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Title page

Effects of 3,4-dimethylpyrazole phosphate (DMPP) on nitrification and the abundance and community composition of soil ammonia oxidizers in three land uses

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Abstract

The application of the nitrification inhibitor, 3,4-dimethylpyrazole-phosphate (DMPP) is considered as an effective strategy to mitigate agricultural nitrogen loss. However, the inhibitory effect of DMPP on nitrification is variable and the importance of the soil microbial community to the variability is poorly understood. In this study, nine soils were collected across three land uses to investigate the impact of DMPP on nitrification and associated dynamics of ammonia oxidizers in a 28-day microcosm incubation. The results showed that the efficacy of DMPP at inhibiting net nitrification rates varied highly from no effect to 63.6% during the first week of incubation. The abundance of ammonia-oxidizing bacteria (AOB), rather than ammonia-oxidizing archaea (AOA) was significantly correlated with nitrate concentrations across three land uses and significantly inhibited by DMPP addition. DMPP had higher efficacy in neutral and alkaline wheat and vegetable soils, compared with pasture soils. Canonical correspondence analysis suggested that soil pH was the most influential factor explaining the community composition of AOB and AOA in the collected soils. However, neither ammonium nitrate nor DMPP addition had a significant effect on the community composition of AOB or AOA during the incubation indicated by non-metric multidimensional scaling ordination. Taken together, our findings indicated that DMPP slowed nitrification by inhibiting the growth of AOB, and DMPP application affected the abundance of AOB more than the ammonia oxidizers community composition.

Keywords:

3,4-dimethylpyrazole-phosphate; ammonia-oxidizing archaea; ammonia-oxidizing bacteria; abundance; community composition

Introduction

Nitrogen (N)-based fertilizer is applied extensively in agriculture as an essential element for plant growth (Gilsanz et al. 2016). According to the prediction of the international fertilizer industry association (IFA), the global demand for N fertilizers will continuously increase and reach approximate161 million metric tonnes in 2018 (IFA, 2014). However, a major concern associated with the use of synthetic N fertilizers is the substantial N losses into the environment and the low nitrogen use efficiency (NUE) (Chen et al. 2008). Furthermore, the rising costs of synthetic N fertilizers have strengthened farmers' commercial incentive to reduce N fertilization and as a consequence N losses (Ernfors et al. 2014). Nitrification is a critical component in the N cycle, which causes N losses in the form of nitrate (NO_3^-) leaching and nitrous oxide (N_2O) emissions from agricultural systems (Wakelin et al. 2014; Chen et al. 2015). Nitrate leaching could trigger waterway eutrophication and drinking water contamination (Philippot et al. 2007). N_2O is a potent greenhouse gas, with about 300-fold greater warming potential than CO_2 on a per molecule basis, and is also involved in destruction of the stratospheric ozone layer (Hu et al. 2015a). Therefore, major efforts have been devoted to decreasing nitrification rates to retain N in the soil for plant uptake (Ernfors et al. 2014; Wakelin et al. 2014).

The incorporation of nitrification inhibitors (NIs) into N fertilizers is widely adopted as an efficiency-enhanced strategy to mitigate N losses through reducing nitrification rates (Akiyama et al. 2009). NIs are chemical compounds capable of modifying the active site of ammonia monooxygenase (AMO) and causing the formation of polypeptides, thus resulting in the inactivation of AMO activity and delay in nitrification (McCarty and Bremner 1989). Ammonia oxidation, as the rate-limiting step of nitrification, is catalyzed by AMO encoded by the *amoA* gene within two distinct groups of nitrifying microbes: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Brochier-Armanet et al. 2008;

Purkhold et al. 2000). Of the available NIs, 3,4-dimethylpyrazole-phosphate (DMPP) has been widely used together with synthetic N fertilizers (Weiske et al. 2001; Di and Cameron 2011; Liu et al. 2015) and animal manure (Hatch et al. 2005; Fangueiro et al. 2009; Maienza et al. 2014). To achieve comparable effects, DMPP requires much lower application rates than other NIs such as dicyandiamide (DCD) (Chaves et al. 2005; Chen et al. 2008). More importantly, no negative effect of DMPP on non-target microorganisms in soils has been reported (Tindaon et al. 2012; Kong et al. 2016). The inhibition of nitrification by DMPP was previously thought to be through indiscriminately binding to the complex of membrane-bound proteins and inactivating the AMO-containing AOB (Kleineidam et al. 2011; Benckiser et al. 2013). This perception, however, was challenged by the recent studies of Dong et al. (2013) and Florio et al. (2014), which provided evidence that DMPP inhibited AOA as well. Therefore, the underlying contribution of AOA and AOB to the DMPP-induced inhibition of nitrification is still in debate.

