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Effects of the Nitrification Inhibitor 3,4-Dimethylpyrazole Phosphate on Nitrification and Nitrifiers in Two Contrasting Agricultural Soils

Xiuzhen Shi,[a] Hang-Wei Hu,[a] Christoph Müller,[b,c] Ji-Zheng He,[a] Deli Chen,[a] Helen Charlotte Suter[a]

Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, Australia[a]; Department of Plant Ecology, Justus Liebig University Giessen, Giessen, Germany[b]; School of Biology and Environmental Sciences and Earth Institute, University College Dublin, Dublin, Ireland[c]

ABSTRACT

The nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) is a powerful tool that can be used to promote nitrogen (N) use efficiency and reduce N losses from agricultural systems by slowing nitrification. Mounting evidence has confirmed the functional importance of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in nitrification and N_2O production; however, their responses to DMPP amendment and the microbial mechanisms underlying the variable efficiencies of DMPP across different soils remain largely unknown. Here we compared the impacts of DMPP on nitrification and the dynamics of ammonia oxidizers between an acidic pasture soil and an alkaline vegetable soil using a ^{15}N tracing and $^{13}CO_2$-DNA–stable-isotope probing (SIP) technique. The results showed that DMPP significantly inhibited nitrification and N_2O production in the vegetable soil only, and the transient inhibition was coupled with a significant decrease in AOB abundance. No significant effects on the community structure of ammonia oxidizers or the abundances of total bacteria and denitrifiers were observed in either soil. The ^{15}N tracing experiment revealed that autotrophic nitrification was the predominant form of nitrification in both soils. The $^{13}CO_2$-DNA–SIP results indicated the involvement of AOB in active nitrification in both soils, but DMPP inhibited the assimilation of $^{13}CO_2$ into AOB only 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.

IMPORTANCE

The combination of the ^{15}N tracing model and $^{13}CO_2$-DNA–SIP technique provides important evidence that the nitrification inhibitor DMPP could effectively inhibit nitrification and nitrous oxide emission in an alkaline soil through influencing the abundance and metabolic activity of AOB. In contrast, DMPP amendment has no significant effect on nitrification or nitrifiers in an acidic soil, potentially owing to the low abundance of AOB and the possible adsorption of DMPP by organic matter. Our findings have direct implications for improved agricultural practices through utilizing the nitrification inhibitor DMPP in appropriate situations, and they emphasize the importance of microbial communities to the efficacy of DMPP.

Nitrification is a microbially mediated process which converts ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-) and significantly influences plant nitrogen (N) availability, nitrate leaching, and nitrous oxide (N_2O) emissions (1, 2). As the first and rate-limiting step of nitrification, ammonia oxidation, is catalyzed by ammonia monooxygenase (AMO), encoded by the *amoA* gene, within ammonia-oxidizing bacteria (AOB) classified as beta- or gammaproteobacteria (1) and ammonia-oxidizing archaea (AOA) belonging to the recently recognized *Thaumarchaeota* phylum (3). Recently, several *Nitrospira* strains belonging to nitrite oxidizers were discovered and cultivated as completely nitrifying bacteria with the capacity to oxidize ammonia to nitrate via nitrite in a single organism (termed comammox) (4, 5). The ammonia oxidation-derived N_2O is an important pathway for soil N_2O production, contributing to the increasing atmospheric N_2O loads (6). N_2O is a potent greenhouse gas, with 298-times-higher warming potential than CO_2 (7), and is also the most important gas triggering the destruction of the stratospheric ozone layer (8). Another ecological concern from nitrification derives from the negative effect of nitrate leaching on the environment, resulting in waterway eutrophication and drinking water contamination (9). Given that fertilizer applications are predicted to increase by 35 to 60% before 2030 (7) and with the majority of N fertilizers being ammoniacal, it is extremely important to develop effective management strategies to mitigate the environmental impact and economic loss from use of N-based fertilizers.

The application of nitrification inhibitors together with N-based fertilizers has been suggested as a practical way to reduce nitrate leaching and N_2O emissions and to promote N use efficiency (NUE) and crop yields in grasslands and croplands (10–12). 3,4-Dimethylpyrazole phosphate (DMPP) was marketed in 1999 and is one of the most effective nitrification inhibitors (13, 14). DMPP has been demonstrated to be effective in increasing NUE (15–17) and crop yields and quality (18–20), and it requires lower application rates than dicyandiamide (DCD) for compara-

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Address correspondence to Hang-Wei Hu, hang-wei.hu@unimelb.edu.au.

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ble efficacy (21). However, the efficiency of DMPP to reduce N losses is highly variable (22). For example, the reduction in N_2O emissions due to DMPP application was reported to vary from no effects (23, 24) to 32% (25) and 45 to 69% (26) in grasslands and from 38 to 75% in intensively fertilized cropping systems (16, 27–29). To date, extensive efforts have been directed toward investigating the main environmental factors causing the variable efficacy of DMPP. These studies have identified temperature (30, 31), soil moisture (23), soil properties (32, 33), fertilizer types (24), and application methods (20) as the key determinants. However, despite the importance of biological activity in mediating the N transformation processes, there is limited research on the potential significance of the soil microbial community.

Several studies have explored the abundance, diversity, and structure of soil microbial communities in response to DMPP addition. The first field-based evidence revealed that DMPP application reduced the abundance of AOB but not that of AOA in bulk and rhizosphere soils (34). This finding was further supported by a series of laboratory microcosms of six pasture soils (15). In contrast, other laboratory incubation studies have found that DMPP application significantly decreased the abundance and transcriptional activity of both AOA and AOB (35, 36). Recently, a change in the community structure of AOB in response to DMPP application was observed (37). To our knowledge, there is no information currently available regarding the metabolic activity and taxonomic identity of AOA and/or AOB in terms of DMPP-induced inhibition of nitrification. DNA–stable-isotope probing (SIP) is a culture-independent technique with wide applicability in microbial ecology, and it is capable of linking microbial function with taxonomic identity (38, 39). The use of DNA-SIP in this study facilitates deep insight into the metabolic response of ammonia oxidizers to DMPP at a functional level, and their relationship to DMPP effectiveness can be further identified.

This study was designed to determine the impact of DMPP on nitrification rates and the abundance, community structure, and metabolic activity of ammonia oxidizers in an acidic pasture soil and an alkaline vegetable soil. Microcosm incubation experiments were established under the same controlled environment with different purposes: (i) a 42-day incubation to understand the effects of DMPP on nitrification activity and the abundances of nitrifying and denitrifying functional assemblages, (ii) a 7-day ^{15}N tracing experiment to investigate the effects of DMPP on the gross rates of simultaneously occurring N transformation processes, and (iii) a 28-day $^{13}CO_2$-DNA–SIP microcosm to explore the impact of DMPP on metabolically active ammonia oxidizers. We hypothesized that (i) DMPP would have contrasting effects on nitrification rates and nitrifier abundance in the acidic pasture soil and the alkaline vegetable soil, (ii) DMPP could inhibit nitrification mainly by targeting the ammonia oxidizers, while having no significant impact on total bacteria and denitrifiers, and (iii) AOA and AOB would exhibit distinctly different responses to DMPP, owing to their different metabolic pathways and ecological niches.

MATERIALS AND METHODS

Site description and soil sampling. Soil samples were collected in August 2014 from two sites: a grazed dairy pasture at Dookie (36°25′S, 145°42′E) and a conventionally managed vegetable farm, growing celery, at Clyde (38°07′S, 145°19′E), Victoria, Australia. Both sites are located in the temperate climate zone, with average annual rainfalls of 551 mm (Dookie) and 819 mm (Clyde) and mean annual temperatures of 20.9°C (Dookie) and 19.4°C (Clyde). The pasture soil at Dookie is classified as silty loam with a pH value of 5.65, while the vegetable soil at Clyde is classified as loamy sand with a pH value of 7.19. At each site, surface soil (0 to 15 cm) was collected, thoroughly mixed, and transported on ice to the laboratory. The soils were then sieved (<2 mm) and stored at 4°C before soil microcosm incubation and physicochemical characterization.

