Effects of dicyandiamide and acetylene on N₂O emissions and ammonia oxidizers in a fluvo-aquic soil applied with urea

Qing Wang^{1,2} · Li-Mei Zhang^{1,2} · Ju-Pei Shen^{1,2} · Shuai Du^{1,2} · Li-Li Han¹ · Ji-Zheng He^{1,2,3}

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Abstract Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are crucial for N₂O emission as they carry out the key step of nitrification. Dicyandiamide (DCD) and acetylene (C₂H₂) are typical nitrification inhibitors (NIs), while the comparative effects of these NIs on N₂O production and ammonia oxidizers' (AOB and AOA) growth are unclear. Four treatments including a control, urea, urea + DCD, and urea + C₂H₂ were set up to investigate their effect of inhibiting soil nitrification, nitrification-related N₂O emission as well as the growth of ammonia oxidizers with a fluvo-aquic soil using microcosms for 28 days. N₂O emission and net nitrification rate increased after the application of urea, but were significantly restrained in urea + NI treatments, while C₂H₂ was more effective in reducing N₂O emission and nitrification rate than DCD. The abundance of AOB, which was significantly correlated with N₂O emission and net nitrification rate, was more inhibited by C₂H₂ than DCD. Furthermore, the application of urea in all the soils had little impact on the AOA community, while obvious shifts of AOB community structure were found compared with the control. All

AOB sequences fell within *Nitrosospora* cluster 3, and the AOA community was clustered to group 1.1b. Collectively, it indicated that application of urea combined with NIs (DCD or C₂H₂) could potentially alter N₂O emission, mainly through regulating the growth of AOB but not AOA in this fluvo-aquic soil.

Keywords Nitrification inhibitor · N₂O emission · *amoA* gene · Net nitrification rate · Ammonia-oxidizing bacteria · Ammonia-oxidizing archaea

Introduction

Nitrous oxide (N₂O) is an important greenhouse gas (GHG) that confers a global warming potential (GWP) 300-fold greater than that of carbon dioxide (CO₂) and is involved in the depletion of the ozone layer (Ravishankara et al. 2009; Butterbach-Bahl et al. 2013). The global N₂O concentration in the atmosphere has increased since the pre-industrial era, partly due to the expansion of agricultural soils and increasing application of mineral nitrogen fertilizer (Davidson 2009; Smith et al. 2012). Agricultural soils are recognized as the major source of N₂O, accounting for nearly 65 % of the atmospheric N₂O emissions (IPCC 2007). N₂O is produced in soils via nitrification and denitrification processes (Wrage et al. 2001), and their respective contributions depend on soil conditions (Nakajima et al. 2005; Braker and Conrad 2011). Additionally, it has been reported that ammonia oxidation, the key step of nitrification, may contribute up to 80 % of the soil N₂O flux, depending on soil types, temperature, and water content (Gdde and Conrad 1999; Zhu et al. 2013; Huang et al. 2014). Therefore, it is necessary to determine the relative contribution of the nitrification process to N₂O emission in agricultural soils.

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✉ Ju-Pei Shen
jpshen@rcees.ac.cn

✉ Ji-Zheng He
jzhe@rcees.ac.cn; jizheng.he@unimelb.edu.au

¹ State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Beijing 100085, China

² University of Chinese Academy of Sciences, Beijing 100049, China

³ Melbourne School of Land and Environment, The University of Melbourne, Parkville, Victoria, Australia

Nitrification is an important nitrogen transformation process in agricultural ecosystems, oxidizing ammonia into nitrate via nitrite and emitting N_2O as a by-product. Ammonia oxidation, catalyzed by ammonia monooxygenase (AMO) encoding with the *amoA* gene, is traditionally thought to be mainly regulated by ammonia-oxidizing bacteria (AOB). However, this view has recently been questioned by the discovery of the *amoA* gene in archaeal groups (Venter et al. 2004; Francis et al. 2005; Kneke et al. 2005). In fact, archaeal *amoA* genes appear to be ubiquitously distributed in soils (Nicol et al. 2008) and they are more abundant than bacterial *amoA* genes in most terrestrial ecosystems (Leininger et al. 2006; He et al. 2007). A positive relationship between potential ammonia oxidation and archaeal *amoA* gene or transcript abundance, but not AOB, has been demonstrated in acidic soils (Gubry-Rangin et al. 2010; Yao et al. 2011; Zhang et al. 2012), suggesting that ammonia-oxidizing archaea (AOA) may play a greater role than AOB in soil ammonia oxidation. However, the relative role of AOB and AOA in ammonia oxidation and N_2O emission is not well understood.