DMPP has been frequently applied in pasture and farming systems, but the inhibitory efficacy was reported to differ largely within and across agricultural systems. For example, studies found that the effect of DMPP on nitrification varied from no effect to 28.2% in grasslands (Fangueiro et al. 2009; Di and Cameron 2012), from 8.6 to 56.6% in cropping systems (Weiske et al. 2001; Liu et al. 2013; Migliorati et al. 2014), and from13.8 to 19.1% in vegetable production systems (Xu et al. 2005). Environmental variables such as temperature and moisture have been widely studied and considered as key factors controlling the efficacy of DMPP at inhibiting nitrification (Azam et al. 2001; Menéndez et al. 2009; 2012). Recently, Shi et al. (2016) suggested that the efficacy of NIs at reducing the nitrification rates was highly correlated with the abundance and activity of AOB. In contrast, McGeough et al. (2016) found that the efficacy of NIs was largely affected by soil properties such as soil clay and organic matter content. To date, there is limited knowledge regarding

the importance of soil microbial community composition to the variable efficacy of DMPP at inhibiting nitrification, which has greatly hindered our ability to assess the efficacy of DMPP in different scenarios.

The main objective of this study was to explore the influence of ammonia oxidizers and land uses on DMPP efficacy. Nine soils across three land uses (pasture, wheat and vegetable) were incubated following fertilizer application in presence and absence of DMPP in a 28-day microcosm incubation at 25°C. The following hypotheses were tested: (1) The abundance and/or community composition of AOA and AOB would respond differently to DMPP application, owing to their different physiological characteristics and enzyme systems for ammonia oxidation; (2) The efficacy of DMPP at inhibiting nitrification would be higher in the wheat and vegetable soils compared to the pasture soils, due to their lower organic matter content.

Materials and methods

Site descriptions

Soils samples were collected in October, 2015 from nine sites across three land uses (pasture, wheat, and vegetable) in Victoria and New South Wales, Australia. Pasture soils were collected from Dookie (P-DK, 36°25’ S, 145°42’ E), Glenormiston (P-GN, 38°15’ S, 142°52’ E) and Finley (P-FL, 35°38’ S, 145°40’ E). Two wheat growing soils were collected from Finley (W-FL_1, 35°33’ S, 145°32’ E; W-FL_2, 35°39’ S, 145°37’ E) and one from Horsham (W-HS, 36°45’ S, 142°07’ E). Vegetable growing soils were collected at Clyde (V-CD, 38°08’ S, 145°20’ E), Werribee (V-WB, 37°56’ S, 144°40’ E) and Perry Bridge (V-PB, 37°59’ S, 147°16’ E). The soils were classified using the Australian Soil Classification (Isbell 2002).

Soil sampling and physicochemical characterization

At each site, four surface soil samples (0-10 cm) were mixed together as a composite sample and passed through a 2 mm sieve. Gravimetric water content was determined by oven-drying sub-samples at 105°C for 24 h. Soil pH was determined using a ratio of 1:5 (fresh soil : water) with an Orion Star A211 pH-meter (Thermo Scientific Inc., Melbourne, Australia). Soil organic matter was determined using the $K_2Cr_2O_7$ wet oxidation and colorimetric method as previously described by Walkley and Black (1934). Soil exchangeable 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, and the solution, filtered through a Whatman 42 filter paper, was measured by a Segmented Flow Analyzer (SAN++, Skalar, Breda, Holland). Total N was determined using the classic Dumas method of combustion on an isotope ratio mass spectrometer (Sercon Hydra, Crewe, UK). Particle size analysis was measured using the sieve and hydrometer procedures. Details of the soil physical and chemical properties are shown in Table 1.