TABLE 1 Basic properties of the two soils at 0 to 15 cm

Property	Pasture soil (Dookie)	Vegetable soil (Clyde)
Location	36°25′S, 145°42′E	38°07′S, 145°19′E
Water content (%)	14.0	12.3
Soil pH (H_2O)	5.65	7.19
Cation-exchange capacity (cmol kg^{-1})	10.2	12.7
Organic matter (%)	5.96	3.11
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 (%)		
Coarse sand (0.2–2 mm)	7	38
Fine sand (0.02–0.2 mm)	52	47
Silt (0.002–0.02 mm)	30	11
Clay (<0.002 mm)	11	4

Soil DNA extraction and physicochemical 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). Soil water content was measured by oven drying subsamples at 105°C for 24 h. Soil pH was measured using a ratio of 1:5 (fresh soil to water) with an Orion Star A211 pH meter (Thermo Scientific Inc., Melbourne, Australia). Soil organic matter was measured using the $K_2Cr_2O_7$ wet oxidation method. Soil NH_4^+-N and NO_3^--N were extracted with 1 M KCl using a ratio of 1:10 (fresh soil to KCl) by shaking at 170 rpm for 1 h, and the filtered solution (Whatman no. 42) was analyzed with a segmented flow analyzer (SAN++; Skalar, Breda, Holland). Total N was measured using the classic Dumas method of combustion on the isotope ratio mass spectrometer (Sercon Hydra, Crewe, United Kingdom). Particle size analysis was done using sieve and hydrometer procedures. Details of the soil physical and chemical properties are shown in Table 1.

Experiment I: soil microcosm incubation. The laboratory soil incubation was carried out in 250-ml plastic vials containing 10 g of soils (oven dry weight equivalent) to investigate the effects of DMPP on the abundances of the key functional genes (*amoA*, *narG*, *nirK*, and *nosZ*) involved in nitrification and denitrification. Three treatments were established with three replicates: (i) control, (ii) NH_4NO_3, and (iii) NH_4NO_3 plus DMPP (1% of applied NH_4^+-N). Nitrogen was applied at rates of 75 mg NH_4^+-N kg^{-1} soil and 75 mg NO_3^--N kg^{-1} soil. The microcosms were incubated at 25°C in the dark for 42 days and maintained at 60% water-filled pore space (WFPS). The vials were aerated every 3 days, and the moisture lost was replenished every 7 days. Destructive samples were collected on days 0, 1, 2, 4, 7, 14, and 42 for determination of mineral N. Samples for molecular analysis were collected on days 2, 7, and 42.

Experiment II: ^{15}N tracing experiment and model. The laboratory ^{15}N tracing experiment was established in 250-ml plastic vials containing 10 g of soils (oven dry weight equivalent) to explore the effects of DMPP on the gross rates of various N transformation processes. Four treatments were applied with three replicates: (i) $^{15}NH_4NO_3$, (ii) $^{15}NH_4NO_3$ plus DMPP (1% of applied NH_4^+-N), (iii) $NH_4{}^{15}NO_3$, and (iv) $NH_4{}^{15}NO_3$ plus DMPP (1% of applied NH_4^+-N). The treatment solutions were evenly sprayed onto the soil surface, providing N at rates of 75 mg

NH_4^+-N kg^{-1} soil and 75 mg NO_3^--N kg^{-1} soil. The ^{15}N-enriched NH_4^+-N and ^{15}N-enriched NO_3^--N were both at 10.0 atom% excess. DMPP was added to the treatment solution at a rate of 1% of the amount of NH_4^+-N. The microcosms were incubated at 25°C in the dark for 14 days and maintained at 60% WFPS. The vials were aerated every 3 days, and the moisture lost was replenished every 7 days. The microcosms were destructively sampled on days 0, 1, 4, and 7 for determination of the concentrations and isotopic signatures of NH_4^+-N and NO_3^--N. The ^{15}N enrichments of the NH_4^+-N and NO_3^--N were determined using the microdiffusion method (40), and the ^{15}N isotopic analyses were carried out on an isotope ratio mass spectrometer (Sercon Hydra, Crewe, United Kingdom). Gas samples for N_2O measurement were collected every 7 h on days 1, 2, 4, 7, 10, and 14 from each vial using a gas-tight syringe and were injected into 12-ml preevacuated exetainers. N_2O analysis was performed on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies Inc., USA) using an electron capture detector (ECD). The N_2O fluxes were calculated based on the linear regression of N_2O concentrations versus time.

The ^{15}N tracing model Ntrace was used to quantify the gross rates of 10 N transformation processes in soils, as shown in Fig. S1 in the supplemental material and previously described (41). The model was used for the first 7 days because thereafter the NH_4^+-N concentrations were too low for the detection of the ^{15}N enrichments. The model included six soil N pools: NH_4^+-N, NO_3^--N, labile organic N (N_{lab}), recalcitrant organic N (N_{rec}), NH_4^+-N fixed to soil particles ($NH4_{ads}$), and stored NO_3^--N ($NO_3^-{}_{sto}$). The concentrations of NH_4^+-N and NO_3^--N and their ^{15}N enrichments (averages ± standard deviations) were used as the input data set for the model. Ten N transformation processes were considered: mineralization of labile N_{lab} to NH_4^+-N (M_{Nlab}), mineralization of N_{rec} to NH_4^+-N (M_{Nrec}), immobilization of NH_4^+ to N_{lab} (I_{NH4_Nlab}), immobilization of NH_4^+ to N_{rec} ($I_{NH4\text{-}Nrec}$), oxidation of N_{rec} to NO_3^--N (O_{Nrec}), oxidation of ammonia to NO_3^--N (O_{NH4}), adsorption of NH_4^+ on cation-exchange sites (A_{NH4}), release of adsorbed NH_4^+ (R_{NH4ads}), immobilization of NO_3^- to N_{rec} (I_{NO3}), and dissimilatory NO_3^- reduction to NH_4^+ (D_{NO3}). Compared with the original model (42), the new model is more efficient in quantifying the cooccurring N transformation rates in soil, based on the Metropolis algorithm, a Markov chain Monte Carlo method (43). The optimization tool allows the calculation of N transformation rates based on zero-order, first-order, or Michaelis-Menten kinetics, enabling more realistic simulation of N dynamics. The new model calculates gross N transformation rates by simultaneously optimizing the kinetic parameters through minimizing the misfit between simulated and observed values. For more detailed information about the ^{15}N tracing model, see references 43 and 42.

Experiment III: DNA-SIP microcosm and SIP gradient fractionation. A DNA-SIP microcosm was established to identify the metabolically active autotrophic ammonia oxidizers in both soils. Briefly, three treatments were applied to soil with three replicates in 160-ml serum bottles containing 10 g soil (oven dry weight equivalent): (i) NH_4NO_3 plus 5% (vol/vol) $^{12}CO_2$, (ii) NH_4NO_3 plus 5% (vol/vol) $^{13}CO_2$ (99 atom%; Sigma-Aldrich Co. St. Louis, MO, USA), and (iii) NH_4NO_3 plus 5% (vol/vol) $^{13}CO_2$ plus DMPP (1% of applied NH_4^+-N). The pairwise comparison between treatments i and ii aimed to identify the active autotrophic ammonia oxidizers, and the comparison between treatments ii and iii was used to investigate the inhibitory effect of DMPP on the metabolic activity of autotrophic ammonia oxidizers. The treatment solutions were evenly sprayed onto the soil to provide rates 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 taken from each treatment on days 0, 14, and 28 for molecular analysis and determination of NH_4^+-N and NO_3^--N.