The application of nitrification inhibitors (NIs) such as dicyandiamide (DCD) and acetylene (C_2H_2) is often used to suppress the nitrification process and N_2O emissions (Di et al. 2010b; Liu et al. 2013; Hink et al. 2016). DCD, one of the most widely used nitrification inhibitors, retards the activities of ammonia oxidizers and thereby reduces nitrification and N_2O (O'Callaghan et al. 2010). C_2H_2 is another effective inhibitor and works on autotrophic nitrification at a low concentration (10–100 Pa) (Zhu et al. 2013). These compounds inhibit nitrification by deactivating ammonia monooxygenase (AMO) and blocking the growth of AOB and AOA (Di et al. 2010a; Hink et al. 2016). DCD or C_2H_2 was reported to be more effective in inhibiting AOA compared with AOB in low-pH soils (Gubry-Rangin et al. 2010; Zhang et al. 2012), and vice versa in nitrogen-rich soils (Di et al. 2009; Jia and Conrad 2009; Dai et al. 2013; Chen et al. 2015). Moreover, by comparing the inhibitory effects of different NIs on the growth of ammonia oxidizers using pure cultures, DCD exhibited a stronger inhibitory effect on AOB than AOA, while nitrapyrin dramatically inhibited the growth of AOA (Shen et al. 2013). Considering the varied effects of NIs, it is therefore necessary to compare the effect of different NIs on ammonia oxidizers in soils, especially in the context of urea application.

The North China Plain, as the second largest agricultural region in China (Liu et al. 2001), is dominated by calcareous fluvo-aquic soil. High levels of N_2O are emitted in this agricultural area, primarily ascribed to the excessive N fertilization (Yan et al. 2003). It has been reported that ammonia oxidation is the main source of N_2O emissions from fluvo-aquic soils following irrigation and N-fertilization (Cui et al. 2012; Huang et al. 2014). Moreover, AOB was found to be the main driving force in ammonia oxidation causing enormous

loss of inorganic fertilizer in these agricultural soils (Shen et al. 2008). We therefore hypothesized that the different responses of N_2O emission after urea and NI addition were mainly ascribed to the differences in the growth of AOB. Laboratory microcosms were set up with the following objectives: (i) to estimate the inhibitory effect of DCD and C_2H_2 in regulating nitrification and N_2O production and (ii) to investigate the respective change of ammonia oxidizers and the relationships with N_2O emission with the application of DCD and C_2H_2 in a fluvo-aquic soil.

Materials and methods

Soil and incubation experiment

Soil used for microcosm construction was collected from an upland field in Luancheng (LC) county, Hebei province (40° 7' 34" N, 119° 11' 27" E) characterized by a moderate climate with average annual rainfall of about 553 mm and a mean annual temperature of 15 °C. The basic soil properties were sandy loam texture (clay/silt/sand, 8:27:65), bulk density 1.28 g cm⁻³, pH 8.0 (1:2.5, soil/water), dissolved organic carbon (DOC) 46 mg C kg⁻¹, electrical conductivity (EC) 0.20 mS cm⁻¹, and total N 2.0 g kg⁻¹, respectively. The freshly collected soil was then sieved through a 2-mm mesh and stored at 4 °C for 1 week.

Fresh soil (30 g dry weight basis) was placed in 250-ml serum bottles and pre-incubated for 5 days at 30 °C to stabilize the microbial activity at 40 % water-filled pore space (WFPS). Soil was adjusted to 55 % WFPS with the following treatments in triplicate: CK, urea (200 mg urea-N kg⁻¹ soil), urea + DCD (30 mg kg⁻¹ soil), and urea + C_2H_2 (0.1 %). Aerobic conditions were maintained by ventilating 20 min at intervals of 3–4 days when water content and C_2H_2 were supplemented.

Gas and soil sampling

Gas samples (20 ml) were collected twice per day (one at 0 h and another at 12 h) from the headspace of bottles at days 1, 2, 3, 4, 7, 10, 15, 20, and 28 after the start of incubation. N_2O concentration was determined with a gas chromatograph (Agilent 7890A). N_2O emissions were calculated as the difference between 0 and 12 h based on the equation reported by Yagi et al. (1996). Three bottles per treatment were destructively sampled at days 3, 7, 15, and 28 for soil samples. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were extracted with 2 M KCl solution and analyzed colorimetrically using a flow analyzer. The soil samples for molecular analysis were immediately stored at -80 °C.

