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SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

# Growth of comammox *Nitrospira* is inhibited by nitrification inhibitors in agricultural soils

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**Abstract**

***Purpose*** The discovery of comammox *Nitrospira* being capable of complete oxidizing ammonia to nitrate radically challenged the conventional concept of two-step nitrification. However, the response of comammox *Nitrospira* to nitrification inhibitors (NIs) and their role in soil nitrification remain largely unknown, which has hindered our ability to predict the efficiency of NIs in agroecosystems.

***Materials and methods*** We evaluated the effect of four NIs, 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin), 3,4-dimethylpyrazole phosphate (DMPP), allylthiourea (ATU) and dicyandiamide (DCD) on the growth of comammox *Nitrospira*, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in two pasture and arable soils.

***Results and discussion*** The amendment of nitrogen fertiliser significantly increased soil nitrate concentrations over time, indicating a sustaining nitrification activity in both soils. The addition of all the four NIs effectively reduced the production of nitrate in both soils, but to varying degrees during incubation. The abundances of comammox *Nitrospira* clade A were significantly increased by addition of nitrogen fertilisers and significantly impeded by the four NIs in the pasture soil, but their abundances were only remarkably hindered by nitrapyrin in the arable soil. All the four NIs obviously inhibited the AOB abundances in both soils. Except for DMPP, the other three NIs effectively suppressed the AOA abundances in both soils.

***Conclusions*** We provided new evidence that growth of comammox *Nitrospira* clade A can be stimulated by nitrogen fertilisers and inhibited by various nitrification inhibitors, suggesting their potential role in nitrification of agricultural soils.

**Keywords** Agricultural soil • Comammox *Nitrospira* • Nitrification inhibitor • Nitrogen use efficiency

## 1 Introduction

The intensive use of nitrogen (N) fertilizers in agro-ecosystems is driven by a growing demand of food for a global human population of more than 11 billion by 2100 (Gerland et al. 2014; Gruber and Galloway 2008). However, nitrogen use efficiency (NUE) is rarely exceeding 50%, as majority of N fertilizers is lost to the environment through nitrate leaching, nitrous oxide ($N_2O$) emission, and ammonia volatilization (Wang et al. 2018a; Zhang et al. 2019). Application of nitrification inhibitors (NIs) together with N-based fertilizers is a practical tool to reduce N losses and to promote plant NUE in order to increase crop yields in agricultural systems ( Dinnes et al. 2002; Subbarao et al. 2006; Di and Cameron 2012; Shi et al. 2016b). Dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP) and 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin, NP) are commercial NIs widely applied in agricultural soils (Shi et al. 2016a; Subbarao et al. 2006) and allylthiourea (ATU) is frequently used in laboratory experiments to differentiate the relative contribution of ammonia oxidizers to nitrification (Lehtovirta-Morley et al. 2013; Shen et al. 2013; Wang and Gu 2014). The underlying mechanisms for NIs are generally attributed to their deactivation of ammonia monooxygenase (AMO), which catalyses the ammonia oxidation process, conversion from ammonia to nitrite, mediated by ammonia-oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) (Subbarao et al. 2009; Shen et al. 2013). However, the outcomes of these NIs are reported to be variable across soils (Shi et al. 2016a, b), primarily owing to their largely unknown effects on nitrogen-cycling microorganisms (Di et al. 2009; Shi et al. 2017).

Nitrification was traditionally considered as a two-step process including ammonia oxidation catalysed by AOA and AOB, and nitrite oxidation catalysed by nitrite-oxidizing bacteria (NOB). This long-held perspective was radically challenged by the recent discovery of complete ammonia oxidizers, namely comammox organisms within the genus *Nitrospira*, which are capable of oxidizing ammonia to nitrate in a single organism (Daims et al. 2015; van

Kessel et al. 2015). The assembled complete genomes of comammox *Nitrospira* harbour the full set of genes encoding AMO and hydroxylamine dehydrogenase for ammonia oxidation, and genes encoding nitrite oxidoreductase for nitrite oxidation (Daims et al. 2015; van Kessel et al. 2015). Since the discovery of comammox *Nitrospira* as a third group of ammonia oxidizers, numerous studies have been carried out to investigate their environmental prevalence in various environmental settings, including agricultural soils, forest soils, paddy soils, freshwater sediments, and wastewater treatment plants (Bartelme et al. 2017; Hu and He 2017; Pjevac et al. 2017; Fowler et al. 2018; Wang et al. 2018b; Yu et al. 2018; Beach and Noguera 2019). It was found that comammox *Nitrospira* are widely prevalent in terrestrial ecosystems and can be more abundant than canonical ammonia oxidizers in some soils (Hu and He 2017; Pjevac et al. 2017), but their role in soil nitrification and responses to NIs remains unknown.

Given the fact that genomes of comammox *Nitrospira* contain the *amoA* gene encoding the enzyme AMO, which is the target of NIs, we hypothesized that growth of comammox organisms can be inhibited by the currently available NIs, but to varying degrees. The study of van Kessel et al. (2015) reported that the addition of ATU significantly inhibited ammonia-dependent carbon fixation of comammox *Nitrospira* in an incubation experiment. As far as we know, no studies have compared the responses of comammox *Nitrospira*, AOA and AOB to NIs in agroecosystems. The objective of this study was therefore to assess the impacts of four NIs on nitrification and the abundance of comammox *Nitrospira*, AOA and AOB in two agricultural soils during a 28-day microcosm incubation. The results of this study can improve our understanding of the possible mechanisms governing the efficacy of NIs on nitrification by including comammox *Nitrospira* as a third group of nitrifiers.