The DNA-SIP gradient fractionation followed the method described previously (39). Briefly, the density gradient centrifugation was performed in 4.9-ml OptiSeal polypropylene seal tubes (Beckman Coulter, Palo Alto, CA, USA) in a VTi90 vertical rotor (Beckman Coulter), subjected to isopycnic centrifugation at 56,200 rpm (228,166 × g_{av}) at 20°C for 24 h. The extracted DNA (1 μg) was loaded into CsCl gradient buffer with an initial density of 1.696 g ml^{-1} in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The gradient buffer was fractionated after centrifugation into 20 equal fractions by displacing the CsCl solution in the tubes with sterilized water using a fraction recovery system (Beckman Coulter) and a syringe pump (New Era Pump Systems, USA). The buoyant density of each fraction was determined by measuring the refractive index of 30-μl aliquots using a hand-held refractometer (Uni-It, Bellingham, WA, USA). DNA was subsequently precipitated from the CsCl gradients by adding two volumes of 30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl, washed twice with ice-cold 70% ethanol, and dissolved in 20 μl of sterilized water. The precipitated DNA was further checked by quantitative PCR (qPCR) analysis to quantify the relative abundance of *amoA* genes in individual fractions.

T-RFLP analysis. Changes in the community structure of AOA and AOB during the 42-day incubation (experiment I) were characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis of the *amoA* genes using the fluorescently labeled primers FAM-CrenamoA23f/CrenamoA616r (44) and FAM-amoA-1F/amoA-2R (45), respectively. The 50-μl PCR mixture contained 2 μl of diluted template DNA (1 to 10 ng), 0.5 μl of each primer (20 μM), 5 μl of 10× NH_4 reaction buffer, 2 μl of $MgCl_2$ solution (50 mM), 2 μl of bovine serum albumin (BSA) (20 mM), 1 μl of deoxynucleoside triphosphate (dNTP) mix (20 mM), and 2.5 units of BioTaq DNA polymerase (Bioline, Sydney, Australia). Thermal cycling conditions were as follows: 95°C for 5 min; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and 10 min at 72°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 200 ng of purified PCR products, 0.1 μl of BSA, 1 μl of 10× NEBuffer, and 5 units of the restriction enzymes RsaI for AOA or MspI for AOB. Digests were incubated at 37°C for 3 h and then denatured at 95°C for 10 min. Terminal restriction fragments (TRFs) were size separated with an ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using a local Southern size calling method and a peak amplitude threshold setting of 50, using Genemapper version 4.0 (Applied Biosystems). TRFs with peak heights comprising less than 2% 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. The relative fluorescence abundances of all TRFs were exported for further analysis.

qPCR analysis. Abundances of the key nitrifying and denitrifying 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 shown in Table S1 in the supplemental material. 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 10-fold serial dilutions of plasmids containing correct inserts of the target 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, and the amplification efficiencies for all qPCR runs ranged between 95 and 105%. We tried to amplify the *nirS* gene from the two examined soils with the universal primers reported previously (46, 47) but failed to obtain any positive amplicons.

Cloning library, sequencing, and phylogenetic analysis. To understand the community composition of the metabolically active ammonia oxidizers, the AOA and AOB *amoA* genes were amplified from the "heavy" fractions of the $^{13}CO_2$ treatment on day 28. The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega), ligated into the pGEM-T Easy vector, and then transformed into *Escherichia coli* JM109 competent cells as per the manufacturer's instructions. Sequencing of clones containing the correct insert was performed by the

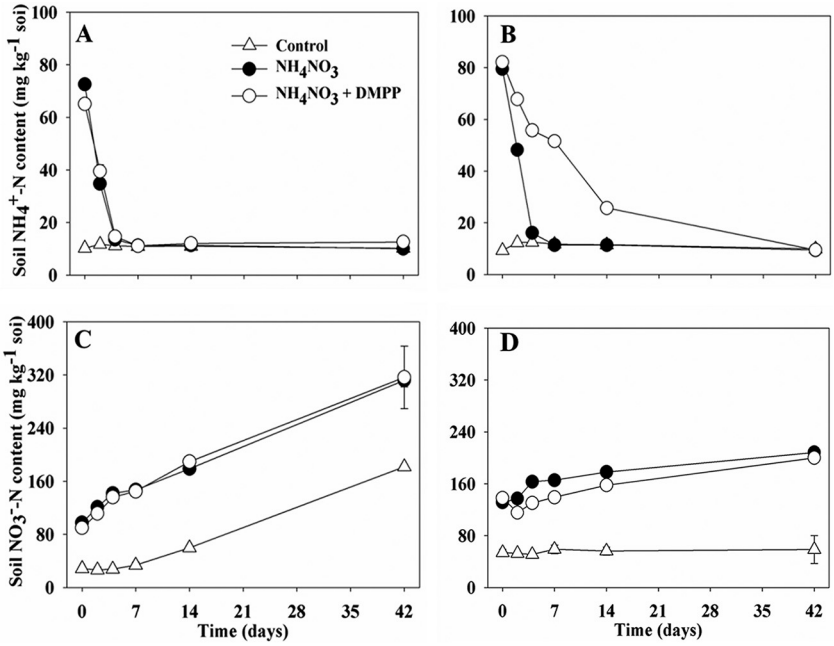

FIG 1 Changes in the NH_4^+-N and NO_3^--N concentrations in the 42-day microcosm incubation of the pasture soil (A and C) and vegetable soil (B and D). Error bars represent standard errors from three replicates.

Macrogen Sequencing Department, South Korea. The obtained sequences were checked for chimeras and then aligned with the reference sequences using MUSCLE implemented in MEGA 6.0. Phylogenetic analysis was performed through constructing a neighbor-joining tree using the Kimura 2-parameter distance with 1,000 replicates to produce Bootstrap values.

Statistical analysis. One-way analysis of variance (ANOVA) based on the Duncan test was conducted for statistical analysis with SPSS 17.0 (IBM, USA). Spearman's correlation analysis was performed to test the relationships between the NO_3^--N concentrations and AOA or AOB abundances. Differences at a P value of <0.05 were considered to be statistically significant. Nonmetric multidimensional scaling (NMDS) was used to visualize the Bray-Curtis dissimilarity matrices based on the T-RFLP data. A permutational multivariate analysis of variance (PERMANOVA) test with 999 permutations was performed to examine whether the community structures were statistically different between treatments by using the adonis function of the Vegan package in the R platform.

Accession number(s). The sequences retrieved in this study have been deposited in GenBank under accession numbers from KT861604 to KT861610 for AOB and KT861611 to KT861615 for AOA.

RESULTS

Experiment I: soil microcosm incubation. During the 42-day microcosm incubation, the concentrations of NH_4^+-N remained largely unchanged in the control treatments of both soils (Fig. 1A and B). In the NH_4NO_3 treatment of the pasture soil, the NH_4^+-N concentrations decreased rapidly from 72.5 ($\pm$1.93) mg kg^{-1} soil to the control treatment level of 11.4 ($\pm$0.27) mg kg^{-1} soil by day 7, and there was no evidence of inhibition by DMPP (Fig. 1A). Similarly the NH_4^+-N concentrations in the vegetable soil decreased to the control level by day 7, but addition of DMPP significantly slowed down the reduction of NH_4^+-N levels, and the NH_4^+-N concentrations gradually reached the control level on day 42 in the NH_4NO_3-plus-DMPP treatment (Fig. 1B).