Soil microcosm incubation

The microcosm incubation was established to assess the effects of DMPP addition on nitrification and nitrifiers in the nine collected soils. Three treatments involving (1) control, (2) NH_4NO_3, and (3) NH_4NO_3 plus DMPP were set up in 250 ml plastic vials containing 20 g of soils (oven dry-weight equivalent) with three replicates. Nitrogen was added at a rate of 75 mg NH_4^+-N kg^{-1} soil and 75 mg NO_3^--N kg^{-1} soil. DMPP was applied in the form of solution at 1% of the applied NH_4^+-N according to the typical recommended rate (Serna et al. 2000; Pasda et al. 2001). All microcosms were pre-incubated for a week at 25°C and then adjusted to 60% water-filled pore space (WFPS) after adding treatment solutions (or deionized water for the control treatment). The final bulk density varied between 1.07 and 1.28 g cm^{-3}. The vials were closed immediately after treatment solutions were applied and incubated at 25°C in the dark for 28 days. During the incubation, the microcosms were aerated through opening

the vials for about three min every three days and moisture loss was replenished every week. Soil samples (10 g) were collected from three vials on days 0, 1, 4, 7, 14 and 28 for determination of exchangeable NH_4^+-N and NO_3^--N. Soils (0.5 g) were collected from the same vials on days 0, 7 and 28 for DNA extraction and molecular analysis.

The equation developed by Persson and Wirén (1995) was used to calculate the net nitrification rates in the first week and for the incubation time (28 days):

$$n_{(d0–d7)} = [(NO_3^-\text{-N})_{d7} - (NO_3^-\text{-N})_{d0}] / 7, \quad (1)$$

$$n_{(d0–d28)} = [(NO_3^-\text{-N})_{d28} - (NO_3^-\text{-N})_{d0}] / 28, \quad (2)$$

where $(NO_3^-\text{-N})_{d0}$, $(NO_3^-\text{-N})_{d7}$ and $(NO_3^-\text{-N})_{d28}$ are the NO_3^--N concentrations in the soil on days 0, 7 and 28, respectively.

DNA extraction and quantitative PCR (qPCR) analysis

Total genomic DNA was isolated from 0.25 g of soil using MoBio PowerSoil™ DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed using the NanoDrop ND2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The abundance of AOB and AOA *amoA* genes were quantified on a Bio-Rad CFX96 Optical Real-Time PCR Detection System (Bio-Rad, Laboratories Inc, Herculers, CA, USA) using the primer sets amoA1F/amoA2R (Rotthauwe et al. 1997) and CrenamoA23f/CrenamoA616r (Tourna et al. 2008), respectively. The 10 μl reaction mixture contained 5 μl of SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.2 μl of each primer (20 μM), and 1-10 ng of template DNA. Thermal cycling conditions for the AOB *amoA* gene were as follows: 30 s at 95°C, 40 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C. As for the AOA *amoA* gene, thermal cycling conditions were: 30 s at 95°C, 40 cycles of 30 s at 95°C, 45 s at 55°C, and 45 s at 72°C. Standard curves were generated using ten-fold serial dilutions of plasmids containing correct inserts of the targeted genes. Melting curve analysis was

157 performed between 75 and 94.5°C at the end of each amplification assay to evaluate the
158 specificity of qPCR products. The amplification efficiencies for all qPCR runs ranged
159 between 95-105%.
160 **Community profiling of the *amoA* gene by terminal restriction fragment length**
161 **polymorphism (T-RFLP) analysis**
162 The community composition of AOB and AOA in soils on days 0 and 7 of the incubation
163 were determined by T-RFLP analysis of the *amoA* genes, using the fluorescently labelled
164 primers FAM-CrenamoA23f/CrenamoA616r and FAM-amoA1F/amoA2R, respectively (Hu
165 et al. 2015b). The 25 μl PCR reaction mixture contained 2 μl of diluted template DNA (1-10
166 ng), 0.3 μl of each primer (20 μM), 2.5 μl of 10 × NH_4 reaction buffer, 1 μl of $MgCl_2$ solution
167 (50 mM), 1 μl of BSA (20 mM), 0.5 μl of dNTP mix (20 mM), and 1.5 units of BioTaq DNA
168 polymerase (Bioline, Sydney, Australia). The thermal cycling conditions were as follows:
169 95°C for 5 mins; 35 cycles of 30 s at 95°C, 45 s at 55°C, 45 s at 72°C, followed by 10 min at
170 72°C. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System
171 (Promega, San Luis Obispo, CA, USA) and quantified using the NanoDrop ND2000c
172 Spectrophotometer.
173 The restriction digestion was carried out in a 10 μl mixture containing 300 ng of purified
174 PCR products, 0.1 μl of BSA, 1 μl of 10 × NEBuffer, and 5 units of the restriction enzymes
175 MspI for AOB or RsaI (BioLabs, Sydney, Australia) for AOA. Digests were incubated at
176 37°C for 3 h, and then denatured for 10 min at 95°C. Terminal restriction fragments (TRFs)
177 were size-separated with an ABI PRISM 3500 Genetic analyzer (Applied Biosystems, CA,
178 USA), and analyzed using a local southern size calling method (peaks > 50 bp) and a peak
179 amplitude threshold setting of 50, using Genemapper version 4.0 (Applied Biosystems).
180 TRFs with peak height comprising less than 1% of the total peak height were removed from
181 the downstream analysis, and peaks that differed by less than 1 bp were combined into the

same TRF (Singh and Thomas 2006; Hu et al. 2015b). The relative fluorescence abundances of all TRFs were exported for the analysis of community composition.