## 2 Materials and methods

### 2.1 Site description and soil sampling

Soil samples were collected during May 2018 (autumn in Australia) from two long-term research farms: a dairy farm site (pasture soil) at Dookie (36°33'S, 145°69'E) and a vegetable farm site (arable soil) at Clyde (38°13′S, 145°33′E), Victoria, Australia. The mean annual rainfall at Dookie is 554 mm, and the mean annual maximum and minimum temperatures are 21 °C and 9 °C, respectively. The Dookie site has been used as a dairy farm for more than 30 years, including around 43 hectares of irrigated pastures with a feeding capacity for 180 cows. The pastures at the Dookie site are a mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The mean annual rainfall at Clyde is 816 mm and the mean annual maximum and minimum temperatures are 20 °C and 10 °C, respectively. Approximately 5 kg soils were collected from the upper 10 cm of soils from each site, placed into plastic buckets, and shipped on ice to the laboratory. The soil samples were sieved through a 2 mm sieve to remove roots and stones, mixed thoroughly and stored at 4 °C prior to construction of soil microcosms.

### 2.2 Soil physicochemical analysis

Soil physicochemical properties were analysed through the Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences platform. Soil moisture content was measured by oven drying 10 ± 0.05 g of soil at 105 °C for 24 h. Soil pH and electrical conductivity were estimated with a soil to water ratio of 1: 5 using an Orion Star A211 pH meter (Thermo Scientific Inc., Melbourne, Australia). Total C and N were determined via the Dumas combustion method on an isotope ratio mass spectrometer (Sercon Hydra, Crewe, UK). Soil $NH_4^+$-N and $NO_3^-$-N were extracted by 1 M potassium chloride (KCl) solution with a soil to solution ratio of 1:5, filtered through a Whatman Grade 42 filter paper (Sigma-Aldrich Co., St.

Louis, MO, USA), and analysed with a segmented flow analyzer (SAN++; Skalar, Breda, Holland). Available phosphorus (P) were measured via the Colwell P test on a segmented flow analyzer (SAN++; Skalar, Breda, Holland). Available sulphur (S) and potassium (K) were extracted with a soil to Mehlich 3 ratio of 1:10 and estimated by Optima 8300 ICP-OES spectrometer (PerkinElmer Inc., Waltham, WA, USA). More detailed information about the soil properties is shown in Table 1.

### 2.3 Soil microcosm incubation

The soil incubation was performed in 250-ml plastic vials containing 20 g soil samples (oven dry weight equivalent). Six treatments were established with three replicates: (i) control; (ii) $(NH_4)_2SO_4$ (100 mg $NH_4^+$-N $kg^{-1}$ soil); (iii) $(NH_4)_2SO_4$ plus nitrapyrin (50 μmol $kg^{-1}$ soil); (iv) $(NH_4)_2SO_4$ plus DMPP (5 μmol $kg^{-1}$ soil); (v) $(NH_4)_2SO_4$ plus ATU (1000 μmol $kg^{-1}$ soil); and (vi) $(NH_4)_2SO_4$ plus DCD (10 μmol $kg^{-1}$ soil). The selected NIs, including DCD, DMPP, nitrapyrin (NP) and ATU, have been widely used in agricultural soils or laboratory experiments to inhibit the conversion of ammonium to nitrate, and the concentrations of NIs used were based on the findings from previous studies (Lehtovirta-Morley et al. 2013; Shen et al. 2013; Wang and Gu 2014; Shi et al. 2016a). All microcosms were incubated in a temperature-controlled incubator at 25 °C in the dark for 28 days. Soil moisture content was maintained at 55% water filled pore space (WFPS) throughout the incubation by weekly adding water to the incubation vials based on the weight changes. Aerobic conditions were maintained twice a week by opening the vials and replenishing. Destructive samples were collected for molecular and mineral N analysis on days 0, 3, 7, 14, 21 and 28.

### 2.4 DNA extraction and quantitative PCR assay

Nucleic acids were extracted from 0.25 g of soil using MoBio PowerSoil DNA isolation kits (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions, with slight modifications as previously described (Hu et al. 2015). The concentrations of extracted DNA were determined using the NanoDrop ND2000c spectrophotometer (NanoDrop Technologies).

All quantitative PCR (qPCR) assays were performed on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The primer sets comaA-244F/comaA-659R, comaB-244F/comaB-659R (Pjevac et al. 2017), CrenamoA23f/CrenamoA616r (Tourna et al. 2008) and amoA1F/amoA2R (Rotthauwe et al. 1997) were used to qualify the *amoA* gene abundances of comammox clade A, clade B, AOA and AOB, respectively (Table S1, Electronic Supplementary Material - ESM). Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed at the end of each qPCR run to check the specificity of amplification products, before identification by agarose gel electrophoresis. Based on the agarose gel results, the qPCR products of comammox clade A yielded single and bright bands with the expected 415 bp target size in both soils. The resultant PCR products were cloned into the pGEM®-T Easy vector and transformed into *Escherichia coli* JM109 competent cells based on the manufacturer's instructions. Positive clones were sent to Macrogen Sequencing Department, South Korea to analyse the sequences containing the correct insert. The obtained sequences were checked for chimeras and aligned with reference sequences using MUSCLE in MEGA 7.0 (Kumar et al. 2016). The results showed that all obtained sequences (based on 54 positive clones) have high similarity (> 95%) with reference sequences of comammox *Nitrospira*. The sequence for comammox *Nitrospira* clade A has been deposited into GenBank with the accession number MK581036. However, we did not obtain

any specific amplification for comammox *Nitrospira* clade B in either pasture or arable soil, and therefore comammox *Nitrospira* clade B were excluded from the downstream analysis. The amplification efficiencies of all *amoA* gene were 85%-99% and the $r^2$ values were > 0.99.