The NO_3^--N concentrations showed a growing trend in all treatments of the pasture soil (Fig. 1C). The average net nitrification rate was 3.7 ($\pm$0.03) mg NO_3^--N kg^{-1} soil day^{-1} in the control treatment during the 42 days of incubation. The NH_4NO_3 and NH_4NO_3-plus-DMPP treatments had higher average net nitrification rates over 42 days, at 5.1 ($\pm$0.66) and 5.4 ($\pm$0.32) mg NO_3^--N kg^{-1} soil day^{-1}, respectively. In contrast, the NO_3^--N concentrations remained unchanged in the control treatment of the vegetable soil and were significantly ($P < 0.05$) higher in the NH_4NO_3 and NH_4NO_3-plus-DMPP treatments (Fig. 1D). DMPP addition significantly ($P < 0.05$) decreased the formation of NO_3^--N at all the sampling points except days 0 and 42 compared to the NH_4NO_3 treatment (Fig. 1D). The average net nitrification rates over 42 days in the vegetable soil were 1.8 ($\pm$0.11) (NH_4NO_3) and 1.4 ($\pm$0.17) (NH_4NO_3 plus DMPP) mg NO_3^--N kg^{-1} soil day^{-1}. Overall, DMPP was capable of inhibiting the conversion from NH_4^+-N to NO_3^--N in the vegetable soil but not in the pasture soil.

The changes in the abundances of the key nitrifying and denitrifying genes were quantified using qPCR analysis (see Fig. S2 and S3 in the supplemental material). For the pasture soil, compared with the control treatment, no significant effect of fertilizer addition on AOA or AOB *amoA* gene abundances was observed (see Fig. S2 in the supplemental material). DMPP application significantly ($P < 0.05$) decreased AOA abundance by 44.9% on day 2 and reduced AOB abundance by 50.7% relative to that with the fertilizer-only treatment on day 42 (see Fig. S2A and B in the supplemental material). For the vegetable soil, the AOA abundance significantly ($P < 0.05$) increased from day 7 to day 42 in the presence of fertilizer, but no significant effect of DMPP could be observed (see Fig. S3A in the supplemental material). The AOB abundance was remarkably higher in the fertilizer-only treatment, ranging from 1.6×10^8 to 2.0×10^8 copies g^{-1} soil, than in the control treatment (ranging from 7.1×10^7 to 1.4×10^8 copies g^{-1}

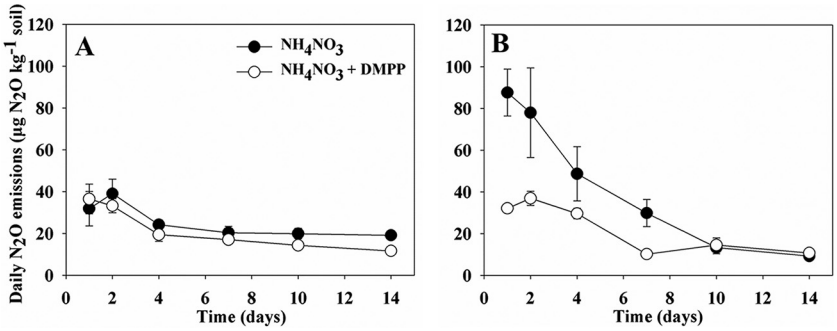

FIG 2 Daily N_2O emissions during the 14-day ^{15}N tracing microcosms in the pasture soil (A) and vegetable soil (B). Error bars represent standard errors from three replicates.

soil), and DMPP addition significantly ($P < 0.05$) decreased AOB abundance by 41.6% relative to that in the fertilizer treatment on day 7 (see Fig. S3B in the supplemental material). For the denitrifying genes *narG*, *nirK*, and *nosZ* and the bacterial 16S rRNA gene in both soils, there was no significant difference in the abundance between treatments, indicating that DMPP addition had no direct influence on these genes (see Fig. S2 and S3 in the supplemental material).

The T-RFLP analysis of the AOA community digested by RsaI yielded two and seven distinct TRFs across all the treatments on day 42, with TRF-201 and TRF-56 as the major genotypes in the pasture and vegetable soils, respectively (see Fig. S4C and D in the supplemental material). For the T-RFLP pattern of the AOB community digested by MspI, TRF-55 and TRF-255 were the most dominant genotypes across treatments in both soils (see Fig. S4A and B in the supplemental material). The NMDS ordinations based on the Bray-Curtis dissimilarity matrices and PerMANOVA analysis suggested that there was no significant difference in the community structure of AOA or AOB regardless of DMPP addition (see Fig. S5 in the supplemental material).

Experiment II: ^{15}N tracing experiment. The daily N_2O emissions from the pasture soil ranged from 19.1 to 39.1 μg N_2O kg^{-1} soil day^{-1} and were significantly ($P < 0.05$) lower than those from the vegetable soil, which ranged from 9.2 to 87.6 μg N_2O kg^{-1} soil day^{-1} (Fig. 2). The cumulative N_2O emission was 329.3 (±14.4) μg N_2O kg^{-1} soil in the pasture soil, compared to 485.4 (±65.2) μg N_2O kg^{-1} soil in the vegetable soil, during the first 2 weeks of incubation. DMPP addition substantially reduced the cumulative N_2O emissions to 265.3 (±14.7) and 270.1 (±5.2) μg N_2O kg^{-1} soil in the pasture soil and vegetable soil, respectively, but the inhibitory effect was significant only in the vegetable soil.

The ^{15}N recovery was in line with changes of the NH_4^+-N and NO_3^--N contents in experiment I (Fig. 3). In the $^{15}NH_4^+$-labeled treatments of the pasture soil, the ^{15}N signature in the NH_4^+ pool declined significantly and the ^{15}N enrichment in the NO_3^- pool increased significantly during the incubation, regardless of DMPP application (Fig. 3A and C). Similarly, the $^{15}NH_4^+$ enrichment decreased sharply in the fertilized treatments in the vegetable soil, but the decline was significantly ($P < 0.05$) delayed by the addition of DMPP (Fig. 3B). Meanwhile, the $^{15}NO_3^-$ enrichment was

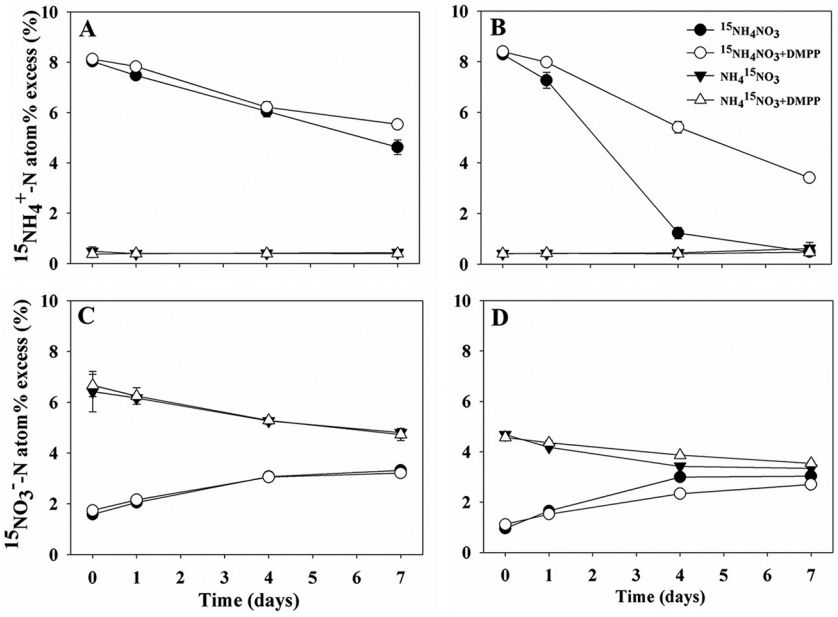

FIG 3 ^{15}N enrichments of NH_4^+-N and NO_3^--N concentrations in the first week of the ^{15}N tracing microcosms of the pasture soil (A and C) and vegetable soil (B and D). Error bars indicate standard deviations from three replicates.