Statistical analysis

The *amoA* gene copy numbers were log-transformed prior to statistical analysis to meet normality assumptions. One-way analysis of variance (ANOVA) was used to compare net nitrification rates and the *amoA* gene abundance across treatments in SPSS 19.0 (IBM, USA). Spearman's correlation was performed to assess the correlations between the *amoA* gene copy numbers and NO_3^--N concentrations. Difference at $P < 0.05$ was considered to be statistically significant. Canonical correspondence analysis was performed with the Monte Carlo permutation test (the number of permutations is 499) to investigate the significant environmental factors influencing the community composition of AOB and AOA based on the T-RFLP data from soils collected on day 0 using CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA). Non-metric multidimensional scaling (NMDS) was used to visualize the Bray-Curtis dissimilarity matrices based on the T-RFLP data from soils collected on day 7 of the incubation. Permutational multivariate analysis of variance (PERMANOVA) was performed to examine the significance of Bray-Curtis dissimilarity between treatments, by using the *adonis* function of the *vegan* package in R platform.

Results

Soil physical and chemical properties

The physical and chemical properties of the soil samples were highly variable across the nine sampling sites (Table 1). Briefly, soil pH values varied from 5.44 in V-PB to 7.96 in V-WB, and mineral N contents shifted between 10.6 and 31.8 μg g^{-1} soil for exchangeable NH_4^+-N and 8.3 and 78.5 μg g^{-1} soil for NO_3^--N. Soil organic matter ranged widely from 1.35 to 7.30% and total N ranged from 0.05 to 0.49%, with higher values recorded in P-FL and P-DK soils.

206 The wide range of the shift in soil properties provided a good opportunity to explore the
207 effect of DMPP across various soil orders and the three land uses.

208 **Dynamics of mineral N and net nitrification rates**

209 In the control treatments, the exchangeable NH_4^+-N concentrations remained low and almost
210 unchanged around a level of 10 μg g^{-1} soil for all the examined soils (Fig. 1). In the NH_4NO_3
211 treatments, the exchangeable NH_4^+-N concentrations decreased over time, with dramatic
212 declines towards the control levels in P-DK, P-FL, W-FL_1, W-HS, V-CD and V-WB soils
213 within two weeks, while DMPP amendment significantly slowed down the decline of
214 exchangeable NH_4^+-N in these soils ($P < 0.05$). However, regardless of DMPP addition, the
215 exchangeable NH_4^+-N concentrations in P-GN, W-FL2, and V-PB soils remained much
216 higher than those in the control treatments until the end of the incubation at day 28.

217 The NO_3^--N concentrations gradually increased in the control microcosms (Fig. 2),
218 indicative of the oxidation of NH_3 derived from the mineralization of native organic N.
219 Addition of NH_4NO_3 significantly increased the NO_3^--N concentrations over time ($P < 0.05$),
220 which coincided with the dramatic declines in exchangeable NH_4^+-N, indicating the
221 occurrence of nitrification. In treatments amended with DMPP, nitrification was evidently
222 reduced by DMPP at certain sampling points. The net nitrification rates for all soils attained
223 the highest values in the NH_4NO_3 treatments in the first week of incubation, and DMPP
224 exerted significant inhibition on the net nitrification rates in P-FL, W-FL_1, W-HS, V-CD and
225 V-WB soils ($P < 0.05$), which varied between 23.4 and 63.6% (Table 2). The net nitrification
226 rates substantially decreased at the end of incubation, and no significant difference was
227 observed in treatments with and without DMPP.

