Effects of a nitrification inhibitor on the metabolic activity of ammonia oxidizers

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Abstract
The nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) is a powerful tool that can be used to reduce N losses from agricultural systems by slowing nitrification and promote nitrogen (N) use efficiency. Mounting evidence has confirmed the functional importance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in nitrification and N_2O production, however, their responses to DMPP amendment and the possible reasons for the variable efficacy of DMPP across different soils are not well known. Here we compared the effects of DMPP on the abundance and metabolic activity of ammonia oxidizers using quantitative PCR and $^{13}CO_2$-DNA-stable isotope probing (SIP) method in an acidic pasture soil and an alkaline vegetable soil. Results showed that DMPP significantly inhibited nitrification in the vegetable soil only, and this was coupled with a significant decrease in AOB abundance. The $^{13}CO_2$-DNA-SIP results indicated the involvement of AOA and AOB in active nitrification in both soils, but DMPP only inhibited the assimilation of $^{13}CO_2$ into AOB in the vegetable soil. Our findings provide evidence that DMPP could effectively inhibit nitrification through impeding the abundance and metabolic activity of AOB in the alkaline vegetable soil, but not in the acidic pasture soil possibly due to the low AOB abundance or the adsorption of DMPP by organic matter.

Key Words
3, 4-dimethylpyrazol phosphate (DMPP), ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), DNA-SIP

Introduction
Ammonia oxidation, the first and rate-limiting step of nitrification, is catalyzed by ammonia monooxygenase (AMO) encoded by the *amoA* gene within ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Purkhold et al. 2000, Brochier-Armanet et al. 2008). Nitrification inhibitors are chemical compounds capable of modifying the active site of AMO and causing the formation of polypeptides, thus resulting in the inactivation of AMO activity and delay in nitrification (McCarty and Bremner 1989). 3,4-dimethylpyrazole-phosphate (DMPP) is one of the most effective nitrification inhibitors (Zerulla et al. 2001, Rowlings et al. 2016). However, the efficacy of DMPP to reduce nitrogen losses is highly variable (Wakelin et al. 2014). Temperature, soil moisture, soil properties, fertilizer types and application methods have been reported as the factors influencing DMPP efficacy. Limited and contradictory results were shown regarding the potential significance of the soil microbial communities (Kleineidam et al. 2011, Florio et al. 2014). To our knowledge, there is no information currently available regarding the metabolic evidence and taxonomic identity of AOA and/or AOB in terms of DMPP-induced inhibition on nitrification. The use of DNA-SIP technique in this study facilitates a deep insight into the response of ammonia oxidisers to DMPP at a functional level, and their relations with DMPP effectiveness can be further identified.

Methods
Site sampling
Soil samples were collected from two sites: a grazed dairy pasture at Dookie (36°25'S, 145°42'E) and a vegetable farm at Clyde (38°07'S, 145°19'E), Victoria, Australia. At each site, soil (0-10cm) was collected, thoroughly mixed, sieved (< 2 mm), and stored at 4°C before soil microcosm incubation. Soil properties are shown in Table. 1.

Table 1. Basic properties of the two soils (0-15 cm)

	Pasture soil	Vegetable soil
Soil pH	5.7	7.2
CEC (cmol kg^{-1})	10.2	12.7
Organic Matter (%)	5.3	4.1
NH_4^+-N (mg kg^{-1})	3.8	5.0
NO_3^--N (mg kg^{-1})	28.5	50.0
Total N (g kg^{-1})	3.8	2.8

Texture	silty loam	loamy sand
Particle size (%)		
Sand (0.02-2 mm)	59	85
Silt (0.002-0.02 mm)	30	11
Clay(< 0.002 mm）	11	4

DNA-SIP microcosm

Three treatments were applied to soil in triplicate 160 mL serum bottles containing 10 g soils (oven dry-weight equivalent): (1) NH_4NO_3 + 5% (v/v) $^{12}CO_2$, (2) NH_4NO_3 + 5% (v/v) $^{13}CO_2$ (99 atom%, Sigma-Aldrich Co. St Louis, MO, USA), (3) NH_4NO_3 + 5% (v/v) $^{13}CO_2$ plus DMPP (1% of applied NH_4^+-N). In each vial, 2 mL of the treatment solutions were evenly sprayed to provide an application rate of 75 mg NH_4^+-N kg^{-1} soil and 75 mg NO_3^--N kg^{-1} soil. The DNA-SIP microcosms were incubated at 25°C in the dark for 28 days, and maintained at 60% WFPS, with CO_2 replenished every 3 days following aeration. Samples were destructively sampled on days 0, 14 and 28 for determination of mineral N and SIP gradient fractionation.

