

# Does the nitrification inhibitor dicyandiamide affect the abundance of ammonia-oxidizing bacteria and archaea in a Hap-Udic Luvisol?

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## Abstract

To date, there are several studies on the responses of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) to nitrification inhibitors, but only a limited number of observations are available for higher than regular application rates. Here, we report the results of a study investigating the responses of AOB and AOA to the nitrification inhibitor, dicyandiamide (DCD). DCD suppressed growth of AOB significantly, whereas there was no significant difference between a single or a double dose of DCD on inhibiting the growth of AOB for 91 days. AOA abundance was stable among treatments, regardless of the addition of a single or a double dose of DCD. When DCD was applied alone, the AOA abundance was not appreciably changed at day 14. The results clearly show that AOA was not sensitive to the nitrification inhibitor DCD under our experimental conditions.

**Keywords:** real-time PCR, ammonium, nitrate, nitrification inhibition rate.

## 1. Introduction

Soil nitrification, one of the key processes of the nitrogen cycle, may enhance the loss of fertilizer nitrogen by leaching and denitrification. Many nitrification inhibitors, such as dicyandiamide (DCD), have been proven to be effective in reducing nitrification rates in soil. DCD suppresses ammonia oxidation by deactivating ammonium monooxygenase (Amberger, 1989). Ammonia oxidation, as the first step in the nitrification process, is often thought to be

the rate-limiting step, and traditionally controlled by ammonia-oxidizing bacteria (AOB) by autotrophic nitrification (Boer and Kowalchuk, 2001). With the discovery of larger numbers of ammonia-oxidizing archaea (AOA) than AOB, a contribution from AOA was expected (He *et al.*, 2007; Leininger *et al.*, 2006). In recent studies, the views on the contribution of AOA in ammonia oxidation were different (Di *et al.*, 2009; Jia and Conrad, 2009; Offre *et al.*, 2009; Tourna *et*

*al.*, 2008). Responses of AOA and AOB to nitrification inhibitors were investigated in several studies, which found that AOB abundance decreased after the application of DCD or DMPP (3,4-dimethylpyrazole phosphate), whereas AOA abundance remained largely unchanged (Di and Cameron, 2011; O'Callaghan *et al.*, 2010).

Much work has gone into the effects of nitrification inhibitors on the soil nitrogen cycle and on crop yields (Cookson and Cornforth, 2002; Pasda *et al.*, 2001; Zhang *et al.*, 2010). However, limited information is available concerning the effects of higher than regular application doses of DCD on the abundance of AOA and AOB. In addition, there are few studies on the responses of AOA and AOB to the nitrification inhibitor DCD.

The goal of this study was to evaluate the effects of different amounts of DCD on the growth of AOA and AOB in brown soil, which is the prevalent agricultural soil in the Liaoning Province of Northeast China. To achieve this goal, two laboratory experiments were constructed to investigate the dynamic changes of soil AOA and AOB populations in response to the addition of DCD by real-time PCR assay.

## 2. Materials and Methods

Surface (0-20 cm) brown soil (Hap-Udic Luvisol, WRB 1998) was collected from the Experimental Station of Shenyang Agricultural University (41°49' N, 123°33' E) in the Liaoning Province of Northeast China. The total carbon and nitrogen were 1.0% and 0.1%, respectively. The particle size distribution was sand = 31%, silt = 49% and clay = 20%. The soil pH in a 1:2.5 soil: water paste was 6.1.

The moist field soil was crushed to pass through a 2 mm sieve and further pre-incubated with 40% water-filled pore space (WFPS) for 2 weeks at 25 °C in the dark.

### 2.1. Experiment 1:

Four treatments were replicated three times: (1) Control (no nitrogen added), (2) N (ammonium sulfate), (3) N+DCD1 (ammonium sulfate + DCD1) and (4) N+DCD2 (ammonium sulfate + DCD2).

The amount of soil in each triplicate is equivalent to 50 g air-dried soil, which was placed in 150 mL centrifuge tubes with lids. The application quantity for ammonium sulfate was 0.5 g N kg<sup>-1</sup> air-dried soil, and for DCD, it was 1% and 2% of N in treatments N+DCD1 and N+DCD2, respectively. DCD, with a purity of 99.5%, was supplied by Shanghai Chemical Institute.