228 **Changes in the abundance of AOB and AOA**

229 The abundance of AOB and AOA in the V-PB soil was below the detection limit of qPCR
230 analysis, therefore is not shown in Fig. 3. The abundance of AOB in the control treatment

varied greatly from 2.62×10^4 copies g^{-1} soil in W-FL_2 soil to 8.13×10^8 copies g^{-1} soil in V-WB soil, and was evidently higher in vegetable soils than in pasture and wheat soils, except for P-FL soil (Fig. 3). Addition of NH_4NO_3 significantly increased AOB abundance in W-HS and V-WB soils on day 7, compared with the control treatments, while DMPP significantly decreased the AOB abundance by 69.1% and 51.4%, respectively. In the NH_4NO_3 treatments, the abundance of AOB was observed to significantly increase in P-GN, P-FL, W-HS, V-CD and V-WB soils on day 28 ($P < 0.05$). However, significant decreases in the AOB abundance due to DMPP addition were only found in W-HS and V-WB soils on day 28 ($P < 0.05$). The AOA abundance was highest in the vegetable soils ranging from 1.55×10^7 to 1.35×10^8 copies g^{-1} soil, and much lower in the pasture and wheat soils ranging between 5.73×10^4 and 2.18×10^6 copies g^{-1} soil (Fig. 4). However, there was no significant difference in the AOA abundance between treatments during the incubation.

Pearson correlation analysis revealed that AOB abundances showed significant correlations with NO_3^--N concentrations in pasture soils ($R^2 = 0.44$, $P < 0.001$), wheat soils ($R^2 = 0.32$, $P < 0.01$) and vegetable soils ($R^2 = 0.31$, $P < 0.05$). However, significant correlation between AOA abundance and NO_3^--N was only observed in pasture soils ($R^2 = 0.30$, $P < 0.01$) (Fig. 5).

Effects of DMPP on the community composition of AOB and AOA

The community composition of AOB and AOA in soils collected on days 0 and 7 of the incubation was analyzed using T-RFLP. After being digested by the MspI enzyme, the AOB *amoA* gene yielded 14 distinct TRFs across land uses. Canonical correspondence analysis (CCA) showed that soil organic matter ($F = 2.24$, $P = 0.002$) and soil pH ($F = 2.02$, $P = 0.002$) were the two most influential soil properties explaining the AOB community composition, with the first two axes of the CCA accounting for 63.9 % of the total variance. The CCA biplots showed that the AOB community from the same land use formed the same cluster (Fig. 6). NMDS ordinations based on the Bray-Curtis dissimilarity matrices revealed a clear

256 divergence of AOB assemblages between different soils (Fig. 7), which was further
257 corroborated by the PERMANOVA analysis (Pseudo-F = 6.7, P < 0.001).
258 The AOA *amoA* gene digested by the RsaI enzyme yielded nine TRFs. Canonical
259 correspondence analysis showed that soil pH (F = 1.49, P = 0.014) and indigenous NO_3^--N (F
260 = 1.46, P = 0.040) were the significant environmental factors explaining the AOA
261 community composition, with the first two axes standing for 52.9 % of the total AOA
262 variance (Fig. 6). NMDS ordinations showed no clear divergence between treatments (Fig. 7).
263 The PERMANOVA analysis suggested that the land use had a significant impact on the AOA
264 community profiling composition (Pseudo-F = 2.4, P < 0.001).
265 **Discussion**
266 **DMPP inhibits nitrification through impairing the abundance of AOB**
267 We found that the DMPP played a significant inhibitory role in the soils with pH ranging
268 between 6.28 and 7.96, whereas no DMPP-related inhibition on nitrification was observed in
269 soils with pH between 5.44 and 5.65 (Tables 1and 2). Recent studies suggested that soil pH is
270 a critical factor driving the niche partitioning of AOB and AOA (as reviewed in Hu et al.
271 2014). On one hand, neutral and slightly alkaline soils were generally considered as
272 favourable habitats for AOB growth (Shen et al. 2008; Jia and Conrad 2009), and AOB could
273 not be capable of oxidizing NH_3 under extremely low soil pH conditions (De Boer and
274 Kowalchuk 2001). The explanation for the inability of AOB to nitrify NH_3 under acid
275 conditions is the exponential ionization of NH_3 to NH_4^+ in acid soils (Hu et al. 2014). In this
276 study, the highest AOB abundance was observed in the vegetable soils V-CD (pH 7.02) and
277 V-WB (pH 7.96) soils, whereas the lowest AOB abundance appeared in P-GN (pH 5.55) and
278 W-FL2 (pH 5.49) soils (Fig. 3). On the other hand, numerous studies indicated that AOA
279 plays a more dominant role in mediating nitrification in acid soils, with comprehensive
280 evidence provided by the $^{13}CO_2$-DNA-SIP and 454 barcoded pyrosequencing techniques

