for 40 s, 72 °C for 40 s, 83 °C for 15 s × 40 cycles	93–97	Rotthauwe et al. 1997
amoA	amoA2R	CCCCTCKGSAAAGCCTTCTTC				
AOA	CrenamoA23f	ATGGTCTGGCTWAGACG	625 bp	95 °C for 3 min × 1 cycle; 95 °C for 30 s, 53 °C for 40 s, 72 °C for 40 s, 83 °C for 15 s × 35 cycles	90–95	Tourna et al. 2008
amoA	CrenamoA616r	GCCATCCATCTGTATGTCCA				
nirK	FlaCu	ATCATGGTCTGCCGCG	474 bp	95 °C for 3 min × 1 cycle; 95 °C for 30 s, 58 °C for 40 s, 72 °C for 40 s, 83 °C for 15 s × 40 cycles	88–93	Hallin and Lindgren 1999
	R3Cu	GCCTCGATCAGRTTGTGGTT				
nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	424 bp	95 °C for 3 min × 1 cycle; 95 °C for 30 s, 58 °C for 40 s, 72 °C for 40 s, 83 °C for 15 s × 40 cycles	91–94	Kloos et al. 2001
	nosZ1622R	CGSACCTTSTTGCCSTYGCG				

3.4 $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentration

During the 21-day incubation, the $\text{NH}_4^+\text{-N}$ concentration was consistently low in the control treatment in both soils (Fig. 4). In the alluvial soil, the $\text{NH}_4^+\text{-N}$ concentration decreased sharply from 148 to 2 mg N kg^{-1} soil at day 7 in the NH_4^+ treatment and DCD addition significantly reduced the rate of NH_4^+ oxidation compared with the NH_4^+ only treatment. In the red soil, the $\text{NH}_4^+\text{-N}$ concentration of NH_4^+ treatment gradually declined to the level of the control treatment at the end of the incubation, and the average decreasing rate was 4.0 mg N kg^{-1} soil day^{-1} . When DCD was added with NH_4^+ , the declining rate of the NH_4^+ concentration was much lower, and less NH_4^+ was lost from the red soil than that from the alluvial soil.

The $\text{NO}_3^-\text{-N}$ concentration in the two soils changed in a different pattern from that of $\text{NH}_4^+\text{-N}$ (Fig. 4). In the alluvial soil, the concentration of $\text{NO}_3^-\text{-N}$ in the NH_4^+ treatment increased sharply and reached the peak in the

first 7 days. The concentration of $\text{NO}_3^-\text{-N}$ in the $\text{NH}_4^+ + \text{DCD}$ treatment was significantly lower than that in the NH_4^+ treatment, but was obviously higher than that in the control treatment ($P < 0.05$). In the red soil, the $\text{NO}_3^-\text{-N}$ concentration increased gradually during the incubation in the NH_4^+ treatment. DCD addition markedly inhibited the production of $\text{NO}_3^-\text{-N}$ which was equal to that of the control treatment for about 14 days in the red soil.

3.5 Quantification of functional gene abundance

3.5.1 Abundance of the AOA and AOB *amoA* genes

After 21 days of incubation, the AOB *amoA* gene abundance in the control treatment was about 1.1×10^7 copies g^{-1} soil in the alluvial soil and 2.8×10^6 copies g^{-1} soil in the red soil, respectively (Fig. 5). The AOB *amoA* gene abundance, strongly increased by the NH_4^+ addition, was 3.1×10^7 copies g^{-1} soil in the alluvial soil and 7.2×10^6 copies g^{-1} soil in the red

Fig. 1 Soil pH at the beginning (0 day) and end (21 days) of microcosm incubation of the alluvial soil (a) and the red soil (b) without NH_4^+ and DCD (control), with NH_4^+ addition (NH_4^+) and with NH_4^+ and DCD