Soil water content was adjusted to reach 60% WFPS by adding distilled water. Ammonium sulfate and nitrification inhibitor were applied in liquid solution. A vacuum method was used to ensure that the solution could mix with the soil well (Cavagnaro *et al.*, 2008). Sample tubes were covered with lids to minimize water loss and weighed at 2-day intervals by adding distilled water to the initial weight. All of the samples were placed in a temperature-controlled incubator (LTI- 1001SD, EYELA, Japan) at 25°C in the dark.

After incubation for 1, 3, 5, 7, 14, 28, 49, 70, 91 or 112 days, a destructive collection of the soil was made with three replicates to observe dynamic changes of mineral nitrogen contents, AOA populations and AOB populations.

### 2.2. Experiment 2:

In addition to the four treatments in Experiment 1, two other treatments (DCD1 and DCD2) were added. The two treatments represent single and double doses of DCD, respectively, without applying ammonium sulfate. The incubation method was the same as in Experiment 1. Soil samples were collected at day 14, when nitrification inhibition rates of DCD were the highest in Experiment 1.

In both experiments, roughly 10 g soil were removed from each tube and immediately stored at  $-60^{\circ}\text{C}$ . Fresh soil samples were immediately analyzed by an AutoAnalyzer III Continuous Flow Analyzer (Bran+Luebbe, Norderstedt, Germany) for  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  contents after extraction with a 2 M KCl solution. Nitrification inhibition rate (%) was calculated as in previous studies (McCarty and Bremner, 1989).

Soil DNA was extracted from 0.5 g soil by using a FastDNA® Spin kit for soil (Qbiogene, Inc., Irvine, CA). The extracted soil DNA was checked for purity with a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

The archaeal and bacterial *amoA* copies were determined by real-time PCR using an iCycler iQ 5 Thermocycler (Bio-Rad, USA) with the fluorescent dye, SYBR-Green I, as previously described (Zhang *et al.*, 2009). The primers targeted the *amoA* gene of the AOB and AOA DNA (Francis *et al.*, 2005; Rotthauwe *et al.*, 1997), and the data were analyzed with iQ™ 5 software (Bio-Rad, USA). All results were expressed and based on the oven-dried soil weight ( $105^{\circ}\text{C}$ , 24 h).

All data were analyzed using SPSS version 16.0. Significant differences ( $p < 0.05$ ) in data obtained between treatments were determined by using an ANOVA followed by a Duncan test.

### 3. Results

#### 3.1. Experiment 1

##### 3.1.1. Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ contents

Without DCD addition, soil content of  $\text{NH}_4^+\text{-N}$  in treatment N declined sharply and was significantly lower than that in treatments N+DCD1 and N+DCD2 at day 7 and until the end of the experiment (Figure 1). Compared to treatment N+DCD1, a double dose of DCD maintained significantly higher levels of soil  $\text{NH}_4^+\text{-N}$  from day 14 to the end of the experiment.

Soil nitrate contents, in treatment N, were substantially increased compared to other treatments from day 14 on. Combining a double dose of DCD with N significantly reduced the amounts of nitrate produced from days 49 to 91, compared to a single dose of DCD.

The nitrification inhibition rate of DCD increased from day 5, reached the peak value at day 14, and then decreased till the end (Table 1). A double dose of DCD could result in a higher nitrification inhibition rate than a single dose of DCD from days 14 to day 91.