(Zhang et al. 2012; Hu et al. 2013). In this study, a higher abundance of AOA than AOB was only found in W-FL_2 soil with the lowest pH of 5.49 among the investigated soils. Although a higher abundance of AOB than AOA was observed in other acid soils including P-GN, W-FL_2 and V-PB soils, previous researches revealed that the numerical advantage at genomic level does not necessarily equal the dominance at functional level (Jia and Conrad 2009). It should be noted that the nitrification rate in P-DK soil was much higher than other acid soils, and could be probably ascribed to the oxidation of NH_3 derived from the mineralization of native organic N, as suggested by the rapid increase in nitrate content in the control treatment. This is probably facilitated by the higher organic N in P-DK soil (Table 2). However, no significantly inhibitory effect of DMPP was observed in these acid soils during the incubation. Taken together, it could be inferred that DMPP has an inhibitory effect on soil nitrification through impairing the AOB abundance favoured by the higher soil pH conditions, whereas DMPP was not efficient at decreasing nitrification in acid soils.

The different responses of AOB and AOA to DMPP inhibition could be ascribed to not only the pH-associated niche differentiation, but the different enzyme systems for nitrification. Firstly, it is conventionally considered that hydroxylamine (NH_2OH) acts as the intermediate of ammonia oxidation ($NH_3 \rightarrow NH_2OH$), catalyzed by the AMO enzyme, and DMPP could inhibit nitrification through inactivation of the AMO activity (Hu et al. 2015a). However, the homologous gene encoding the hydroxylamine oxidoreductase (HAO), which is a necessity for the oxidation of NH_2OH, has not been identified in AOA genomes (Tourna et al. 2011). It is speculated that AOA could possess a unique way of nitrification, unlike AOB. One alternative is that AOA could produce nitroxyl hydride (HNO) as an intermediate of ammonia oxidation ($NH_3 \rightarrow HNO$) (Walker et al. 2010). Secondly, the annotated AOA *amo* sequences were more similar to the genes encoding bacterial particulate methane monooxygenases (pMMO) rather than AOB *amoA* genes, indicative of functional differences

306 in AMO between the AOA and AOB, which is responsible for the oxidation of NH_3 to
307 NH_2OH (Walker et al. 2010). An early study reported that DMPP reduced AMO activity,
308 while pMMO stayed unimpaired in an agricultural crop rotation (Weiske et al. 2001). Thirdly,
309 the possibility of heterotrophic growth has been suggested for AOA (Tourna et al. 2011),
310 such as group I.1c, well-known as a major lineage of AOA (Lehtovirta et al. 2009). The
311 potential of heterotrophic nitrification was further suggested by Hu et al. (2013) who found
312 that the group I.1c abundance had a significant positive correlation with soil organic C.
313 Therefore, DMPP could inhibit nitrification by inhibiting AOB growth, and the inefficiency
314 of DMPP on AOA-mediated nitrification could be caused by the different enzyme systems
315 and the mixotrophic growth of AOA.

316 **Environmental and soil factors influencing DMPP efficacy**

317 It has been more than a decade since the earliest report regarding the use of DMPP to regulate
318 N supply through maintaining N as NH_4^+ and reducing NO_3^- leaching, consequently
319 improving NUE (Serna et al. 2000; Zerulla et al. 2001), decreasing greenhouse gas emissions
320 and increasing crop yields (Pasda et al. 2001; Weiske et al. 2001). Given these benefits
321 derived from DMPP application, the factors influencing the efficacy of DMPP have attracted
322 considerable research interest. Soil temperature has been considered as one of the most
323 important factors controlling DMPP efficacy, because temperature has the great potential to
324 impact the mobility of DMPP and the degradation rate of the DMPP in soils (Azam et al.
325 2001; Irigoyen et al. 2003). In our study, DMPP slowed down the decline of exchangeable
326 NH_4^+-N in one or two weeks, and the longest extension was observed in W-FL_1 and W-HS
327 soils till the end of incubation at 25°C (Figs. 1 and 2), which was comparable with previous
328 studies that showed DMPP retained exchangeable NH_4^+-N for eight days at 30°C, and 40
329 days at 20°C (Irigoyen et al. 2003; Menéndez et al. 2012). However, regardless of DMPP
330 addition, the exchangeable NH_4^+-N concentration remained at high levels in P-GN, W-FL_2

and V-PB soils till day 28. The different changes in exchangeable NH_4^+-N under the same temperature indicate that there are other critical factors influencing the efficacy of DMPP in the retention of NH_4^+-N.