TABLE 2 Gross N transformation rates based on the ^{15}N tracing model

Parameter	Pasture soil			Vegetable soil		
		Rate, mg kg^{-1} day^{-1} (mean ± SD)			Rate, mg kg^{-1} day^{-1} (mean ± SD)	
	Kinetics[a]	Without DMPP	With DMPP	Kinetics	Without DMPP	With DMPP
M_{Nlab}	1	0.82 ± 0.15	0.61 ± 0.37	1	0.85 ± 0.29	1.33 ± 0.31
M_{Nrec}	0	4.47 ± 0.41	3.82 ± 0.43	0	6.69 ± 0.43	5.38 ± 0.31
$I_{NH4\text{-}Nlab}$	1	0.07 ± 0.03	0.07 ± 0.05	1	0.10 ± 0.03	0.39 ± 0.18
$I_{NH4\text{-}Nrec}$	1	0.16 ± 0.08	0.33 ± 0.20	1	0.09 ± 0.03	0.28 ± 0.08
O_{Nrec}	0	0.06 ± 0.04	0.18 ± 0.04	0	0.06 ± 0.01	0.09 ± 0.04
O_{NH4}	1	5.75 ± 0.21	6.36 ± 0.13	2	15.71 ± 0.70	12.24 ± 0.52
A_{NH4}	1	8.27 ± 0.85	6.79 ± 0.61	1	2.18 ± 0.16	2.85 ± 0.30
R_{NH4a}	1	0.96 ± 0.59	2.27 ± 0.50	1	0.00 ± 0.00	0.01 ± 0.00
I_{NO3}	1	0.12 ± 0.10	0.04 ± 0.03	1	0.01 ± 0.00	0.02 ± 0.01
D_{NO3}	1	0.02 ± 0.01	0.06 ± 0.01	1	0.02 ± 0.00	0.01 ± 0.00

[a] 0, zero order; 1, first order; 2, Michaelis-Menten.

significantly ($P < 0.05$) lower in the treatment with DMPP after day 1 (Fig. 3D). In the $^{15}NO_3^-$-labeled treatments of both soils, there was no significant $^{15}NH_4^+$ enrichment in treatments with or without DMPP addition (Fig. 3A and B), indicating that DMPP had no effect on the process of dissimilatory nitrate reduction to ammonium. DMPP addition significantly ($P < 0.05$) delayed the decline of $^{15}NO_3^-$ enrichment levels in the vegetable soil (Fig. 3D), but no such inhibitory effect was observed in the pasture soil (Fig. 3C).

Gross N transformation rates were calculated based on the ^{15}N tracing model to investigate the effects of DMPP on various N transformation processes during the first week of incubation (Table 2). The main gross N transformation processes occurring in both soils were oxidation of NH_4^+-N to NO_3^--N (O_{NH4}), mineralization of N_{rec} to NH_4^+-N (M_{Nrec}), and adsorption of NH_4^+ on cation-exchange sites (A_{NH4}). The modeled data generally fit the observed data well (see Fig. S6 in the supplemental material), and r^2 values were ≥0.97. Compared with O_{NH4} (autotrophic nitrification), oxidation of N_{rec} to NO_3^- (O_{Nrec}; heterotrophic nitrification) was a minor proportion, indicating the dominant role of autotrophic nitrification in total nitrification in both soils. DMPP decreased the gross autotrophic nitrification rate by 22% in the vegetable soil, but no such inhibitory effect of DMPP was observed in the pasture soil. For both soils, M_{Nrec} and total gross rates of mineralization ($M_{Nrec} + M_{Nlab}$) tended to be higher as a result of DMPP addition. At the same time, the total immobilization of NH_4^+ ($I_{NH4\text{-}Nlab} + I_{NH4\text{-}Nrec}$) substantially decreased when DMPP was added. Additionally, DMPP decreased the initial adsorption of NH_4^+ to the fixed pool (A_{NH4}) by only 18%, while it increased the release from fixed NH_4^+ pool (R_{NH4}) by 136% in the pasture soil, indicating the replacement of NH_4^+ from fixed cation-exchange sites by other molecules in the pasture soil.

Experiment III: $^{13}CO_2$-DNA–SIP microcosm and ^{13}C-labeled functionally active ammonia oxidizers. In the 28-day DNA-SIP incubation, fertilization had a significant and positive effect on AOB abundance in the vegetable soil, and DMPP addition significantly decreased the abundances of AOB in both soils on day 28 (Fig. 4A), whereas no significant inhibitory effect of DMPP on AOA abundance could be observed in either soil (Fig. 4B).

Isopycnic gradient centrifugation was carried out with genomic DNA extracted from each microcosm to identify the metabolically active nitrifiers incorporating $^{13}CO_2$ during the DNA-SIP incubation. AOB abundance in the $^{12}CO_2$ microcosms for all the sampling time points peaked only in the light fractions around a buoyant density of 1.72 to 1.73 g ml^{-1} in both soils (Fig. 5). After 28 days of incubation, the majority of the AOB community was detected in the "heavy" SIP fractions with a buoyant density of 1.73 to 1.76 g ml^{-1} in the $^{13}CO_2$ microcosms of both soils (Fig. 5E and F), indicating assimilation of $^{13}CO_2$ into the genomic DNA of AOB. A clear shift of the $^{13}CO_2$ peak toward the light fractions was observed in the DMPP treatment in the vegetable soil on day 28 (Fig. 5F), but the inhibitory effect of DMPP on the assimilation of $^{13}CO_2$ into AOB *amoA* gene was very weak in the pasture soil (Fig. 5E). Compared with the $^{12}CO_2$ treatment, the peak of AOA community clearly shifted toward the heavy SIP fractions after 28-day incubation in the presence of $^{13}CO_2$, but DMPP had no obvious

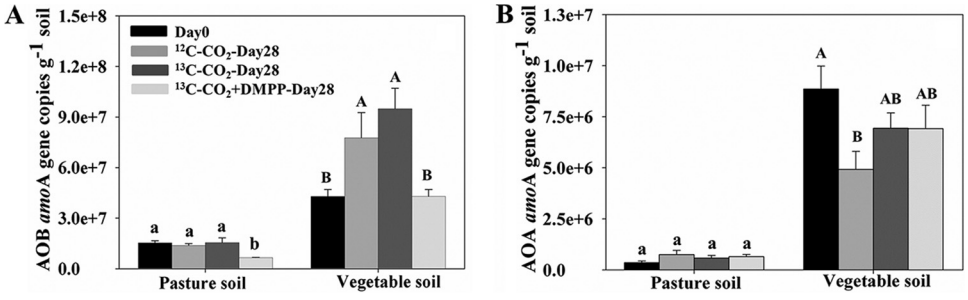

FIG 4 Changes in the AOB (A) and AOA (B) *amoA* gene copies across treatments (day 0 and $^{12}CO_2$, $^{13}CO_2$, and $^{13}CO_2$ plus DMPP on day 28) in the DNA-SIP microcosms of the pasture soil and vegetable soil. Error bars represent standard errors from three replicates. Different letters above the bars indicate a significant difference ($P < 0.05$).