### 2.5 Statistical analysis

Two-way analysis of variance (ANOVA) based on the Duncan test was conducted to analyse the differences in ammonium and nitrate concentrations, the abundance of comammox *Nitrospira* clade A, AOA and AOB across different treatments and sampling times with SPSS Statistics 25 (IBM, USA). Differences at a P value of < 0.05 were statistically significant.

## 3 Results

### 3.1 Changes in soil mineral N in microcosm incubation

The $NH_4^+$-N concentrations remained largely unchanged during incubation in the control treatments of both soils (Fig. 1A and 1B). The $NH_4^+$-N concentrations in the $(NH_4)_2SO_4$ treatment rapidly reduced from 55.43 (± 4.71) to 0.65 (± 0.10) mg $kg^{-1}$ and from 78.83 (± 2.68) to 0.88 (± 0.04) mg $kg^{-1}$ soil in the control treatment from day 0 to day 3 in the pasture and arable soils, respectively (Fig. 1A and 1B). The addition of nitrapyrin and DMPP significantly ($P < 0.001$) increased the $NH_4^+$-N concentrations in the pasture soil and slowed down the reduction of the $NH_4^+$-N concentrations in the arable soil. No significant differences ($P > 0.05$) in the temporal changes of the $NH_4^+$-N concentrations in the $(NH_4)_2SO_4$, $(NH_4)_2SO_4$ + ATU and $(NH_4)_2SO_4$ + DCD was observed (Table 2).

In the control microcosms, the $NO_3^-$-N concentrations continuously increased over time, indicating a sustaining nitrification activity in both soils (Fig. 1C and 1D). In the $(NH_4)_2SO_4$ treatment, the $NO_3^-$-N concentrations sharply increased and had significantly ($P < 0.001$) higher net nitrification rates of ~ 9.36 and ~ 4.64 mg $NO_3^-$-N $kg^{-1}$ soil per day than the control

treatments of ~5.86 and ~ 1.44 mg $NO_3^-$-N $kg^{-1}$ soil per day in the pasture and arable soils, respectively. The addition of all the four NIs effectively reduced the production of $NO_3^-$-N in both soils, but to varying degrees during incubation. The addition of nitrapyrin showed the most significant effect on the inhibition of nitrification in both soils. Net nitrification rates in the other three treatments with NIs also had significantly ($P < 0.001$) lower values than that in the $(NH_4)_2SO_4$ treatment (Table 2).

### 3.2 Changes of the comammox *Nitrospira amoA* gene abundance

In the control treatments, the comammox *Nitrospira* clade A *amoA* gene abundance ranged between 4.32 to 9.26 × $10^7$ copies $g^{-1}$ soil in the pasture soil (Fig. 2A), and 1.77 to 3.28 × $10^7$ copies $g^{-1}$ soil in the arable soil (Fig. 2B). Addition of $(NH_4)_2SO_4$ significantly ($P < 0.05$) increased the comammox *Nitrospira* clade A *amoA* gene abundance from 6.08 × $10^7$ at day 7 to 5.50 × $10^7$ at day 28 in the pasture soil (Fig. 2A), and from 2.37 × $10^7$ at day 7 to 3.06 × $10^7$ at day 28 in the arable soil (Fig. 2B), compared to the control treatment (Table 2).

The comammox *Nitrospira* clade A abundance showed different responses to the four NIs in both soils. Compared with the $(NH_4)_2SO_4$ treatment, the comammox *Nitrospira* clade A abundance in the $(NH_4)_2SO_4$ + nitrapyrin treatment of the pasture and arable soils significantly ($P < 0.05$) declined from 5.59 to 3.91 × $10^7$ copies $g^{-1}$ soil and from 1.56 to 1.71 × $10^7$ copies $g^{-1}$ soil, respectively (Fig. 2A and 2B). The other three NIs showed different effects on the comammox *Nitrospira* clade A in the pasture and arable soils. In the pasture soil, comammox *Nitrospira* clade A abundance was significantly ($P < 0.05$) reduced in the presence of ATU and DCD, and significantly ($P < 0.05$) decreased from day 14 to day 28 after the addition of DMPP (Fig. 2A). In the arable soil, no obvious inhibitory effect of DCD on the growth of comammox *Nitrospira* clade A was observed, and comammox *Nitrospira* clade A abundance even tended to increase in the DMPP and ATU treatments (Fig. 2B).