SIP gradient fractionation

The DNA-SIP gradient fractionation followed the method of Zhang et al. (2012). Briefly, the density gradient centrifugation was performed in 4.9-mL OptiSeal Polypropylene seal tubes and subjected to isopycnic centrifugation at 56,200 rpm (228,166 g_{av}) at 20°C for 24 h. The centrifuged DNA was fractionated into 20 equal fractions using a fraction recovery system (Beckman Coulter) and a syringe pump (New Era Pump Systems, USA). The buoyant density was determined using a hand-held refractometer. DNA was subsequently precipitated from the CsCl gradients and dissolved in 20 μL of sterilized water. The precipitated DNAs were further checked by qPCR analysis to quantify the relative abundance of *amoA* genes in individual fractions.

Mineral N assay

Soil NH_4^+-N and NO_3^--N were extracted using a ratio of 1:5 (fresh soil : 1 M KCl, w/v) by shaking at 180 rpm for 1 h, filtered through a Whatman 42 filter paper and measured by a Segmented Flow Analyzer (SAN++, Skalar, Breda, Holland).

Quantification of amoA genes

Abundances of *amoA* genes were quantified on a Bio-Rad CFX96 Optical Real-Time PCR Detection System (Bio-Rad, Laboratories Inc, Herculers, CA, USA) using the primers reported previously. The 10 μL reaction mixture contained 5 μL of SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.4 μL of each primer (10 μM), and 2 μL of template DNA. Standard curves were generated using ten-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed to evaluate the specificity of qPCR product. The amplification efficiencies ranged between 95-105%.

Statistical analysis

One-way analysis of variance (ANOVA) based on Duncan-test was conducted for statistical analysis by using SPSS 17.0 (IBM, USA). Differences at $P < 0.05$ were considered to be statistically significant.

Results

No significant effects of ^{13}C-CO_2 or DMPP on the concentrations of NH_4^+-N and NO_3^--N could be observed in the pasture soil (Fig. 1). While DMPP addition significantly slowed down the decline of the NH_4^+-N concentrations and reduced the increase of the NO_3^--N concentrations in the vegetable soil.

The AOB abundance in the pasture soil varying between 6.6×10^6-1.5×10^7 copies g^{-1} soil was significantly lower than that in the vegetable soil varying between 4.3- 9.5×10^7 copies g^{-1} soil ($P < 0.05$). Fertilization had a significant positive effect on AOB abundance in the vegetable soil. DMPP addition was observed to significantly inhibit the growth of AOB in both soils on day 28 (Fig. 2A). AOA abundance ranged between 3.5-7.5 $\times 10^5$ copies g^{-1} soil in the pasture soil to 4.9-8.9 $\times 10^6$ copies g^{-1} soil in the vegetable soil and no significant inhibitory effect of DMPP on the AOA abundance was observed (Fig. 2B).

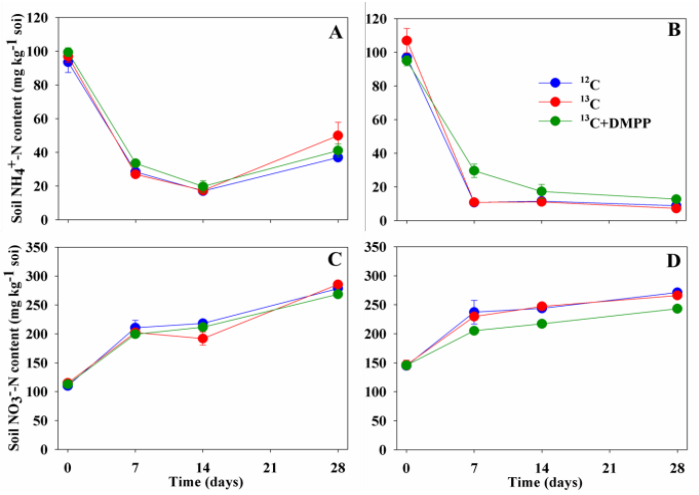

Fig.1 Changes in the NH_4^+-N and NO_3^--N content across three treatments (^{12}C-CO_2, ^{13}C-CO_2 and ^{13}C-CO_2 + DMPP) in the 28-day DNA-SIP microcosms of the pasture soil (A, C) and vegetable soil (B, D). Error bars represent standard errors of three replicates.

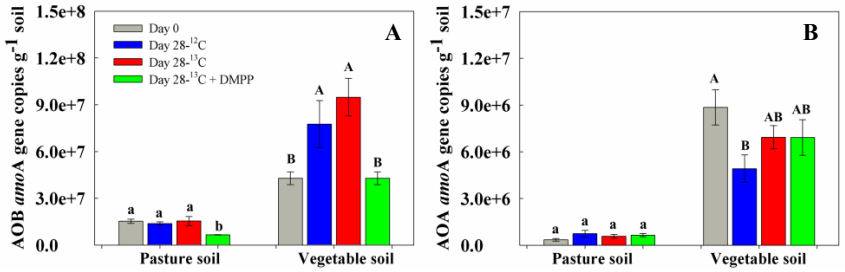

Fig.2 Changes in the AOB (A) and AOA (B) *amoA* gene copies across treatments (Day 0, ^{12}C-CO_2, ^{13}C-CO_2 and ^{13}C-CO_2+DMPP on day 28) in the DNA-SIP microcosms of the pasture soil and vegetable soil. Error bars represent standard errors of three replicates. Different letters above the bars indicate significant differences ($P < 0.05$) among treatments within the same soil.