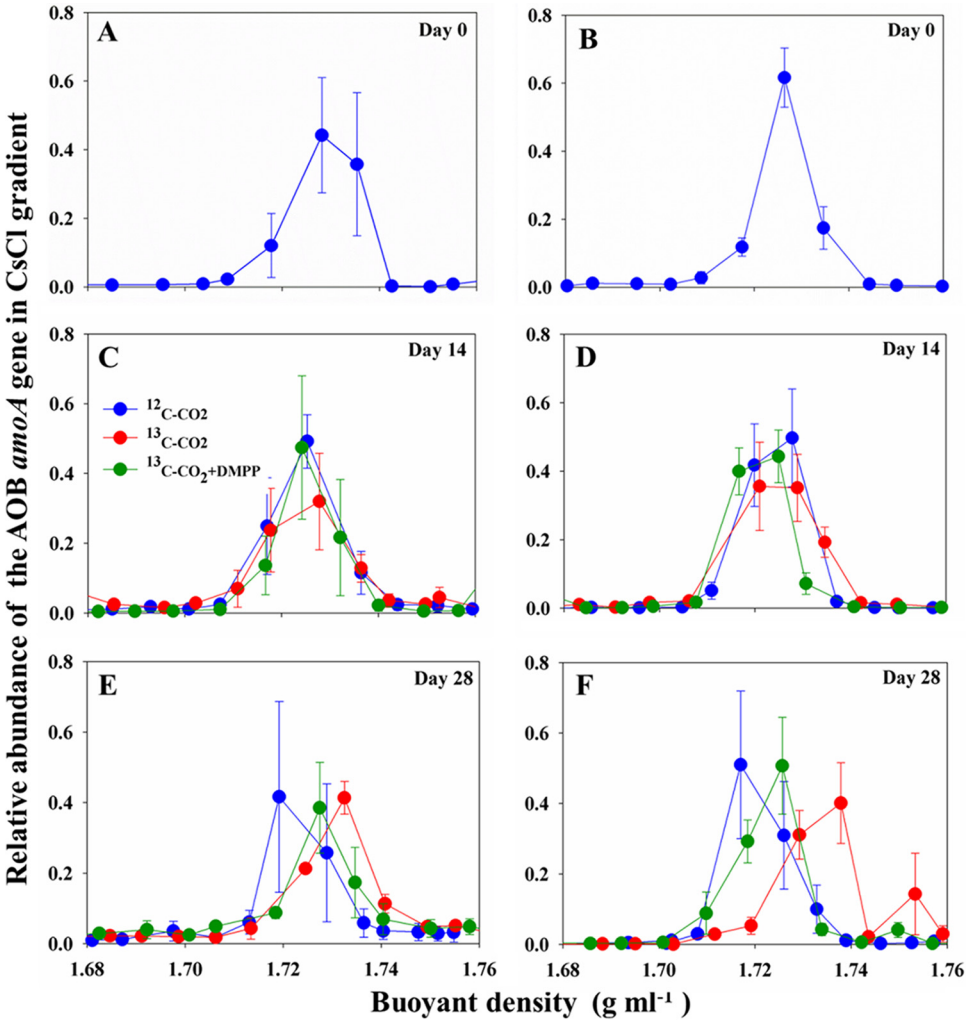

FIG 5 Quantitative distribution of the relative abundances of the AOB *amoA* genes retrieved from different treatments ($^{12}CO_2$, $^{13}CO_2$, and $^{13}CO_2$ plus DMPP) in the 28-day DNA-SIP microcosms of the pasture soil (A, C, and E) and vegetable soil (B, D, and F). Error bars represent standard errors from three replicates.

inhibitory effect on the metabolic activity of AOA in either soil (Fig. 6).

Given that DMPP addition had no significant effect on the metabolically active AOA or AOB community in the pasture soil, phylogenetic analysis of the *amoA* gene was conducted only from the heavy SIP regions of the $^{13}CO_2$ microcosms in the vegetable soil on day 28. The results showed that the active AOB community (based on 25 positive clones) belonged to the *Nitrosospira* lineage (Fig. 7), with 67% of ^{13}C-labeled AOB affiliated within an unclassified cluster (cluster X) and 17% within cluster 9. In terms of the active AOA community (based on 20 positive clones), the *Nitrosopumilus* lineage accounted for 84% of the obtained AOA *amoA* gene sequences and the *Nitrososphaera* lineage for 16% (Fig. 8).

DISCUSSION

Contrasting effects of DMPP on nitrification in the acidic pasture and alkaline vegetable soils. In the vegetable soil, the decrease in NH_4^+-N content was accompanied by the increase in NO_3^--N content in the NH_4NO_3 treatment, suggesting the occurrence of nitrification during the incubation. The inhibitory effect of DMPP on the conversion from NH_4^+-N to NO_3^--N in the vegetable soil was observed until the end of incubation (Fig. 1B and D). In contrast, in the pasture soil, the added NH_4^+-N was rapidly nitrified in the first week and then remained almost unchanged, while NO_3^--N concentrations maintained a substantial increase (Fig. 1A and C). As revealed by the ^{15}N tracing model, heterotrophic nitrification played a minor role compared with autotrophic nitrification in the acidic pasture soil (Table 2); therefore, the observed large increase in NO_3^--N concentrations could have originated from the oxidation of NH_3 derived from the mineralization of native organic N, which was much higher in the pasture soil than in the vegetable soil (Table 1).

The inhibitory effects of DMPP on ammonia oxidation were much less in the acidic pasture soil than in the alkaline vegetable soil. Previous studies also suggested that DMPP had no significant effect on NH_4^+ retention in a pasture soil (26, 41). One possible explanation is that DMPP, as a type of heterocyclic compound, can be adsorbed onto soil particles with high levels of clay, silt, and organic matter (48), thereby leading to low DMPP availability in soils (30, 49). Pasture soils are generally characterized by high levels of organic matter as a consequence of a large amount of

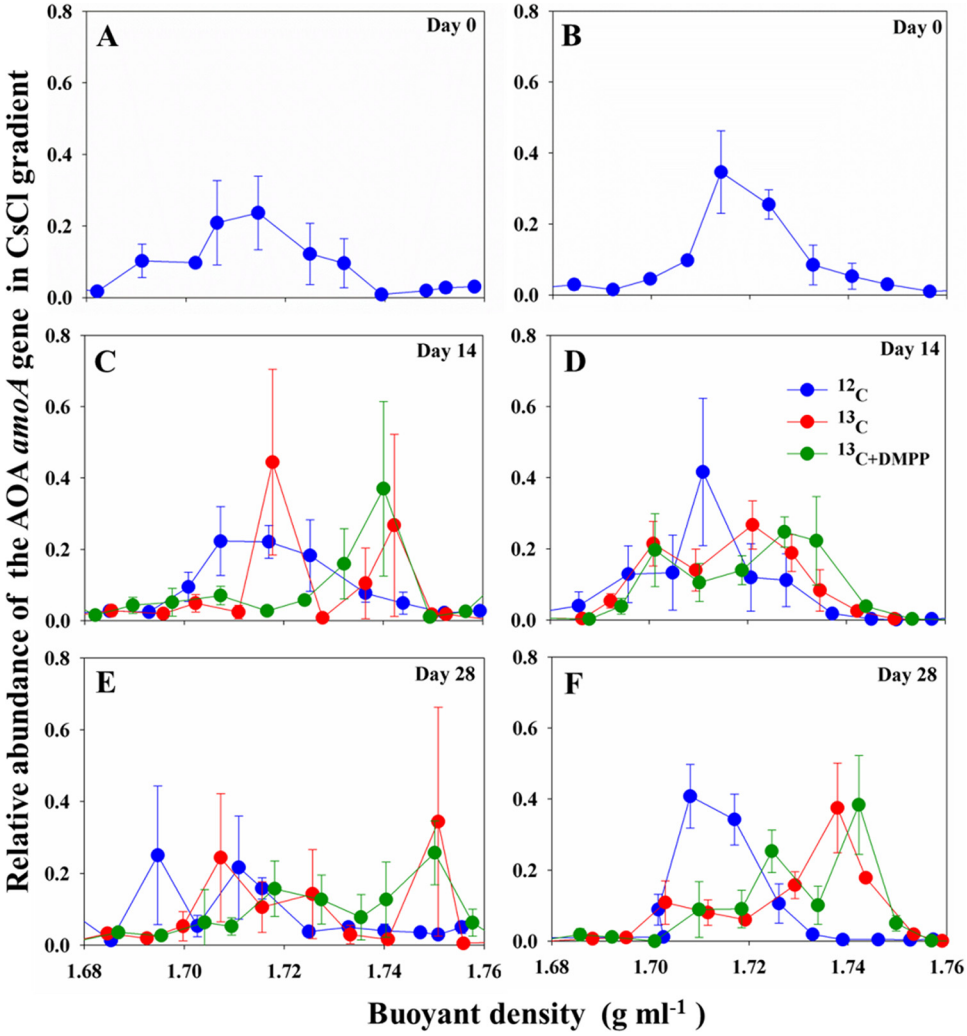

FIG 6 Quantitative distribution of the relative abundances of the AOA *amoA* genes retrieved from different treatments ($^{12}CO_2$, $^{13}CO_2$, and $^{13}CO_2$ plus DMPP) in the 28-day DNA-SIP microcosms of the pasture soil (A, C, and E) and vegetable soil (B, D, and F). Error bars represent standard errors from three replicates.