($\text{NH}_4^+ + \text{DCD}$). Data are means with standard deviations ($n = 3$). Different letters above the bars denote significant difference and the same letters denote no significant difference

Fig. 2 Dynamics of N₂O emission rates in the alluvial soil and the red soil without NH₄⁺ and DCD addition (control), with NH₄⁺ addition (NH₄⁺), and with NH₄⁺ and DCD (NH₄⁺ + DCD). Data were presented as mean values with standard errors ($n = 3$)

soil, respectively, which was significantly greater than those of the respective control treatments ($P < 0.05$). However, the application of DCD with NH₄⁺ showed an obvious effect of inhibition on AOB growth in both soils ($P < 0.05$).

Unlike AOB, the AOA *amoA* gene abundance showed no significant difference among the treatments in the alluvial soil ($P > 0.05$). In the red soil, the AOA *amoA* gene abundance in the control treatment at day 21 was higher than that in other treatments (Fig. 5). By contrast, the AOA *amoA* gene abundance dramatically ($P < 0.05$) decreased in the NH₄⁺ treatment in the red soil, suggesting a possible inhibitory effect of high NH₄⁺ concentration on the AOA growth in this soil. There is no significant difference in the AOA *amoA* gene abundance between the NH₄⁺ and NH₄⁺ + DCD treatments ($P > 0.05$).

3.5.2 Denitrifier functional gene abundance

The *nirK* gene abundance in the control treatment was about 1.6×10^7 copies g⁻¹ soil in the alluvial soil and 3.4×10^7 copies g⁻¹ soil in the red soil, respectively (Fig. 5). In the

alluvial soil, the application of NH₄⁺ significantly increased the *nirK* gene abundance, reaching 2.4×10^7 copies g⁻¹ soil, and DCD addition strongly reduced the *nirK* gene abundance to 1.8×10^7 copies g⁻¹ soil ($P < 0.05$). The *nosZ* gene abundance in the alluvial soil was about 1.25×10^7 copies g⁻¹ soil, and did not significantly respond to the NH₄⁺ and DCD addition. In the red soil, the NH₄⁺ addition significantly reduced denitrifier functional genes abundance (*nirK* and *nosZ*). The *nirK* and *nosZ* genes abundance in the NH₄⁺ + DCD treatment was considerably lower than that in the control treatment, but significantly greater than that in the NH₄⁺ treatment.

4 Discussion

In the alluvial soil, soil pH remained stable among all the treatments (Fig. 1). In the red soil, soil pH decreased sharply in the NH₄⁺ treatment in our study, because NH₄⁺ is converted to NO₃⁻ via NO₂⁻ by nitrifiers in the nitrification process during which the H⁺ ions could be released, consequently

Fig. 3 The total N₂O emissions during the 21-day microcosm incubation of the alluvial soil (a) and the red soil (b) without NH₄⁺ and DCD (control), with NH₄⁺ addition (NH₄⁺) and with NH₄⁺ and DCD (NH₄⁺ +

DCD). Data are means with standard deviations ($n = 3$). Different letters above the bars denote significant difference and the same letters denote no significant difference

Fig. 4 The dynamics of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ during the 21 days' incubation in the alluvial soil (a, b) and the red soil (c, d) without NH_4^+ and DCD (control), with NH_4^+ addition (NH_4^+), and with NH_4^+ and DCD (NH_4^+ + DCD). Data are means with standard deviations ($n = 3$)

leading to a decrease in soil pH. Soil pH slightly declined after DCD was applied with NH_4^+ , probably due to an incomplete inhibition of nitrification by DCD.