### 3.3 Changes of the AOA and AOB *amoA* gene abundance

The AOA *amoA* gene abundance was significantly increased ($P < 0.05$) after adding $(NH_4)_2SO_4$ in both soils (Fig. 3A and 3B). Compared with the $(NH_4)_2SO_4$ treatment, only DMPP had no significant ($P > 0.05$) inhibitory effect on the AOA abundance in both soils (Table 2). The addition of nitrapyrin significantly ($P < 0.05$) impeded the growth of AOA from day 14 to day 28 in the pasture soil (Fig. 3A) and the AOA abundance was significantly ($P < 0.05$) lower in the $(NH_4)_2SO_4$ + nitrapyrin treatment than in the $(NH_4)_2SO_4$ treatment during the first three weeks of incubation in the arable soil (Fig. 3B). The other two NIs (ATU and DCD) significantly ($P < 0.05$) reduced the AOA abundance in both soils throughout the incubation (Table 2).

The $(NH_4)_2SO_4$ application in both soils significantly ($P < 0.05$) increased the AOB *amoA* gene abundances, compared with the control treatments, in both soils during the 28-day incubation. All the four NIs significantly ($P < 0.05$) inhibited the growth of AOB in both soils but to varying degrees during the incubation (Fig. 3C and 3D, Table 2).

## 4 Discussion

This study, for the first time, demonstrated that comammox *Nitrospira* clade A can be effectively impeded by diverse NIs (nitrapyrin, DMPP, ATU and DCD) in agricultural soils. Previous studies focused on the responses of AOA and AOB to various NIs without taking comammox *Nitrospira* into consideration, because comammox *Nitrospira* were recently discovered in 2015 (Daims et al. 2015; van Kessel et al. 2015). A large body of studies suggested that most NIs are non-selective and can suppress either AOA or AOB *amoA* gene abundances depending on which nitrifying group is more functionally dominant during the nitrification process (Jia and Conrad 2009; Offre et al. 2009; Zhang et al. 2012). For example, AOB were effectively inhibited by acetylene in agricultural soils functionally dominated by

AOB (Jia and Conrad 2009), while DCD significantly inhibited the growth and activity of AOA in acidic soils thought to be dominated by AOA (Zhang et al. 2012). Although the responses of comammox *Nitrospira* clade A to some NIs (DMPP, ATU and DCD) in the two tested soils were not consistent due to different soil properties, all the NIs were effective in impeding the growth of comammox *Nitrospira* clade A, accompanied by significant reduction in net nitrification rates in the treatments with NIs, compared to the $(NH_4)_2SO_4$ treatment. The results indicated that comammox *Nitrospira* might be involved in the active nitrification of agricultural soils with high N input, and are sensitive to the currently available NIs, albeit the inhibitory effects on comammox *Nitrospira* are soil dependent. However, the main soil factors affecting the responses of comammox *Nitrospira* to NIs are required to be further identified by incubating multiple types of agricultural soils.

Previous studies revealed that soil ammonia availability is an important factor affecting the abundances and activities of AOA and AOB, and further influencing their responses to NIs (He et al. 2012; Prosser and Nicol 2012; Hu et al. 2014). Based on the ionization equilibrium ($NH_4^+ \leftrightarrow NH_3 + H^+$; $pK_a$ = 9.25 at 25℃) (He et al. 2012; Hu et al. 2014), we found that comammox *Nitrospira* clade A might be involved in nitrification in the soils with theoretical ammonia concentrations ranging from 159 nM to 893 nM in the pasture soil and from 408 nM to 781 nM in the arable soil. To date, comammox *N. inopinata*, isolated from aquatic systems, was the only kinetically characterized comammox species and its apparent half-saturation constant of $K_{m\ (NH3)}$ is around 63 nM (Kits et al. 2017), which is remarkably lower than the theoretical soil ammonia concentrations in this study. This finding suggests that, contrasting to the previous perception that comommox *Nitrospira* live an oligotrophic lifestyle (Daims et al. 2015; van Kessel et al. 2015), comammox *Nitrospira* in the soil environment might be more favoured under nitrogen-rich conditions, which was also supported by recent findings that the abundance of comammox *Nitrospira* increased in response to added N sources in acidic forest

soils (Shi et al. 2018). Thus, further research regarding the $K_{m\ (NH3)}$ of comammox *Nitrospira* is required to obtain a better understanding of the ecological niches of this new group of ammonia oxidizers in terrestrial ecosystems.

Apart from nitrapyrin, the other three NIs did not inhibit the abundance of comammox *Nitrospira* clade A in the arable soil. The concentrations of some NIs which can inhibit AOA or AOB in most environments probably have little influence on comammox *Nitrospira* clade A, and the inhibitory effects are dependent on soil properties. Acid soil may be a favourable habitat for the growth of comammox *Nitrospira* clade A, which influences their responses to diverse NIs. The pH value rapidly decreased from 6.90 to 6.01 and even to 5.49 after the addition of $(NH_4)_2SO_4$ fertilizer in the pasture soil, whereas the pH values remained neutral levels between 7.13 and 6.94 in the arable soil (Fig. S1, Electronic Supplementary Material - ESM). Previous studies considered that soil pH is a main factor driving the niche differentiation between AOA and AOB (Hu et al. 2014; Li et al. 2018) and the range of soil pH directly affects the response of canonical ammonia oxidisers to inhibitors (Shi et al. 2016a, b). Therefore, comammox *Nitrospira* may be not very sensitive to some inhibitors in neutral arable soils.