##### 3.1.2. Abundance of soil AOB and AOA

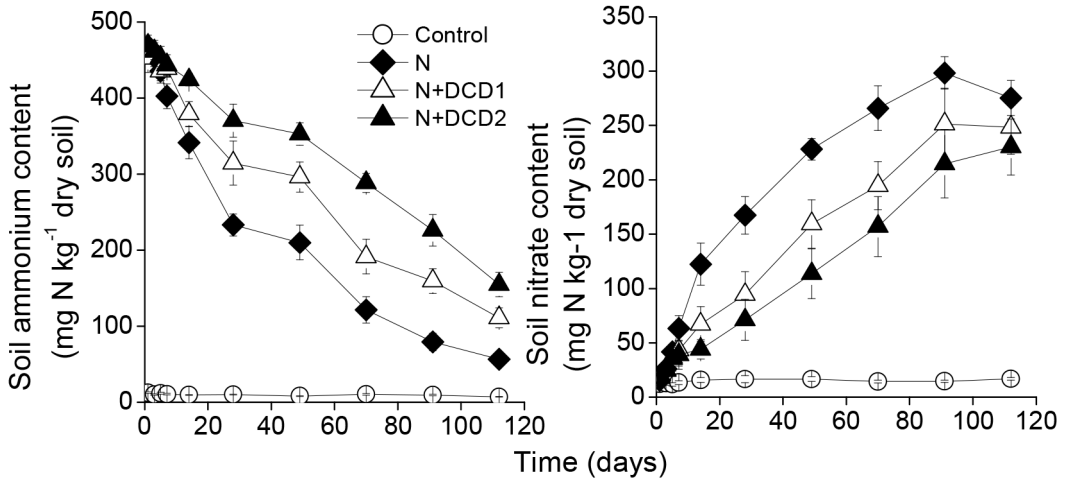
The application of N significantly stimulated AOB growth in the  $\text{NH}_3$  substrate in treatment N (Figure 2). At its peak at day 14, the copies of *amoA* in AOB in treatment N were 3.47 times higher than those in the Control. DCD inhibited the growth of AOB significantly from day 7. There were no significant differences between the single and double doses of DCD, except at day 28.

In contrast to AOB, the growth of AOA was not stimulated by the addition of N (Figure 2), and the archaeal *amoA* copies in the treatments that received N were lower than those in the Control at days 7 and 14. DCD addition did not affect AOA populations.

Ratios of AOA to AOB ranged from 3.58 to 40.10 among all treatments during the whole incubation period. This indicated that AOA populations were more abundant than AOB in the tested soil. The maximum value of AOA/AOB occurred in the Control at day 112, and the minimum value was found in treatment N at day 7.

#### 3.2. Experiment 2

The mineral nitrogen content after DCD addition only was similar to that in the Control (Table 2). DCD had similar effects on mineral nitrogen content in Experiment 2 and Experiment 1.



**Figure 1.** Dynamic changes of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  contents in the soil during the incubation period.

**Table 1.** Nitrification inhibition rate of DCD (%)

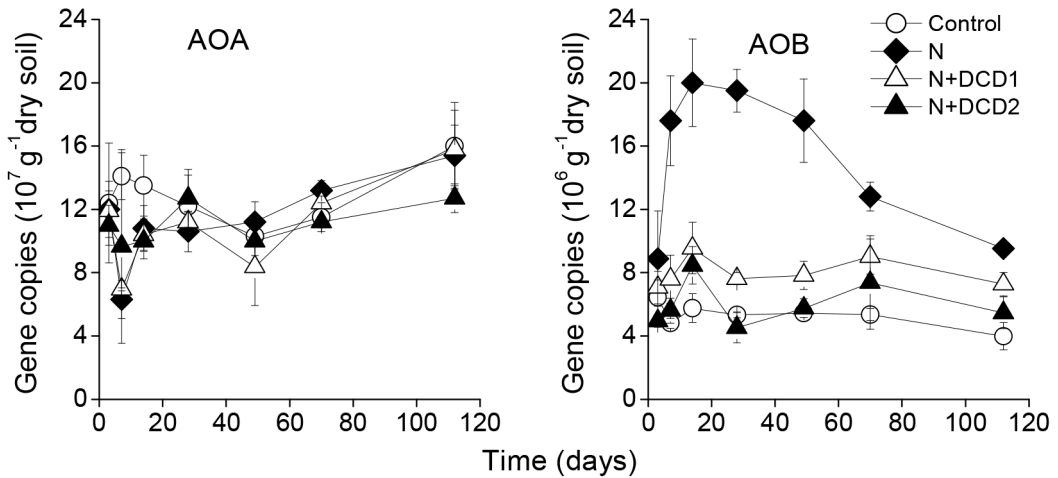
Treatments <sup>a</sup>	Incubation time (day)									
	3	5	7	14	28	49	70	91	112	
N+DCD1	31±3 <sup>b</sup>	31±5	49±6	54±5	50±9	34±8	30±2	18±6	12±3	
N+DCD2	41±8	34±4	57±8	76±1	65±7	55±8	45±7	31±7	18±7	

a. Treatments N+DCD1 and N+DCD2 represent ammonium sulfate combined with single and double doses of DCD, combined with ammonium sulfate, respectively. b. Mean ± SD (n=3).

had similar effects on mineral nitrogen content in Experiment 2 and Experiment 1. Among the Control, DCD1 and DCD2 treatments, there were no significant differences on the archaeal amoA copies (Table 2). In treatments N and N+DCD2, AOA populations were significantly lower than those in the Control.