plant debris incorporation (25). In this study, levels of the organic matter, clay, and silt components were much higher in the pasture soil than in the vegetable soil (Table 1). The poor inhibitory effect of DMPP in the pasture soil with high clay and silt contents is also supported by the previous findings that the inhibitory effect of DMPP was lower in soils with high proportions of clay and silt (30, 32, 33). Similar to the case for DMPP, the adsorption of other nitrification inhibitors, such as DCD and nitrapyrin, to soil organic matter has been recognized as an important factor influencing the inhibition efficacy (50, 51). Meanwhile, our study indicated that DMPP markedly decreased the initial adsorption of NH_4^+ to the fixed pool but increased the release of NH_4^+ to the fixed pool in the pasture soil (Table 2). It could be inferred that the adsorption of DMPP to cation-exchange sites resulted in the low DMPP availability for inhibiting ammonia oxidation and reducing N_2O emissions in the pasture soil.

As ammonia (NH_3) rather than NH_4^+ serves as the direct substrate for ammonia oxidizers, recent studies have shown that AOA thrive better in acidic soils with low availability of NH_3 derived from mineralization of organic N (52, 53), while AOB prefer high and inorganic NH_4^+ inputs in alkaline soils (54). The difference in substrate preferences could affect the differentiation of AOA and AOB in soils and further affects the efficacy of DMPP across different soils. In this study, the microcosm incubation was performed with a high level of NH_4^+-N, which might favor the growth of AOB rather than the oligotrophic AOA. 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 $NH_4^+ \leftrightarrows NH_3 + H^+$ ($pK_a = 9.25$), NH_3 concentrations above 1 μM (1.93 μM) in the pasture soil were available only on the first day of incubation and then declined sharply to 339 nM on day 14. Therefore, the growth of AOB may be retarded by the lower availability of NH_3 in the pasture soil. In contrast, the NH_3 concentrations were calculated to be between 7.58 and 112 μM in the vegetable soil, which is markedly higher than the threshold concentration required by cultivated AOB (above 1 μM near neutral pH [55]). In our study, the AOB abundance remained 1 to 2 orders of magnitude lower in the pasture soil than in the vegetable soil (Fig. 4). Considering that AOB are the target of DMPP inhi-

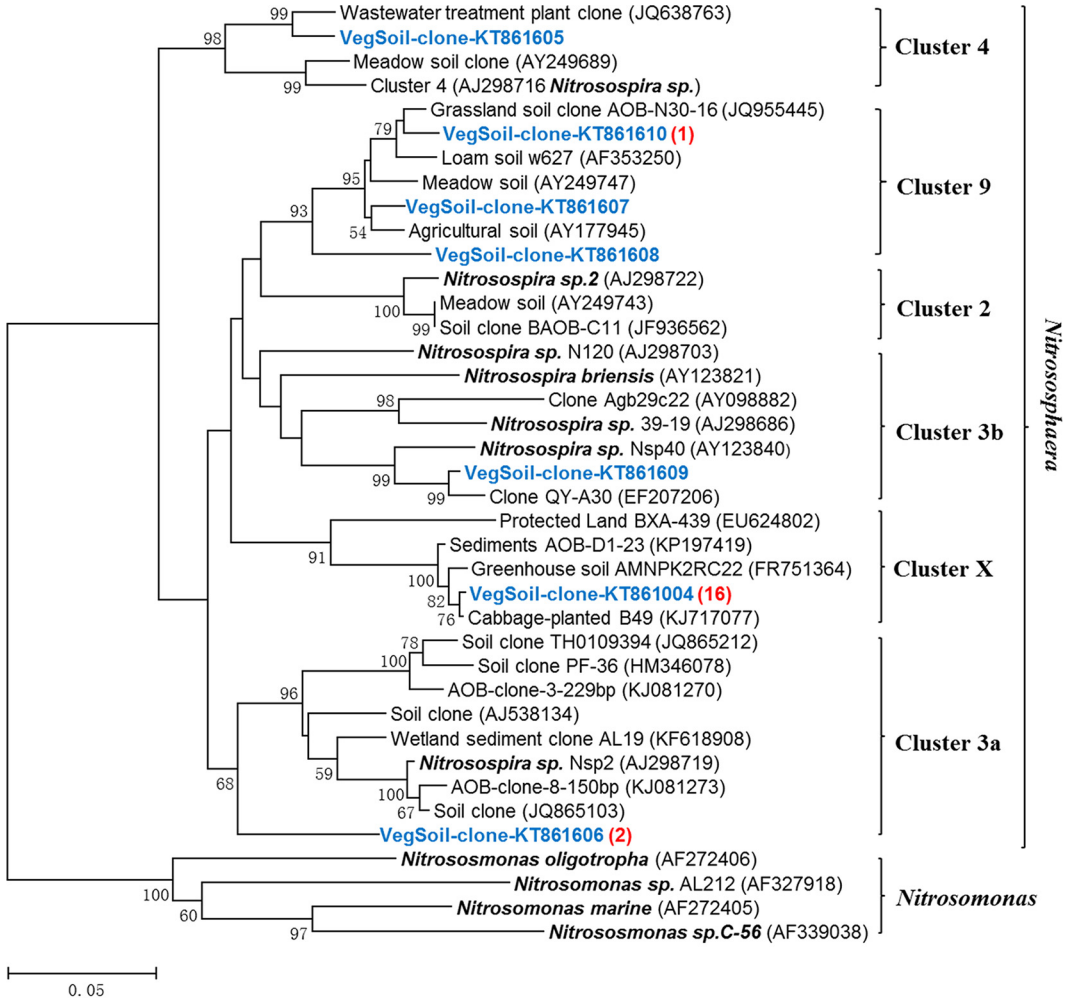

FIG 7 Phylogenetic analysis of the AOB *amoA* gene retrieved from the DNA-SIP heavy fractions in the vegetable soil. The sequences identified in this study are highlighted in blue, and the red numbers represents the numbers of sequences with >98% sequence identity. Numbers at the nodes indicate the levels of bootstrap support based on the neighbor-joining analysis of 1,000 resampled data sets. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (>50%) are indicated at branch points.

bition as suggested by the DNA-SIP result, the intrinsically low population size of AOB might contribute to the ineffectiveness of DMPP in the acidic pasture soil. On the other hand, despite the lower abundance, AOA could be the dominant nitrifiers mediating nitrification in the pasture soil. It should be noted that the numerical advantage does not necessarily mean functional dominance. For example, AOB were demonstrated as the main active nitrifiers responsible for soil nitrification, although AOA outweighed AOB in an agricultural soil (56). The inability of DMPP to inhibit AOA, as demonstrated by the result of DNA-SIP (Fig. 6) further explains the ineffectiveness of DMPP in the pasture soil.

Effects of DMPP on the abundance, community composition, and metabolic activity of ammonia oxidizers. Our study found that the abundance of AOB and not that of AOA was significantly decreased after DMPP application, which is in line with previous findings (57, 58). DMPP had no significant effect on the community structure of ammonia oxidizers in this study, as also found in previous research in a field experiment (34) and a pot experiment (58). Phylogenetic analyses of the AOB *amoA* genes retrieved from the $^{13}CO_2$-labeled heavy fractions in DNA-SIP microcosms revealed that the active AOB community was dominated by *Nitrosospira*, which has been suggested to play a predominant role in soils in AOB-based nitrification (56, 58). The major active AOA community responsible for ammonia oxidation in the vegetable soil belonged to *Nitrosopumilus*, consistent with previous findings that the growth of *Nitrosopumilus* was associated with autotrophic nitrification in soil microcosms (39, 59). *Nitrososphaera* accounted for a minority of the active AOA community in this study, as previous studies reported that the *Nitrososphaera* has the capacity to participate in autotrophic ammonia oxidation (60).