The NIs ATU and DCD significantly inhibited AOA and AOB abundance but cannot inhibit comammox *Nitrospira* clade A in the arable soil. The different responses between comammox *Nitrospira* and canonical nitrifiers to NIs could be ascribed to the unique genomic structures and metabolic versatility of comammox *Nitrospira*. For example, besides the inputs of ammonium from soils, comammox *Nitrospira* may use an alternative substrate to catalyse ammonia oxidation. Comammox *Nitrospira* harbour the genes encoding enzymes to generate energy from cyanate and urea degradation, indicative of their genetic potential to utilize cyanate and/or urea as the substrates for nitrification (Hu and He 2017; Koch et al. 2019). Comammox *Nitrospira* harbour enzymes that regulate the pathways involved in degradation of various carbon compounds suggestive of their potential to grow mixotrophically (Palomo et

al. 2018). Therefore, we assume that comammox *Nitrospira* may also use substrates other than ammonia to support the process of nitrification, which could explain why they are not sensitive to NIs in the arable soil as the currently NIs are mainly targeting the AMO of ammonia oxidizers. Apart from soil pH, ammonia availability and genomic characteristics, other factors, such as soil clay which can adsorb a certain amount of NI (Roco and Blu 2006), may also affect the NI efficacy to hamper the abundance of comammox *Nitrospira*.

## 5 Conclusions

Our studies provided new evidence that growth of comammox *Nitrospira* clade A can be stimulated by nitrogen fertilisers and inhibited by various NIs, suggesting their potential role in nitrification of agricultural soils. Notably, the inhibitory efficacy of different NIs on comammox *Nitrospira* is soil dependent, which may be attributed to the different soil properties that can influence the absorption and degradation of NIs. These findings are essential to a better mechanistic understanding of the variable efficiency of NIs in different soils, and have implications for refining agricultural management practices to promote N use efficiency and reduce N losses in agriculture.

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**Figure legends**

**Fig. 1** Changes in the ammonium and nitrate concentrations during the microcosm incubation of the pasture soil (A and C) and the arable soil (B and D). Error bars indicate standard errors of triplicate samples

**Fig. 2** Changes in the comammox *Nitrospira* clade A *amoA* gene abundance during the microcosm incubation of the pasture soil (A) and arable soil (B). Error bars indicate standard errors of triplicate samples

**Fig. 3** Changes in the AOA and AOB *amoA* gene abundance during the microcosm incubation of the pasture soil (A and C) and arable soil (B and D). Error bars indicate standard errors of triplicate samples

