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 composition 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 oxidizer 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 approximately 161 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

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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 the following 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 from 13.8 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, and (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

Soil 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-FL₁, 35° 33' S, 145° 32' E; W-FL₂, 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 K₂Cr₂O₇ wet oxidation and colorimetric method as previously described by Walkley and Black (1934). Soil exchangeable NH₄⁺-N and NO₃⁻-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

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	77	45	49	60	85	41	90

the nine collected soils. Three treatments involving (1) control, (2) NH₄NO₃, and (3) NH₄NO₃ 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 NH₄⁺-N kg⁻¹ soil and 75 mg NO₃⁻-N kg⁻¹ soil. DMPP was applied in the form of solution at 1 % of the applied NH₄⁺-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 every 3 days for 3 min 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 the determination of exchangeable NH₄⁺-N and NO₃⁻-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 over the incubation time (28 days).

$$n(d_0-d_7) = [(NO_3^- - N)_{d7} - (NO_3^- - N)_{d0}] / 7, \quad (1)$$

$$n(d_0-d_{28}) = [(NO_3^- - N)_{d28} - (NO_3^- - N)_{d0}] / 28, \quad (2)$$

where (NO₃⁻-N)_{d0}, (NO₃⁻-N)_{d7}, and (NO₃⁻-N)_{d28} are the NO₃⁻-N concentrations in the soil on days 0, 7, and 28, respectively.

DNA extraction and quantitative PCR 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, Hercules, 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 tenfold serial dilutions of plasmids containing correct inserts of the targeted genes. Melting curve analysis was performed between 75 and 94.5 °C at the end of each amplification assay to evaluate the specificity of quantitative PCR (qPCR) products. The amplification efficiency for all qPCR runs ranged between 95 and 105 %.

Community profiling of the *amoA* gene by terminal restriction fragment length polymorphism analysis

The community composition of AOA and AOB in soils on days 0 and 7 of the incubation were determined by terminal restriction fragment length polymorphism (T-RFLP) analysis

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

of the *amoA* genes, using the fluorescently labeled primers FAM-CrenamoA23f/CrenamoA616r and FAM-amoA1F/amoA2R, respectively (Hu et al. 2015b). The 25 μl PCR reaction mixture contained 2 μl of diluted template DNA (1–10 ng), 0.3 μl of each primer (20 μM), 2.5 μl of $\times 10$ NH_4 reaction buffer, 1 μl of MgCl_2 solution (50 mM), 1 μl of BSA (20 mM), 0.5 μl of dNTP mix (20 mM), and 1.5 u of BioTaq DNA polymerase (Bioline, Sydney, Australia). The thermal cycling conditions were as follows: 95 $^\circ\text{C}$ for 5 min, 35 cycles of 30 s at 95 $^\circ\text{C}$, 45 s at 55 $^\circ\text{C}$, 45 s at 72 $^\circ\text{C}$, followed by 10 min at 72 $^\circ\text{C}$. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA) and quantified using the NanoDrop ND2000c Spectrophotometer.

The restriction digestion was carried out in a 10 μl mixture containing 300 ng of purified PCR products, 0.1 μl of BSA, 1 μl of $\times 10$ NE buffer, and 5 u of the restriction enzymes *MspI* for AOB or *RsaI* (BioLabs, Sydney, Australia) for AOA. Digests

were incubated at 37 $^\circ\text{C}$ for 3 h and then denatured for 10 min at 95 $^\circ\text{C}$. Terminal restriction fragments (TRFs) were size separated with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) and analyzed using a local southern size-calling method (peaks >50 bp) and a peak amplitude threshold setting of 50, using GeneMapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 1 % of the total peak height were removed from 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.

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

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 $31.8 \mu\text{g g}^{-1}$ soil for exchangeable NH_4^+ -N and 8.3 and $78.5 \mu\text{g g}^{-1}$ 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-DK and P-FL soils. The wide range of the shift in soil properties

provided a good opportunity to explore the effect of DMPP across various soil orders and the three land uses.