DNA extraction and quantitative PCR

DNA was extracted from 0.4 g frozen soil (dry weight) with a MoBio Powersoil™ DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's protocol. DNA quality was checked on 1 % (w/v) agarose gel.

Quantification of AOB and AOA *amoA* genes was determined using amoA-1F/amoA-2R (Rotthauwe et al. 1997) and CrenamoA23F/CrenamoA616R (Tourna et al. 2008), respectively. All reactions were carried out on an iCycler iQ5 Thermocycler (Bio-Rad, USA). Each 25- μ l reaction included 12.5 μ l 2 x SuperMix (Bio-Rad, USA), 0.5 μ l DMSO, 0.5 μ M of each primer, 2 μ l tenfold diluted DNA, and 7 μ l sterilized deionized water. The thermocycling conditions for AOA and AOB were initial denaturation at 95 °C for 4 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 50 s, and data collection at 83 °C for 15 s. Product specificity was confirmed by 1.5 % agarose gel and melt curve analysis. Plasmids as prepared previously (Long et al. 2012) were used as standards for AOA and AOB quantitative PCR (qPCR). The efficiencies for AOB and AOA *amoA* amplifications ranged from 95.0 to 97 % with a correlation coefficient (R^2) of 0.997 for both genes.

Terminal restriction fragment length polymorphism analysis of *amoA* gene fragments

For terminal restriction fragment length polymorphism (T-RFLP) analysis, PCR amplification was conducted using the fluorescently labeled primers FAM-amoA-1F/amoA-2R and FAM-CrenamoA23F/CrenamoA616R, respectively. The 50 μ l PCRs contained 25 μ l Premix EX Taq™ (Takara Biotechnology, Dalian, China), 1 μ l DMSO, 1 μ M of each primer, 2 μ l tenfold diluted DNA, and 21 μ l sterilized deionized water. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, extension at 72 °C for 50 s, followed by a final elongation of 72 °C for 8 min. The PCR products were first purified (QiAGEN Gel Extraction Kit, Hilden, Germany) and then were digested for 1 h with 5 U *RsaI* for AOB and *HpyCH4V* for AOA at 37 °C, respectively. The size and relative abundance of terminal restriction fragments (TRFs) were determined by an ABI 3730xl DNA analyzer. T-RFLP profiles were determined using GeneMapper 2.0 software (Applied Biosystems).

Clone and sequencing

AOB and AOA clone libraries were constructed from the CK treatment. PCR amplification was performed using the primers amoA-1F/amoA-2R and CrenamoA23F/CrenamoA616R without fluorescent labeling. PCR products for AOB and AOA libraries were purified and ligated into

pEasy-T3 vector (TransGen Biotech), and then transformed into cells of *Escherichia coli* JM109 according to the manual. A total of 50 AOB clones and 49 AOA clones were selected for sequencing using an ABI 3730 sequencer (Applied Biosystems).

Sequence analysis

All sequences were analyzed with MEGA 5.0 and the wrong fragments were removed. Similarity of sequences was calculated with QIIME, and operational taxonomy units (OTUs) were defined as sharing 97 % similarity. The representative sequences of OTUs and the most similar sequences obtained by BLAST were used for the phylogenetic tree. The phylogenetic analysis was conducted using MEGA 5.0, and neighbor-joining trees were constructed by performing 1000 replicates to produce bootstrap values. AOA and AOB *amoA* gene sequences retrieved in this study have been submitted to GenBank under accession numbers of KX181608 to KX181621 for AOA and KX181622 to KX181637 for AOB, respectively.

Statistical analyses

The statistical analysis was performed using SPSS 19.0 software (IBM Co., Armonk, NY, USA). The Duncan test of one-way ANOVA was conducted to test the differences in means of N₂O flux and NH₄⁺ and NO₃[−] concentrations among the treatments; $P < 0.05$ level was considered significant. Principal component analysis (PCA) was performed to investigate the effects of urea and NI treatments on the ammonia oxidizer community structure within CANOCO 5.0 (Microcomputer Power). Correlations of *amoA* gene copies with N₂O emission rate and net nitrification rate were assessed by Pearson's correlation analysis.