Figures

**Fig. 1**

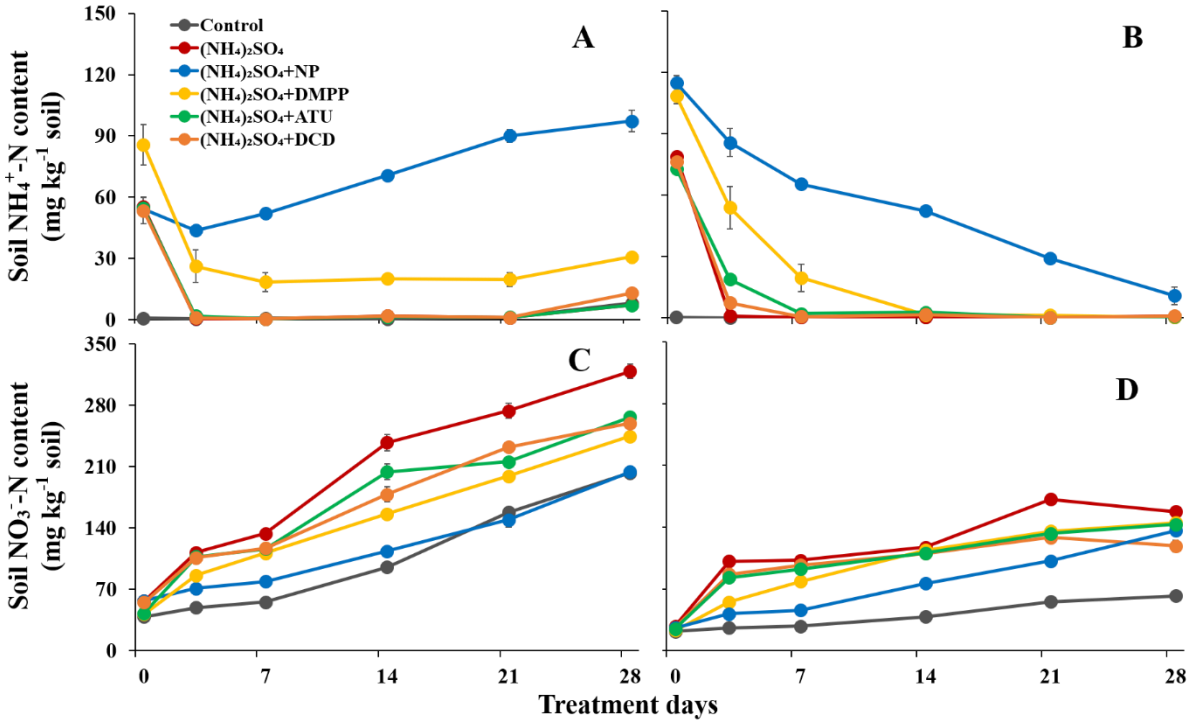

**Fig. 2**

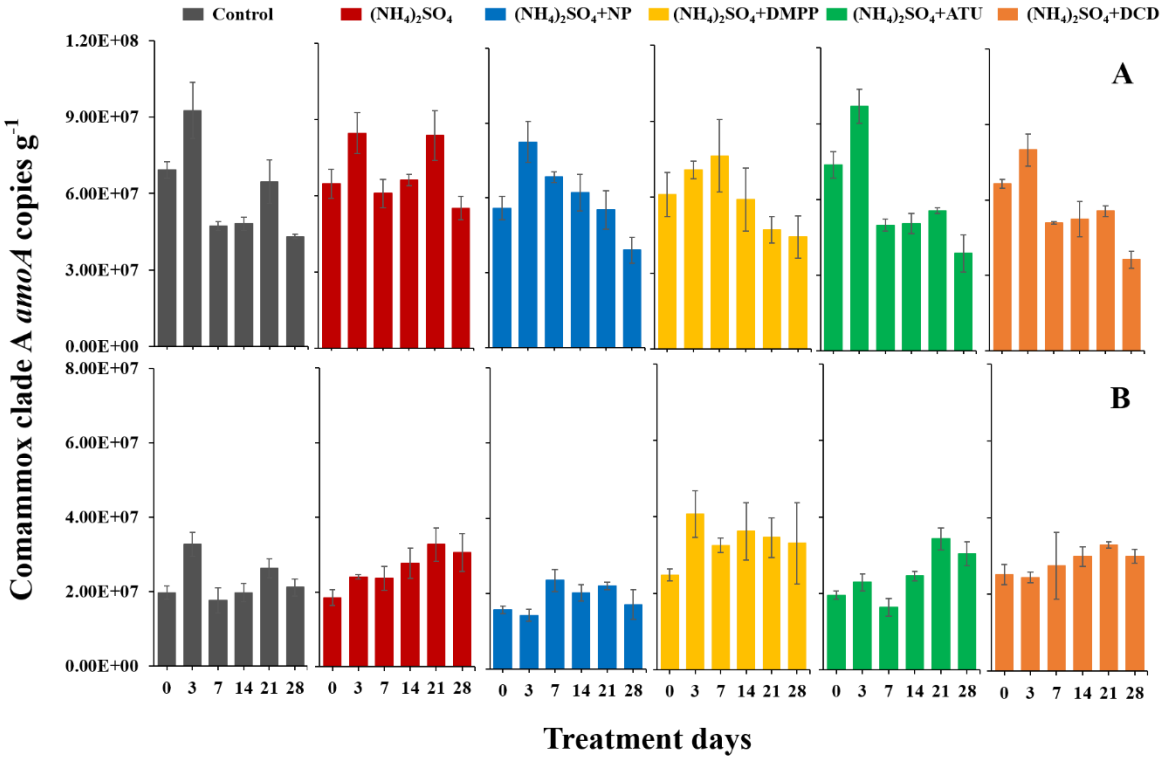
Control
(NH4)2SO4
(NH4)2SO4+NP
(NH4)2SO4+DMPP
(NH4)2SO4+ATU
(NH4)2SO4+DCD
Comammox clade A amoA copies g-1
1.20E+08
9.00E+07
6.00E+07
3.00E+07
0.00E+00
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
A
B
0 3 7 14 21 28
Treatment days

**Fig. 3**

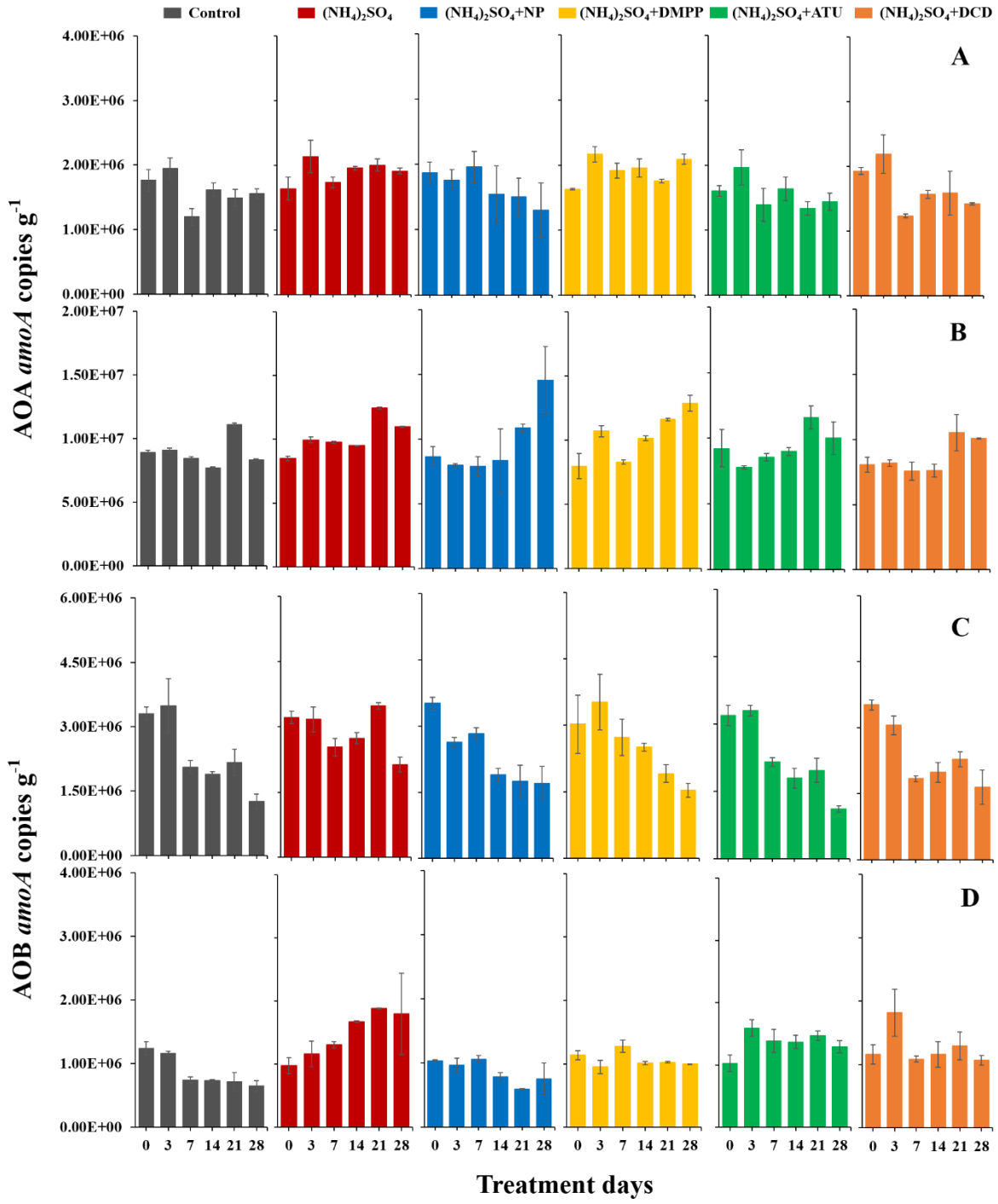
Control
(NH4)2SO4
(NH4)2SO4+NP
(NH4)2SO4+DMPP
(NH4)2SO4+ATU
(NH4)2SO4+DCD
A
B
C
D
AOA amoA copies g-1
AOB amoA copies g-1
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
2.00E+07
1.50E+07
1.00E+07
5.00E+06
6.00E+06
4.50E+06
3.00E+06
1.50E+06
0 3 7 14 21 28
Treatment days