Results from this study clearly showed that NH_4^+ addition considerably increased N_2O production from the two examined soils, but to different extents. The total N_2O emissions from the alluvial soil and the red soil were stimulated by 287.9 and 84.9 ng $\text{N}_2\text{O-N g}^{-1}$ soil after the NH_4^+ application, respectively (Fig. 2). The reductions caused by DCD addition in total N_2O emissions from these two soils followed the similar pattern as N_2O stimulation (19.0 and 10.7 ng $\text{N}_2\text{O-N g}^{-1}$ soil for the alluvial and red soils, respectively). Total N_2O emissions were positively correlated to the AOB *amoA* gene abundance ($R^2 = 0.94$, $P < 0.001$) and the *nirK* gene abundance ($R^2 = 0.64$, $P < 0.001$) in the alluvial soil. Moreover, we only observed a significantly positive relationship between the AOB *amoA* gene abundance and total N_2O emissions in the red soil ($R^2 = 0.74$, $P < 0.001$) (figures not shown). These results are in line with previous findings that distinct responses of N_2O after urea and nitrification inhibitors application in arable soils are mainly attributed to AOB growth (Cui et al. 2013). Similar results have been also reported in grassland soils (Di et al. 2010, 2014; Dai et al. 2013).

Although AOB population abundance was different in the two soils, we found that NH_4^+ addition increased AOB abundance and the application of DCD significantly inhibited its

growth (Fig. 5). These results are in agreement with the findings from various grassland soils (Di et al. 2010, 2014). In the alluvial soil, the AOA *amoA* gene abundance did not respond to the NH_4^+ and DCD application, which was also consistent with previous conclusions (Jia and Conrad 2009; O'Callaghan et al. 2010). By contrast, the AOA *amoA* gene abundance increased in the control treatment in the red soil because AOA can live well in acidic soils with low concentration of NH_3 derived from mineralization of organic N (Levičnik-Höfferle et al. 2012; Zhang et al. 2012; Wang et al. 2016). However, the application of NH_4^+ remarkably reduced the AOA abundance in the red soil. This finding agrees with several studies which demonstrated that AOB grew in high urine-treated soils, where AOA was inhibited (Di et al. 2010, 2014). These results therefore support the hypothesis that the available amount of NH_4^+ is the key factor in influencing the growth of AOB and AOA in various soils (Di et al. 2010; Verhamme et al. 2011).

The application of NIs with mineral N fertilizers has shown the potential to reduce denitrification-derived N_2O emissions (Di and Cameron 2008; Lan et al. 2013; Ruser and Schulz 2015), but it is poorly known whether the associated denitrifying functional microbes will be affected by NIs. In the alluvial soil, the *nirK* gene abundance significantly increased after the addition of NH_4^+ , but the application of DCD with NH_4^+ inhibited its growth compared to the NH_4^+

Fig. 5 Copy numbers of the AOA *amoA* (a, e), AOB *amoA* (b, f), *nirK* (c, g), and *nosZ* (d, h) genes at the beginning (0 day) and end (21 days) of laboratory incubation in the alluvial soil (a–d) and the red soil (e–h) without NH_4^+ and DCD (control), with NH_4^+ addition (NH_4^+) and with

NH_4^+ and DCD (NH_4^+ + DCD). Data are means with standard deviations ($n = 3$). Data are means with standard deviations ($n = 3$). Different letters above the bars denote significant difference and the same letters denote no significant difference

treatment. Overall, the dynamics of the *nirK* gene abundance were similar with that of AOB *amoA* gene. Genomes analysis finds that AOB contain the *nirK* gene which is related to the

pathway known as nitrifier denitrification (Kim et al. 2010). Moreover, the observed obvious reduction in N_2O production by DCD in the alluvial soil is probably due to its inhibitory