The effect of soil properties on DMPP efficacy was first reported by Barth et al. (2001) through a short-term incubation experiment, suggesting that soil textural properties, specifically the proportion of clay, are important factors influencing the binding behaviour of positively charged DMPP. In this study, the inhibitory effects of DMPP on net nitrification rates were generally higher in the soils with clay fractions less than 10% (W-HS, V-CD and P-FL soils). This is well supported by the previous findings that the inhibitory effects of DMPP were lower in soils with high proportions of clay (Wu et al. 2007; Barth et al. 2008), probably because more DMPP is adsorbed by soil components, resulting in less amount of DMPP to inhibit nitrification (Roco and Blu 2006). However, DMPP inhibited nitrification by 40.9% in V-WB soil in the first week, even with 36% clay content in this study (Tables 1 and 2). As suggested by McGeough et al. (2016), clay sorption capacity was not only dependent on the proportion of clay fraction, but also the type of clay minerals. Therfore, the proportion and type of clay in soils should be determined to better understand their effects on DMPP efficiencies in future studies. Apart from clay fractions, soil organic matter is suggested as another important soil component involved in the sorption of organic compounds (Kelliher et al. 2008; McGeough et al. 2016). In this study, the soil organic matter content is generally higher in the pasture soils than in the wheat and vegetable soils (Table 1), consequently, the higher inhibitory effect of DMPP on net nitrification found in the latter two soils could be due to the lower organic matter (Table 2). Recently, Marsden et al. (2016) reported that the sorption of DMPP was higher in the soil with higher amount of organic matter with the ^{14}C labelled DMPP applied in three distinct soils. The sorption phenomenon by soil organic matter also applies to other NIs, as a meta-analysis conducted by

Kelliher et al. (2008) showed that the amount of DCD sorption onto soil organic matter soils is a critical factor determining DCD efficacy. A recent study showed that the efficiency of nitrapyrin, diminished over the long-term addition of soil organic matter (Fisk et al. 2015).

Conclusions

In conclusion, through analysis of functional gene abundance and community composition analysis in microcosm incubation of nine soils across three land uses, we found that only AOB was significantly decreased by DMPP addition, accompanied by the decreased net nitrification rates. The effectiveness of DMPP at reducing nitrification was higher in neutral and alkaline soils wheat and vegetable soils than in pasture soils, indicating DMPP could play an inhibitory role in nitrification through impairing AOB growth. We provided evidence that the community composition of ammonia oxidizers were more resilient than gene abundance in response to DMPP addition. Our results indicated that soil clay fractions and organic matter content could be critical factors influencing DMPP efficacy. Further studies are needed to determine the changes in metabolic activities of AOB and AOA after DMPP addition to provide more direct evidence.

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550 **Table 1** The physical and chemical properties for the soil samples collected from nine sites in three land uses in Australia

Soil properties	P-DK	P-GN	P-FL	W-FL_1	W-FL_2	W-HS	V-CD	V-WB	V-PB
Land use	Pasture	Pasture	Pasture	Wheat	Wheat	Wheat	Vegetable	Vegetable	Vegetable
Location	Dookie	Glenormiston	Finley	Finley	Finley	Horsham	Clyde	Werribee	Perry Bridge
Mean annual temperature (°C)	20.9	19.1	22.9	22.9	22.9	22.7	19.9	20.5	20.3
Mean annual rainfall (mm)	550	779	430	430	430	414	812	538	628
Soil Order	Chromosol	Dermosol	Sodosol	Sodosol	Sodosol	Vertosol	Podosol	Chromosol	Podosol
pH	5.65	5.55	6.28	6.37	5.49	6.37	7.02	7.96	5.44
Soil organic matter (%)	5.96	5.27	7.30	5.33	2.61	3.67	3.11	1.73	1.35
Exchangeable NH_4^+-N (μg g^{-1} soil)	17.7	12.1	21.0	31.8	15.8	12.0	12.4	14.0	10.6
NO_3^--N (μg g^{-1} soil)	37.8	9.3	36.9	78.5	16.7	8.3	32.3	27.9	9.0
Total N (%)	0.40	0.21	0.49	0.31	0.19	0.20	0.30	0.17	0.05
Particle size (%)									
Clay (< 0.002 mm)	11	19	8	11	40	3	4	36	5
Silt (0.002-0.02 mm)	30	14	15	44	11	37	11	23	5

Sand (0.02-2 mm)	59	67	78	45	49	60	85	41	90

551 **Table 2** The average net nitrification rates of the three treatments across the three land uses
552 during the first week of incubation and the 28-day of incubation