**Table 1** Basic characteristics of the two soils in this study

| **Property** | **Pasture soil （Dookie）** | **Arable soil (Clyde)** |
|---|---|---|
| Water content (%) | 29.4 | 10.2 |
| Soil pH ($H_2O$) | 6.90 | 7.13 |
| Total C (g $kg^{-1}$) | 35 | 25 |
| Total N (g $kg^{-1}$) | 3.8 | 2.9 |
| $NH_4^+$-N (mg $kg^{-1}$) | 0.82 | 0.17 |
| $NO_3^-$-N (mg $kg^{-1}$) | 38.60 | 21.83 |
| Sulphur (mg $kg^{-1}$) | 22 | 45 |
| Phosphorus (mg $kg^{-1}$) | 196 | 675 |
| Potassium (mg $kg^{-1}$) | 348 | 129 |
| Texture | Silty loam | Loamy sand |

**Table 2** Two-way ANOVA analysis of mineral N concentrations and gene abundances

| Factor | Source of variation | $NH_4^+$-N conc. | | $NO_3^-$-N conc. | | Coma clade A *amoA* copies | | AOA *amoA* copies | | AOB *amoA* copies | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PS | AS | PS | AS | PS | AS | PS | AS | PS | AS |
| Control VS $(NH_4)_2SO_4$ | T | 127.94** | 35.77** | 1161.83** | 4685.68** | 5.27* | 3.21 | 15.38* | 68.87** | 12.84* | 17.30** |
| | D | 119.27** | 34.18** | 687.27** | 615.95** | 11.14** | 3.46* | 3.78* | 39.81** | 12.65** | 0.33 |
| | T×D | 124.23** | 34.21** | 49.54** | 195.06** | 1.76 | 2.43 | 1.63 | 7.62** | 3.06* | 4.51* |
| $(NH_4)_2SO_4$ VS NP | T | 1591.35** | 293.01** | 640.42** | 1092.99** | 5.93* | 17.91** | 3.21 | 0.60 | 44.47** | 23.55** |
| | D | 60.40** | 96.16** | 451.04** | 837.85** | 7.54** | 3.04* | 0.47 | 5.42* | 5.84* | 0.49 |
| | T×D | 95.98** | 16.51** | 49.87** | 69.77** | 2.05 | 1.39 | 1.25 | 1.92 | 9.23** | 3.20* |
| $(NH_4)_2SO_4$ VS DMPP | T | 79.66** | 30.73** | 283.15** | 175.72** | 3.99 | 6.29* | 0.08 | 0.16 | 12.96* | 11.47* |
| | D | 60.42** | 92.23** | 620.07** | 611.22** | 2.53 | 1.51 | 4.45* | 33.22** | 2.82* | 1.39 |
| | T×D | 0.66 | 7.86** | 18.65** | 19.74** | 2.15 | 0.55 | 0.90 | 5.52* | 3.66* | 2.21 |
| $(NH_4)_2SO_4$ VS ATU | T | 0.00 | 1.22 | 87.37** | 166.74** | 6.22* | 10.78* | 6.79* | 26.30** | 18.35** | 2.01 |
| | D | 346.02** | 104.99** | 556.97** | 1043.04** | 14.10** | 2.16 | 3.70* | 12.68** | 16.39** | 1.25 |
| | T×D | 0.10 | 1.86 | 7.57** | 19.89** | 3.77* | 1.18 | 2.04 | 0.92 | 3.41* | 2.30 |
| $(NH_4)_2SO_4$ VS DCD | T | 0.27 | 0.14 | 104.36** | 107.04** | 15.02* | 2.51 | 12.66* | 5.47* | 35.66** | 0.76 |
| | D | 168.52** | 113.18** | 611.73** | 391.51** | 10.49** | 1.96 | 2.32 | 6.81** | 23.17** | 2.29 |
| | T×D | 0.63 | 0.25 | 12.54** | 17.10** | 1.85 | 2.01 | 0.93 | 0.91 | 6.17* | 1.34 |