effect on the process of nitrifier denitrification mediated by AOB, because AOA appears not be able to produce N_2O via nitrifier denitrification process for lacking a known NO reductase (Hu et al. 2015). Further study is needed to explore the role of AOB in N_2O emission via nitrifier denitrification process under different soil conditions. In the alluvial soil, the *nosZ* gene did not respond to the NH_4^+ and NH_4^+ + DCD treatments. In contrary, the abundances of *nirK* and *nosZ* genes significantly decreased in the NH_4^+ and NH_4^+ + DCD treatments probably due to pH decrease in the red soil. It has been shown that low pH can inhibit the activity of nitrogen oxide reductase, which is directly involved in the N_2O reductions (Čuhel et al. 2010; Bakken et al. 2012; Huang et al. 2014b). However, the abundances of *nirK* and *nosZ* genes in the NH_4^+ + DCD treatment were higher than that in the NH_4^+ only treatment. This might because DCD slowed down the nitrification rate and led to a small decrease in pH in the NH_4^+ + DCD treatment. Therefore, there is a need for more research on the N_2O reducing microbial communities affected by pH which may help in finding mitigation solutions for N_2O emissions from soils.

5 Conclusions

Our results revealed that the extent of the increase and decrease of N_2O production caused by ammonium sulfate and DCD addition was significantly different between the alluvial soil and red soil. Ammonium addition increased the abundance of AOB and *nirK* genes, but decreased by DCD in the alluvial soil. DCD affected the abundance of the *nirK* gene, probably because it is always harbored by ammonia oxidizers. In the red soil, the high concentration of ammonium-N significantly increased the AOB abundance but reduced the growth of AOA. Moreover, denitrifier community abundance was obviously reduced in the ammonium-treated soils probably due to soil acidification. More research is needed to confirm these deductions in the future.

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References

- Arp DJ, Stein LY (2003) Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Crit Rev Biochem Mol* 38:471–495
- Bakken LR, Bergaust L, Liu BB, Frostegard A (2012) Regulation of denitrification at the cellular level: a clue to the understanding of N_2O emissions from soils. *Philos T R Soc B* 367:1226–1234
- Čuhel J, Miloslav Š, Ronnie JL, David B (2010) Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. *Appl Environ Microbiol* 76:1870–1878
- Cui PY, Fan FL, Yin C, Li ZJ, Song AL, Wan YF, Liang YC (2013) Urea- and nitrapyrin-affected N_2O emission is coupled mainly with ammonia oxidizing bacteria growth in microcosms of three typical Chinese arable soils. *Soil Biol Biochem* 66:214–221
- Dai Y, Di HJ, Cameron KC, He JZ (2013) Effects of nitrogen application rate and a nitrification inhibitor dicyandiamide on ammonia oxidizers and N_2O emissions in a grazed pasture soil. *Sci Total Environ* 465:125–135
- Davidson EA (2009) The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nat Geosci* 2:659–662
- Davidson EA, Kanter D (2014) Inventories and scenarios of nitrous oxide emissions. *Environ Res Lett* 9:105012
- Del Grosso SJ, Parton WJ, Mosier AR, Walsh MK, Ojima DS, Thornton PE (2006) DAYCENT national-scale simulations of nitrous oxide emissions from cropped soils in the United States. *J Environ Qual* 35:1451–1460
- Di HJ, Cameron KC (2008) Sources of nitrous oxide from ^{15}N -labelled animal urine and urea fertiliser with and without a nitrification inhibitor, dicyandiamide (DCD). *Aust J Soil Res* 46:76–82
- Di HJ, Cameron KC, Sherlock RR, Shen JP, He JZ, Winefield CS (2010) Nitrous oxide emissions from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia-oxidizing bacteria and archaea. *J Soils Sediments* 10: 943–954
- Di HJ, Cameron KC, Podolyan A, Robinson A (2014) Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. *Soil Biol Biochem* 73:59–68
- Firestone MK, Davidson EA (1989) Microbiological basis of NO and N_2O production and consumption in soils. In: Andreae MO, Schimel DS (eds) Exchange of trace gases between terrestrial ecosystems and the atmosphere. John Wiley & Sons, Chichester, pp 7–21
- Galloway JN, Townsend AR, Erismann JW, Bekunda M, Cai ZC, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320:889–892
- Hallin S, Lindgren PE (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Appl Environ Microbiol* 65:1652–1657
- Henry S, Bru D, Stres B, Hallet S, Philippot L (2006) Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl Environ Microbiol* 72:5181–5189
- Hu HW, Chen DL, He JZ (2015) Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiol Rev* 39:729–749
- Huang T, Gao B, Hu XK, Lu X, Well R, Christie P, Bakken LR, Ju XT (2014a) Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. *Sci Rep* 4: 3950
- Huang Y, Li YY, Yao HY (2014b) Nitrate enhances N_2O emission more than ammonium in a highly acidic soil. *J Soils Sediments* 14:146–154
- IPCC (2007) Mitigation of climate change. In: Merz B et al (eds) Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, UK
- Jia Z, Conrad R (2009) Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environ Microbiol* 11: 1658–1671
- Kim SW, Miyahara M, Fushinobu S, Wakagi T, Shoun H (2010) Nitrous oxide emission from nitrifying activated sludge dependent on denitrification by ammonia-oxidizing bacteria. *Bioresour Technol* 101:3958–3963