The AOB abundance in the $^{12}CO_2$ microcosms peaked in the light fractions around a buoyant density of 1.72-1.73 g mL^{-1}. The majority of the AOB communities were detected in the 'heavy' SIP fractions with a buoyant density of 1.73-1.76 g mL^{-1} of the ^{13}C-CO_2 microcosms in both soils 28 (Fig. 3). A clear shift of the peak towards the light fractions was observed in the DMPP treatment in the vegetable soil on day 28 (Figure 3E and 3F), while DMPP had no obvious inhibitory effect on the metabolic activity of AOA in both soils (data not shown).

Discussion and Conclusion

Our study found that the abundance of AOB rather than that of AOA was significantly decreased by DMPP application, which is in line with the previous findings (Kleineidam et al., 2011). DNA-SIP incubation revealed DMPP effectively decreased the relative abundance of the AOB in the heavy fractions of CsCl gradients, indicating the inhibitory effect of DMPP on ammonia oxidation carried out by AOB. This microbial mechanism was well supported by the observations that the growth of AOB was inhibited by other nitrification inhibitors as acetylene and nitropyrin in soil microcosms where AOB dominated the nitrification activity (O'Callaghan et al., 2010; Wakelin et al., 2014). However, DMPP showed no evidently inhibitory effect on AOA indicated by the DNA-SIP results, indicating the different metabolic pathways for ammonia oxidation existed in AOA.

In this study, the NH_4^+-N concentrations varied from 17.0 to 96.9 mg kg^{-1} soil in the pasture soil and from 7.22 to 107 mg kg^{-1} soil in the vegetable soil during the DNA-SIP incubation. According to the ionization equilibrium $NH_4^+ \fallingdotseq NH_3 + H^+$ (pK_a =9.25), NH_3 concentrations were calculated

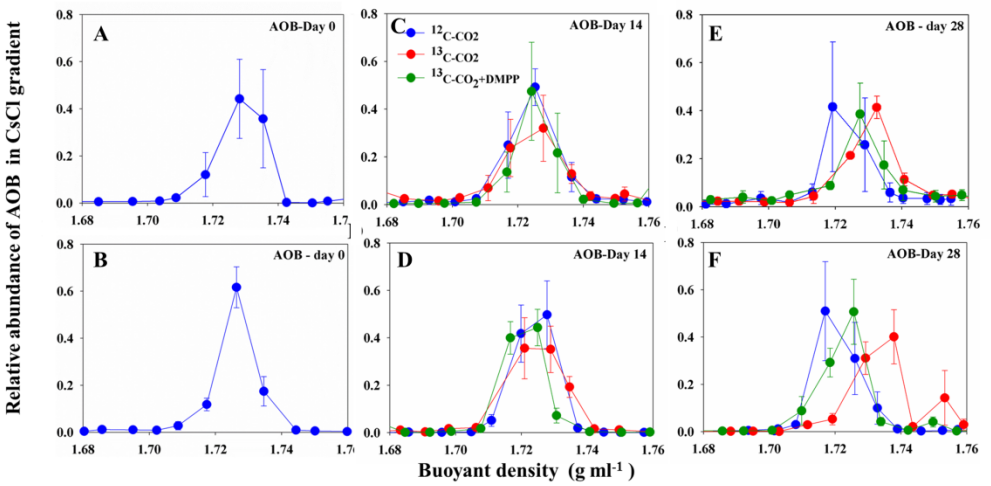

Fig.3 Relative abundance of the AOB *amoA* genes retrieved from different treatments (^{12}C-CO_2, ^{13}C-CO_2 and ^{13}C-CO_2 + DMPP) in DNA-SIP microcosms of the pasture soil (A, C, E) and vegetable soil (B, D, F) on days 0, 14 and 28. Error bars represent standard errors of three replicates.

Between 7.58 to 112 μmol in the vegetable soil, which are markedly higher than the threshold concentration required by cultivated AOB (above 1 μM near neutral pH; Bollmann et al., 2002). By contrast, NH_3 concentrations above 1 μM (1.93 μmol) were only available on the first day of incubation in the pasture soil, then declined sharply to 339 nmol on day 14. Therefore the growth of AOB might be retarded by the lower availability of NH_3 in the pasture soil. In our study, the AOB abundance remained 1-2 orders of magnitude lower in the pasture soil than in the vegetable soil. Given that AOB is the inhibition target of DMPP suggested by DNA-SIP results, the intrinsically low population size of AOB might contribute to the ineffectiveness of DMPP in the acidic pasture soil.

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