Dynamics of mineral N and net nitrification rates

In the control treatments, the exchangeable NH_4^+ -N concentrations remained low and almost unchanged around a level of $10 \mu\text{g g}^{-1}$ soil for all the examined soils (Fig. 1). In the NH_4NO_3 treatments, the exchangeable NH_4^+ -N concentrations decreased over time, with dramatic declines towards the control levels in P-DK, P-FL, W-FL₁, W-HS, V-CD, and V-WB soils within 2 weeks, while DMPP amendment significantly slowed down the decline of exchangeable NH_4^+ -N in these soils ($P < 0.05$). However, regardless of DMPP addition, the exchangeable NH_4^+ -N concentrations in P-GN, W-FL₂, and V-PB soils remained much higher than those in the control treatments until the end of the incubation on day 28.

The NO_3^- -N concentrations gradually increased in the control microcosms (Fig. 2), indicative of the oxidation of NH_3 derived from the mineralization of native organic N. Addition of NH_4NO_3 significantly increased the NO_3^- -N concentrations over time ($P < 0.05$), which coincided with the dramatic declines in exchangeable NH_4^+ -N, indicating the occurrence of nitrification. In treatments amended with DMPP, nitrification was evidently reduced by DMPP at certain sampling points. The net nitrification rates for all soils attained the

highest values in the NH_4NO_3 treatments in the first week of incubation, and DMPP exerted significant inhibition on the net nitrification rates in P-FL, W-FL₁, W-HS, V-CD, and V-WB soils ($P < 0.05$), which varied between 23.4 and 63.6 % (Table 2). The net nitrification rates substantially decreased at the end of incubation, and no significant difference was observed in treatments with or without DMPP.

Changes in the abundance of AOB and AOA

The abundance of AOB and AOA in the V-PB soil was below the detection limit of qPCR analysis; therefore, it 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-FL₂ 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

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

Soils	Time intervals	Net nitrification rates			Percent 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

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

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

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's correlation analysis revealed that the AOB abundance 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 divergence of AOB assemblages between different soils (Fig. 7), which was further corroborated by the PERMANOVA analysis (pseudo- $F = 6.7$, $P < 0.001$).

The AOA *amoA* gene digested by the RsaI enzyme yielded nine TRFs. Canonical correspondence analysis showed that soil pH ($F = 1.49$, $P = 0.014$) and indigenous NO_3^- -N ($F = 1.46$, $P = 0.040$) were the significant environmental factors explaining the AOA community composition, with the first two axes standing for 52.9 % of the

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

total AOA variance (Fig. 6). NMDS ordinations showed no clear divergence between treatments (Fig. 7). The PERMANOVA analysis suggested that the land use had a significant impact on the AOA community profiling composition (pseudo- $F = 2.4$, $P < 0.001$).

Discussion

DMPP inhibits nitrification through impairing the abundance of AOB

We found that the DMPP played a significant inhibitory role in the soils with pH ranging between 6.28 and 7.96, whereas no DMPP-related inhibition on nitrification was observed in soils with pH between 5.44 and 5.65 (Tables 1 and 2). Recent studies suggested that soil pH is a critical factor driving the niche partitioning of AOB and AOA (as detailed reviewed by Hu et al. 2014). On one hand, neutral and slightly alkaline

soils were generally considered as favorable habitats for AOB growth (Shen et al. 2008; Jia and Conrad 2009), and AOB could not be capable of oxidizing NH_3 under extremely low soil pH conditions (De Boer and Kowalchuk 2001). The explanation for the inability of AOB to nitrify NH_3 under acid conditions is the exponential ionization of NH_3 to NH_4^+ in acid soils (Hu et al. 2014). In this study, the highest AOB abundance was observed in the vegetable soils V-CD (pH 7.02) and V-WB (pH 7.96) soils, whereas the lowest AOB abundance appeared in P-GN (pH 5.55) and W-FL2 (pH 5.49) soils (Fig. 3). On the other hand, numerous studies indicated that AOA plays a more dominant role in mediating nitrification in acid soils, with comprehensive evidence provided by the $^{13}\text{CO}_2$ -DNA-SIP and 454 bar-coded 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-FL₂ soil with the lowest pH of 5.49 among the investigated soils. Despite that a higher abundance of AOB than AOA was observed in other acid soils including P-DK, P-