The data indicates F value. Significance levels (P): *$P < 0.05$; **$P < 0.001$. T, treatment; D, day; PS, pasture soil; AS, vegetable soil

Supplementary Material
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SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

# Growth of comammox *Nitrospira* is inhibited by nitrification inhibitors in agricultural soils

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**Table S1** Polymerase chain reaction (PCR) primer sets used in this study

| Target gene | Primer name | Primer sequence (5'-3') | Amplification conditions | Length (bp) | References |
|---|---|---|---|---|---|
| Comammox *Nitrospira* clade A *amoA* | comaA-244f_a | TACAACTGGGTGAACTA | 95°C for 10 min, 40 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 1 min | 415 | Pjevac et al. (2017) |
| | comaA-244f_b | TATAACTGGGTGAACTA | | | |
| | comaA-244f_c | TACAATTGGGTGAACTA | | | |
| | comaA-244f_d | TACAACTGGGTCAACTA | | | |
| | comaA-244f_e | TACAACTGGGTCAATTA | | | |
| | comaA-244f_f | TATAACTGGGTCAATTA | | | |
| | comaA-659r_a | AGATCATGGTGCTATG | | | |
| | comaA-659r_b | AAATCATGGTGCTATG | | | |
| | comaA-659r_c | AGATCATGGTGCTGTG | | | |
| | comaA-659r_d | AAATCATGGTGCTGTG | | | |
| | comaA-659r_e | AGATCATCGTGCTGTG | | | |
| | comaA-659r_f | AAATCATCGTGCTGTG | | | |
| Comammox *Nitrospira* clade B *amoA* | comaB-244f_a | TAYTTCTGGACGTTCTA | 95°C for 10 min, 40 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 1 min | 415 | Pjevac et al. (2017) |
| | comaB-244f_b | TAYTTCTGGACATTCTA | | | |
| | comaB-244f_c | TACTTCTGGACTTTCTA | | | |
| | comaB-244f_d | TAYTTCTGGACGTTTTA | | | |
| | comaB-244f_e | TAYTTCTGGACATTTTA | | | |
| | comaB-244f_f | TACTTCTGGACCTTCTA | | | |
| | comaB-659r_a | ARATCCAGACGGTGTG | | | |
| | comaB-659r_b | ARATCCAAACGGTGTG | | | |
| | comaB-659r_c | ARATCCAGACAGTGTG | | | |
| | comaB-659r_d | ARATCCAAACAGTGTG | | | |
| | comaB-659r_e | AGATCCAGACTGTGTG | | | |
| | comaB-659r_f | AGATCCAAACAGTGTG | | | |
| AOA *amoA* | CrenamoA23f | ATGGTCTGGCTWAGACG | 95°C for 10 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min | 629 | Tourna et al. (2008) |
| | CrenamoA616r | GCCATCCATCTGTATGTCCA | | | |
| AOB *amoA* | amoA-1F | GGGGTTTCTACTGGTGGT | 95°C for 10 min, 35 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min | 491 | Rotthauwe et al. (1997) |
| | amoA-2R | CCCCTCKGSAAAGCCTTCTTC | | | |

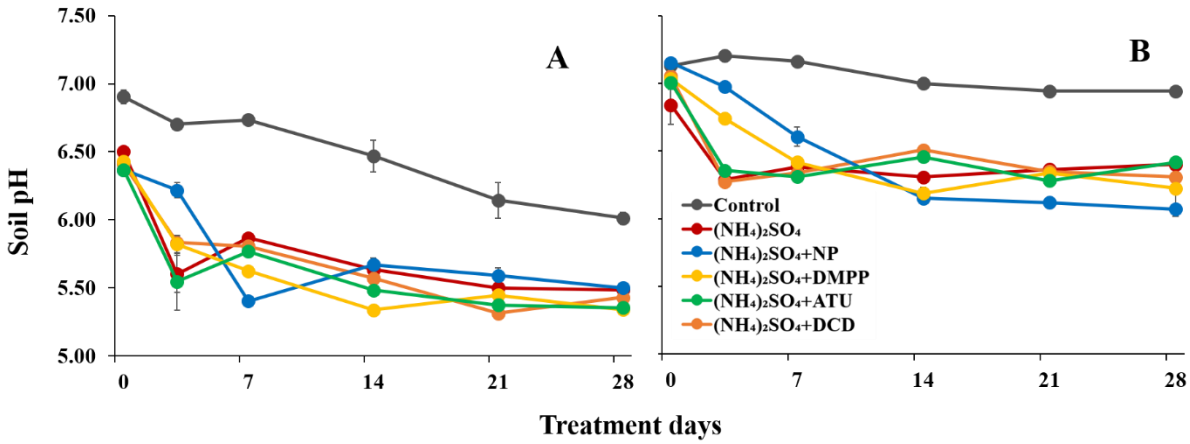


**Fig. S1** Changes in soil pH during incubation trial of the pasture soil (A) and the arable soil (B). Error bars indicate standard errors of triplicate sample