#### 4. Discussion

In Experiment 1, the nitrification inhibition rates showed that DCD was most effective from day 7 to day 28. Degradation and/or leaching of DCD are the reasons why the inhibition effect was weak at the end (Weiske *et al.*, 2001).



**Figure 2.** Dynamic changes in the abundance of bacterial (AOB) and archaeal (AOA) ammonia oxidizers in the soil during the incubation period.

**Table 2.** The mineral nitrogen contents and copies of *amoA* in AOA in the soil after 14 days

Treatments <sup>a</sup>	NH <sub>4</sub> <sup>+</sup> (mg kg <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> (mg kg <sup>-1</sup> )	AOA <i>amoA</i> copies (10 <sup>7</sup> g <sup>-1</sup> dry soil)
Control	11.99±0.47 <sup>b</sup>	11.62±0.80 <sup>a</sup>	9.95±1.26 <sup>bc</sup>
DCD1	12.41±0.44 <sup>a</sup>	10.90±1.11 <sup>a</sup>	9.31±1.38 <sup>b</sup>
DCD2	11.94±0.88 <sup>a</sup>	10.72±1.09 <sup>a</sup>	11.10±1.05 <sup>c</sup>
N	350.72±19.78 <sup>b</sup>	119.63±9.73 <sup>d</sup>	6.79±0.29 <sup>a</sup>
N+DCD1	372.58±23.74 <sup>b</sup>	65.81±4.61 <sup>c</sup>	9.08±0.64 <sup>b</sup>
N+DCD2	405.05±25.33 <sup>c</sup>	52.27±6.14 <sup>b</sup>	6.62±0.37 <sup>a</sup>

a. DCD1 and DCD2 represent single and double doses of DCD alone, respectively. For other abbreviations, see Figure 1.

b. Mean ± SD (n=3). Values within the same column, followed by the same letter, do not differ at  $p < 0.05$ .

Rapid growth of AOB was observed in Experiment 1 when N was added. Similar effects of ammonium on copies of *amoA* in AOB were also obtained in laboratorial and field conditions (Jia and Conrad, 2009; Okano *et al.*, 2004). Therefore, applied ammonium could supply ammonia as a substrate for AOB growth. In contrast, AOA growth seems not only to be stimulated but also suppressed by the addition of ammonium on days 7 and 14.

Different responses between AOB and AOA to the addition of N showed different preferences for N conditions. It has been substantiated that AOB growth is best in high nitrogen soil conditions, whereas AOA may be particularly adapted to low nitrogen environments (Di *et al.*, 2010; Erguder *et al.*, 2009; Schleper, 2010). Further, AOA may grow heterotrophically or mixotrophically; therefore, ammonia oxidation is not its sole or main energy source (Di *et al.*, 2010; Nicol and Schleper, 2006).

High ratios of AOA to AOB showed that AOA were more abundant than AOB in the agricultural soil, supporting many earlier findings in agricultural soils (He *et al.*, 2007; Leininger *et al.*, 2006).

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When N was applied with nitrification inhibitors in Experiment 1, the AOB *amoA* copies decreased significantly compared to those with treatment N alone. However, AOA populations were not inhibited by DCD, even when DCD was applied as a double dose. Based on the responses of AOA to DCD in Experiment 2, AOA populations were not suppressed by DCD. This finding indicated that decreases in AOA populations in treatment N+DCD2 were mainly caused by the addition of N.

Our findings are in accordance with results of previous studies where DCD inhibited AOB *amoA*

copies, whereas AOA remained unchanged (Di and Cameron, 2011; O'Callaghan *et al.*, 2010). As reported in a previous study, AOA populations were more stable than AOB counterparts (O'Callaghan *et al.*, 2010). However, AOA populations increased slightly with the application of DMPP in the root-rhizosphere complex under field conditions (Kleineidam *et al.*, 2011). This increase may be due to different lifestyles and cellular biochemistries. The susceptibility to inhibitory compounds of bacteria and archaea was different (Kleineidam *et al.*, 2011; Schleper *et al.*, 2005; Valentine, 2007).

The AOB growth was still found in the soil after a double dose of DCD, substantiating that DCD inhibits but does not kill the two ammonia oxidizers.