Soils	Time intervals	Net nitrification rates			% Inhibition by DMPP
		Control	NH_4NO_3	NH_4NO_3 + DMPP	
P-DK	d_0–d_7	3.22 ± 0.14 a	11.16 ± 0.52 b	11.61 ± 0.21 b	nil
	d_0–d_{28}	3.15 ± 0.12 a	5.26 ± 0.15 b	5.10 ± 0.07 b	nil
P-GN	d_0–d_7	0.78 ± 0.26 a	2.58 ± 0.14 b	2.71 ± 0.35 b	nil
	d_0–d_{28}	0.87 ± 0.09 a	1.63 ± 0.27 b	1.39 ± 0.02 b	nil
P-FL	d_0–d_7	2.88 ± 0.14 a	11.90 ± 0.15 c	9.12 ± 0.34 b	23.4
	d_0–d_{28}	2.28 ± 0.01 a	4.50 ± 0.02 b	4.18 ± 0.24 b	nil
W-FL_1	d_0–d_7	2.37 ± 0.62 a	4.37 ± 0.30 b	2.42 ± 0.99 a	44.6
	d_0–d_{28}	1.66 ± 0.32 a	3.14 ± 0.14 b	2.97 ± 0.34 b	nil
W-FL_2	d_0–d_7	1.88 ± 0.17 a	3.64 ± 0.08 b	2.88 ± 0.40 b	20.9
	d_0–d_{28}	1.27 ± 0.05 a	2.41 ± 0.05 b	2.42 ± 0.06 b	nil
W-HS	d_0–d_7	1.62 ± 0.12 a	9.42 ± 0.16 b	3.43 ± 0.95 a	63.6
	d_0–d_{28}	1.62 ± 0.06 a	3.56 ± 0.16 b	3.37 ± 0.25 b	nil
V-CD	d_0–d_7	2.08 ± 0.22 a	11.70 ± 0.26 c	8.76 ± 0.67 b	25.1
	d_0–d_{28}	2.22 ± 0.07 a	4.12 ± 0.08 b	4.00 ± 0.08 b	nil
V-WB	d_0–d_7	5.11 ± 0.47 a	14.06 ± 0.87 c	8.31 ± 0.62 b	40.9
	d_0–d_{28}	2.67 ± 0.15 a	4.54 ± 0.21 b	4.13 ± 0.17 b	nil
V-PB	d_0–d_7	0.30 ± 0.12 a	0.63 ± 0.20 a	0.63 ± 0.11 a	nil
	d_0–d_{28}	0.42 ± 0.02 a	0.56 ± 0.19 a	0.57 ± 0.03 a	nil

553 The rates were expressed as mean ± standard errors. The unit is μg g^{-1} soil day^{-1}. Different
554 letters in the same row denote significant difference at the $P < 0.05$ level; nil denotes no
555 observed inhibition derived from DMPP addition.

556 **Figure legends**

557 **Fig. 1** Changes in the exchangeable NH_4^+-N concentrations of the nine soils in the three land
558 uses (pasture, wheat and vegetable) during the 28-day microcosm incubation. Error bars
559 represent standard errors of three replicates.

560 **Fig. 2** Changes in the NO_3^--N concentrations of the nine soils in the three land uses (pasture,
561 wheat and vegetable) during the soil microcosm incubation. Error bars represent standard
562 errors of three replicates.

563 **Fig. 3** Changes in the AOB abundance during the 28-day microcosm incubation of soils in
564 the three land uses (pasture, wheat and vegetable) during the 28-day microcosm incubation.
565 Error bars represent standard errors of three replicates. Note: Y axes scales differ between
566 charts.

567 **Fig. 4** Changes in the AOA abundance during the 28-day microcosm incubation of soils in
568 the three land uses (pasture, wheat and vegetable) during the 28-day microcosm incubation.
569 Error bars represent standard errors of three replicates. Note: Y axes scales differ between
570 charts.

571 **Fig. 5** Relationship between AOB or AOA abundance and the NO_3^--N concentrations during
572 the 28-day microcosm incubation.

573 **Fig. 6** CCA ordination biplots indicating the relationship between the community
574 composition AOB or AOA and soil properties in the three land uses (pasture, wheat and
575 vegetable) before incubation. Arrows indicate the direction and magnitude of measurable
576 variables associated with the microbial community composition.

577 **Fig. 7** Nonmetric multi-dimensional scaling ordinations based on the Bray-Curtis
578 dissimilarity matrices of the T-RFLP data of AOB digested using the MspI enzyme and AOA

digested using the RsaI enzyme across different treatments (C, control; F, NH_4NO_3; FD, NH_4NO_3 + DMPP) and land uses (P, pasture; W, wheat; V, vegetable) on day 7 of the incubation.