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

GN, and V-PB soils, previous research 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 of 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 favored 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 also the different enzyme systems for nitrification. Firstly, it is conventionally considered that hydroxylamine (NH_2OH) acts as the intermediate of ammonia oxidation ($\text{NH}_3 \rightarrow \text{NH}_2\text{OH}$), catalyzed by the AMO enzyme, and DMPP could inhibit nitrification through inactivation of the AMO activity (Benckiser et al. 2013). However, the

Fig. 6 CCA ordination biplots indicating the relationship between the community composition AOB or AOA and soil properties in the three land uses (pasture, wheat, and vegetable) before incubation. Arrows

indicate the direction and magnitude of measurable variables associated with the microbial community composition

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 ($\text{NH}_3 \rightarrow \text{HNO}$) (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 in AMO between AOA and AOB, which is responsible for the oxidation of NH_3 to NH_2OH (Walker et al. 2010). An early study reported that DMPP reduced AMO activity, while MMO stayed unimpaired in an agricultural crop rotation (Weiske et al. 2001). Therefore, DMPP could not inhibit the AOA-mediated nitrification. Thirdly, the possibility of heterotrophic growth has been suggested for AOA (Tourna et al. 2011), such as group I.1c, well known as a major lineage of AOA (Lehtovirta et al. 2009). The potential of heterotrophic nitrification was further suggested by Hu et al. (2013), who found that the group I.1c abundance had a significant positive correlation with soil organic C. Therefore, DMPP could inhibit nitrification by inhibiting AOB growth, and the inefficiency of DMPP on AOA-mediated nitrification could be caused by the different enzyme systems and the mixotrophic growth of AOA.

Environmental and edaphic factors influencing DMPP efficacy

It has been more than a decade since the earliest report regarding the use of DMPP to regulate N supply through maintaining N as NH_4^+ and reducing NO_3^- leaching, consequently improving NUE (Serna et al. 2000; Zerulla et al. 2001), decreasing greenhouse gas emissions, and increasing crop yields (Pasda et al. 2001; Weiske et al. 2001). Given these benefits

derived from DMPP application, the factors influencing the efficacy of DMPP have attracted considerable research interest. Soil temperature has been considered as one of the most important factors controlling DMPP efficacy, because temperature has the great potential to impact the mobility of DMPP and the degradation rate of the DMPP in soils (Azam et al. 2001; Irigoyen et al. 2003). In our study, DMPP slowed down the decline of exchangeable $\text{NH}_4^+\text{-N}$ in 1 or 2 weeks; the longest extension was observed in W-FL₁ and W-HS soils till the end of incubation at 25 °C (Figs. 1 and 2), which was comparable with previous studies that showed that DMPP retained exchangeable $\text{NH}_4^+\text{-N}$ for 8 days at 30 °C and 40 days at 20 °C (Irigoyen et al. 2003; Menéndez et al. 2012). However, regardless of DMPP addition, the $\text{NH}_4^+\text{-N}$ remained at high levels in P-GN, W-FL₂, and V-PB soils till day 28. The different changes in exchangeable $\text{NH}_4^+\text{-N}$ under the same temperature indicate that indigenous nitrification rates in different soils are more critical factor influencing the potential of DMPP to retain $\text{NH}_4^+\text{-N}$.

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 behavior 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

Fig. 7 Non-metric multidimensional scaling ordinations based on the Bray-Curtis 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

was not only dependent on the proportion of clay fraction but also on the type of clay minerals. Therefore, the proportion and type of clay in soils should be determined to better understand their effects on DMPP efficacy 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 -labeled 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 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 that 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 involving more soil types under field conditions should be incorporated into future research on the efficacy of DMPP at inhibiting nitrification and nitrifier activity.

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