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### References

- Amberger, A., 1989. Research on Dicyandiamide as a Nitrification Inhibitor and Future Outlook. Communications in Soil Science and Plant Analysis. 20, 1933-1955.
- Boer, W.D., Kowalchuk, G.A., 2001. Nitrification in acid soils: micro-organisms and mechanisms. Soil Biology & Biochemistry. 33, 853-866.

- Cavagnaro, T.R., Jackson, L.E., Hristova, K., Scow, K.M. 2008. Short-term population dynamics of ammonia oxidizing bacteria in an agricultural soil. *Applied Soil Ecology*. 40, 13-18.
- Cookson, W.R., Cornforth, I.S. 2002. Dicyandiamide slows nitrification in dairy cattle urine patches: effects on soil solution composition, soil pH and pasture yield. *Soil Biology & Biochemistry*. 34, 1461-1465.
- Di, H.J., Cameron, K.C. 2011. Inhibition of ammonium oxidation by a liquid formulation of 3,4-Dimethylpyrazole phosphate (DMPP) compared with a dicyandiamide (DCD) solution in six new Zealand grazed grassland soils. *Journal of Soils and Sediments* 11, 1032-1039.
- Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z. 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience*. 2, 621-624.
- Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z. 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiology Ecology*. 72, 386-394.
- Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M., Verstraete, W. 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiology Reviews*. 33, 855-869.
- Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences of the United States of America*. 102, 14683-14688.
- He, J., Shen, J., Zhang, L., Zhu, Y., Zheng, Y., Xu, M., Di, H.J. 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environmental Microbiology*. 9, 2364-2374.
- Jia, Z.J., Conrad, R., 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology*. 11, 1658-1671.
- Kleineidam, K., Kosmrlj, K., Kublik, S., Palmer, I., Pfab, H., Ruser, R., Fiedler, S., Schloter, M. 2011. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on ammonia-oxidizing bacteria and archaea in rhizosphere and bulk soil. *Chemosphere*. 84, 182-186.
- Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*. 442, 806-809.
- McCarty, G.W., Bremner, J.M. 1989. Inhibition of Nitrification in Soil by Heterocyclic Nitrogen-Compounds. *Biology and Fertility of Soils*. 8, 204-211.
- Nicol, G.W., Schleper, C. 2006. Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *TRENDS in Microbiology*. 14, 207-212.
- O'Callaghan, M., Gerard, E.M., Carter, P.E., 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 Biology & Biochemistry*. 42, 1425-1436.
- Offre, P., Prosser, J.I., Nicol, G.W. 2009. Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. *FEMS Microbiology Ecology*. 70, 99-108.

- Okano, Y., Hristova, K.R., Leutenegger, C.M., Jackson, L.E., Denison, R.F., Gebreyesus, B., Lebauer, D., Scow, K.M. 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Applied and Environmental Microbiology*. 70, 1008-1016.
- Pasda, G., Hahndel, R., Zerulla, W. 2001. Effect of fertilizers with the new nitrification inhibitor DMPP (3,4-dimethylpyrazole phosphate) on yield and quality of agricultural and horticultural crops. *Biology and Fertility of Soils*. 34, 85-97.
- Rotthauwe, J.H., Witzel, K.P., Liesack, W. 1997. The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*. 63, 4704-4712.
- Schleper, C., 2010. Ammonia oxidation: different niches for bacteria and archaea? *The ISME Journal*. 4, 1092-1094.
- Schleper, C., Jurgens, G., Jonuscheit, M. 2005. Genomic studies of uncultivated archaea. *Nature Reviews Microbiology*. 3, 479-488.
- Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I. 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environmental Microbiology*. 10, 1357-1364.
- Valentine, D.L. 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nature Reviews Microbiology*. 5, 316-323.
- Weiske, A., Benckiser, G., Herbert, T., Ottow, J.C.G. 2001. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. *Biology and Fertility of Soils* 34,109-117.
- Zhang, L., Wu, Z., Jiang, Y., Chen, L., Song, Y., Wang, L., Xie, J., Ma, X. 2010. Fate of applied urea N-15 in a soil-maize system as affected by urease inhibitor and nitrification inhibitor. *Plant Soil and Environment*. 56, 8-15.
- Zhang, L.M., Wang, M., Prosser, J.I., Zheng, Y.M., He, J.Z. 2009. Altitude ammonia-oxidizing bacteria and archaea in soils of Mount Everest. *FEMS Microbiology. Ecology*. 70, 208-217.