While the impact of DMPP addition on the abundance and community structure of ammonia oxidizers has been investigated recently (15, 37), to our knowledge, our study shows for the first time that DMPP can strongly influence the metabolic activity of soil ammonia oxidizers. The DNA-SIP incubation revealed that DMPP effectively decreased the relative abundance of AOB in the heavy fractions of CsCl gradients, indicating the inhibitory effect of DMPP on ammonia oxidation carried out by AOB. This finding is well supported by observations that the growth of AOB is inhibited by the nitrification inhibitors acetylene and DCD in soil mi-

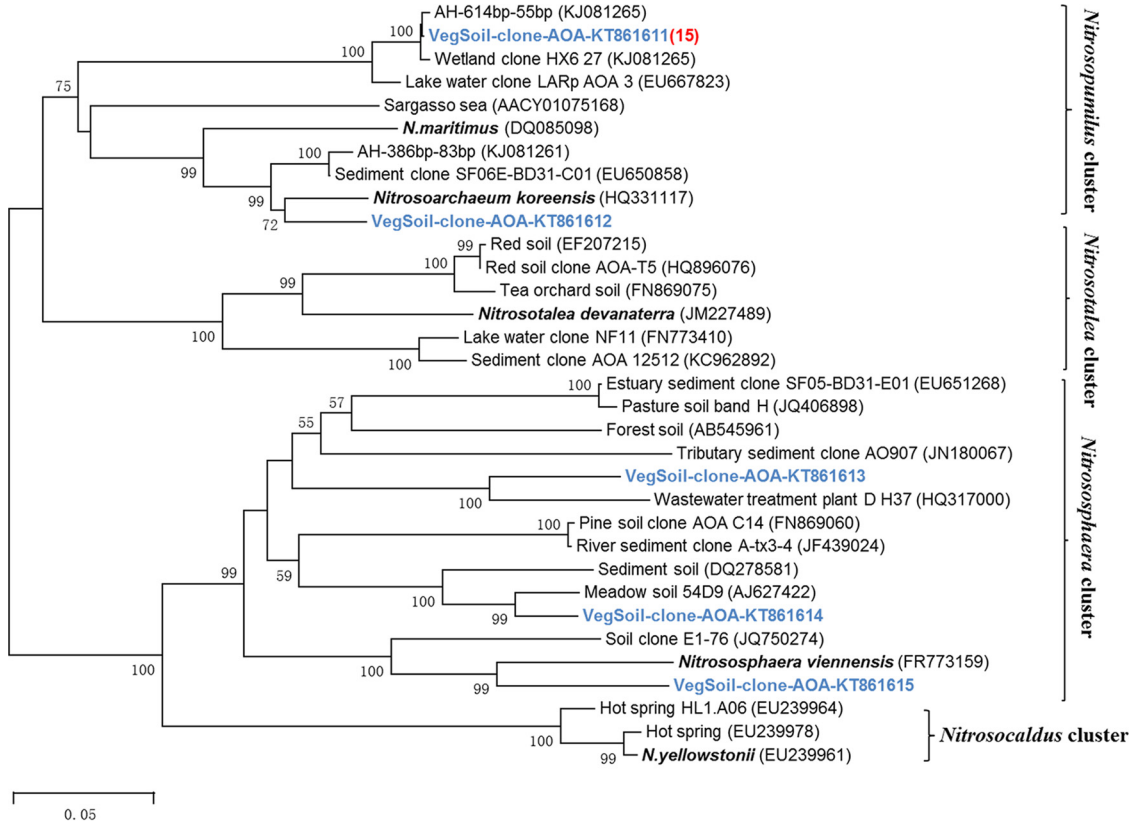

FIG 8 Phylogenetic analysis of the AOA *amoA* gene sequences retrieved from the genomic DNA in the vegetable soil. The sequences identified in this study are highlighted in blue, and the red number represents the numbers of the sequences with >98% sequence identity. Numbers at the nodes indicate the levels of bootstrap support based on the neighbor-joining analysis of 1,000 resampled data sets. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (>50%) are indicated at branch points.

crocosms where AOB dominate the nitrification activity (22, 61). However, DMPP showed no evident inhibitory effect on AOA as indicated by the DNA-SIP results, reinforcing the view that different metabolic pathways for ammonia oxidation exist in AOA (62, 63).

Effects of DMPP on other N transformation processes. It is of fundamental importance to evaluate the effects of DMPP on various N transformation processes for the purpose of a wide application of DMPP in agriculture systems to reduce N losses. Gross transformation rates based on the ^{15}N tracing model showed that DMPP increased the gross immobilization rates of NH_4^+, which is in line with previous studies of DCD (64, 65). This is probably due to the higher concentrations of NH_4^+ facilitated by DMPP application and further favored by the preference of soil microorganisms for NH_4^+-N assimilation relative to that of NO_3^--N (66). At the same time, gross mineralization rates of NH_4^+ decreased in the presence of DMPP in both soils. The most likely cause for the decreased mineralization rate is the feedback regulation when NH_4^+ is already at high concentrations after amendment of DMPP (67).

The application of nitrification inhibitors with N fertilizers has been shown to have the potential to reduce denitrification-derived N_2O emissions (68, 69). We showed that DMPP addition substantially decreased the cumulative N_2O emissions in the first 2 weeks after application by 19.4% and 44.4% in the pasture soil and vegetable soils, respectively, but it is still largely unknown whether the reduction is associated with the inhibitory effects on the denitrifying functional guilds. In our study, the abundances of the *nar*G and *nirK* genes showed no significant changes in response to DMPP application. Earlier assays of denitrifying enzyme activity showed that DMPP had no impact on the nitrate reductase (NaR) (70). However, decreases in the NaR and nitrite reductase activities were found when DMPP was applied together with urea in a 2-year field experiment carried out in a rice and rape rotation system (71). The *nirS* gene abundance significantly decreased on day 49 after DMPP was applied with urea in a soybean-corn rotation system (57). For the *nosZ* gene, there was no DMPP-caused significant change in the abundance in this study. In contrast, a previous study revealed that adding DMPP to cattle effluent significantly decreased the *nosZ* gene at both genomic and mRNA levels compared with cattle that in effluent 1 week after application (36). As suggested by another study, nitrate addition reduced the *nosZ* gene abundance and thus enhanced denitrification-derived N_2O emissions (72). The contrasting results could be explained by the different forms of N fertilizer provided and environmental conditions, particularly given that ammonia oxidizers have the genetic potential to utilize urea directly as a substrate for nitrification (73). The observed significant decrease in N_2O emission in the vegetable soil is mostly likely caused by the inhibitory effect of DMPP on the nitrifier denitrification pathway mediated by AOB, because AOA are considered not to be capable of nitrifier

denitrification due to the lack of genes encoding nitric oxide reductase (6).

Conclusions. In conclusion, using combined approaches with the ^{15}N tracing model, molecular techniques, and DNA-SIP, we provide strong evidence that DMPP application temporarily decreased the autotrophic nitrification rate in an alkaline vegetable soil, most likely through inhibiting the growth and metabolic activity of AOB. Our results suggest that DMPP was less effective at inhibiting ammonia oxidation in the acid pasture soil, which is likely to be attributed to the intrinsically limited growth of AOB under low-pH conditions and the high rate of adsorption of DMPP by organic matter and clay. Our findings suggest that the incorporation of microbial dynamics information could increase the capacity for predicting the effectiveness of DMPP for more informed incorporation of DMPP into N management strategies. Given the inherent limitation of soil microcosm experiments, further research is highly desirable to verify these results under field conditions by covering various soil and climate factors.

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