- Kloos K, Mergel A, Rosch C, Bothe H (2001) Denitrification within the genus *Azospirillum* and other associative bacteria. *Aust J Plant Physiol* 28:991–998
- Lan T, Han Y, Roelcke M, Nieder R, Cai Z (2013) Effects of the nitrification inhibitor dicyandiamide (DCD) on gross N transformation rates and mitigating N₂O emission in paddy soils. *Soil Biol Biochem* 67:174–182
- Levičnik-Höfferle S, Nicol GW, Ausec L, Mandic-Mulec I, Prosser JI (2012) Stimulation of thaumarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen. *FEMS Microbiol Ecol* 80:114–123
- Merino P, Estavillo JM, Besga G, Pinto M, Gonzalez-Murua C (2001) Nitrification and denitrification derived N₂O production from a grassland soil under application of DCD and Actilith F2. *Nutr Cycl Agroecosys* 60:9–14
- O'Callaghan M, Gerard EM, Carter PE, Lardner R, Sarathchandra U, Burch G, Ghani A, Bell N (2010) Effect of the nitrification inhibitor dicyandiamide (DCD) on microbial communities in a pasture soil amended with bovine urine. *Soil Biol Biochem* 42:1425–1436
- Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 63:4704–4712
- Ruser R, Schulz R (2015) The effect of nitrification inhibitors on the nitrous oxide (N₂O) release from agricultural soils—a review. *J plant Nutri. Soil Sci* 178:171–188
- Shaw LJ, Nicol GW, Smith Z, Fear J, Prosser JI, Baggs EM (2006) *Nitrosospora* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ Microbiol* 8:214–222
- Stieglmeier M, Mooshammer M, Kitzler B, Wanek W, Zechmeister-Boltenstern S, Richter A, Schleper C (2014) Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J* 8:1135–1146
- Subbarao GV, Ito O, Sahrawat KL, Berry WL, Nakahara K, Ishikawa T, Watanabe T, Suenaga K, Rondon M, Rao IM (2006) Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. *Crit Rev Plant Sci* 25:303–335
- Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environ Microbiol* 10:1357–1364
- USDA (1994) Keys to soil taxonomy, 6th edn. United States Department of Agriculture, Soil Conservation Service, USA, p. 306
- Verhamme DT, Prosser JI, Nicol GW (2011) Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J* 5:1067–1071
- Wang Q, Zhang LM, Shen JP, Du S, Han LL, He JZ (2016) Nitrogen fertiliser-induced changes in N₂O emissions are attributed more to ammonia-oxidising bacteria rather than archaea as revealed using 1-octyne and acetylene inhibitors in two arable soils. *Biol Fertil Soils* 52:1163–1171
- Wrage N, Velthof GL, van Beusichem ML, Oenema O (2001) Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol Biochem* 33:1723–1732
- Zhang LM, HW H, Shen JP, He JZ (2012) Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *ISME J* 6:1032–1045