Results

Soil N₂O emissions

The dynamics of N₂O varied significantly, with large differences among the treatments ($P < 0.05$, Fig. 1a). N₂O emission was very low throughout the incubation period in the control treatment, while only application of urea substantially increased N₂O emission ($P < 0.01$), reaching a peak of 110.0 ng N g^{−1} soil day^{−1} at day 3, and then declined sharply to a low level. When applying urea combined with DCD or C₂H₂, N₂O emissions declined dramatically, with the peaks being 4.5 and 2.4 ng N g^{−1} soil day^{−1}, respectively (Fig. 1a). After 7 days' incubation, the N₂O emissions were not significantly different among the treatments. The cumulative N₂O emission from the urea treatment was 22.7 times higher than

Fig. 1 Dynamics of N₂O emission rate (a) and total N₂O emission (b) in the fluvo-aquic soil without urea and NIs (DCD or C₂H₂) addition (CK), with urea addition only (Urea), with urea plus dicyandiamide (Urea + DCD), and with urea plus acetylene (Urea + C₂H₂). Data are means with standard deviations ($n = 3$). The letters *a*, *b*, and *c* indicate the statistical significance among different treatments

that from the control (Fig. 1b). Compared to the urea treatment, the cumulative N₂O emissions were reduced by 86.7 and 93.0 % with urea + DCD and urea + C₂H₂ treatments, respectively, and the inhibitory effect of C₂H₂ was much stronger than DCD.

NH₄⁺-N and NO₃⁻-N concentrations

In the control treatment, NH₄⁺-N concentration remained low during the incubation period, indicating the slow nitrification process (Fig. 2a). With the hydrolysis of urea, the NH₄⁺-N concentration reached a peak at day 3, ranging from 72.7 to 181.7 mg N kg⁻¹ soil in the urea and urea + NIs treatments. The NH₄⁺-N concentration of the urea treatment was almost completely consumed in the first 7 days. In the urea + DCD treatment, the NH₄⁺-N concentration gradually decreased to

Fig. 2 Dynamics of NH₄⁺ (a) and NO₃⁻ (b) in the fluvo-aquic soil without urea and NIs (DCD or C₂H₂) (CK), with urea addition only (Urea), with urea plus dicyandiamide (Urea + DCD), and with urea plus acetylene (Urea + C₂H₂). Data are presented as mean values with standard deviations ($n = 3$)

25.0 mg N kg⁻¹ soil at the end of incubation. On the other hand, the NH₄⁺-N concentration remained at a higher level 3 days after applying urea, indicating that C₂H₂ completely inhibited the nitrification process.

The NO₃⁻-N concentration changed conversely to that of the NH₄⁺-N concentration (Fig. 2b). The NO₃⁻-N concentration of the control treatment increased slightly from 129.4 at day 3 to 147.2 mg N kg⁻¹ soil at day 28. In the urea treatment, the concentration of NO₃⁻ reached the peak at day 7 and then stabilized at about 297.5 mg N kg⁻¹ soil. In the urea + DCD treatment, the NO₃⁻ concentration gradually increased during the incubation, which was much higher than that in the control treatment ($P < 0.05$). In the urea + C₂H₂ treatment, the NO₃⁻-N concentration remained stable at about 125.0 mg N kg⁻¹ soil during the whole incubation.

Net nitrification rates were significantly different among all the treatments (Table 1). The net nitrification rate increased by 40.0- and 9.6-fold in the + urea compared with the control

Table 1 Net nitrification rate (mean $\pm$ SE) among the four treatments during incubation

Interval	Net nitrification rate (mg N kg ⁻¹ day ⁻¹)			
	CK	Urea	Urea + DCD	Urea + C ₂ H ₂
Day 0–3	0.72 $\pm$ 0.29b	28.56 $\pm$ 2.7a	6.94 $\pm$ 0.82a	Undetected
Day 3–7	1.92 $\pm$ 0.64a	20.36 $\pm$ 0.89b	7.8 $\pm$ 0.78a	Undetected
Day 7–15	0.27 $\pm$ 0.07c	0.22 $\pm$ 0.08c	4.37 $\pm$ 0.73b	Undetected
Day 15–28	0.6 $\pm$ 0.05b	0.56 $\pm$ 0.19c	4.4 $\pm$ 0.21b	Undetected

The different letters in the same column denote significant difference along the incubation period ($P < 0.05$)

treatment at the interval of day 0–3 and day 3–7, respectively. Moreover, the net nitrification rates in the urea + DCD treatment were 9.7- and 4.0-fold higher than that in the control treatment at the interval of day 0–3 and day 3–7, respectively. Nevertheless, nitrification was not detected during the incubation in the urea + C₂H₂ treatment.

Abundance of AOB and AOA *amoA* genes

AOB abundance was in the range of 4.13 to 95×10^5 copies g⁻¹ soil (Fig. 3a), which was much less than that of AOA. AOB abundance in the control treatment, which changed little before 15 days and remained constant between 2.15 and 2.58×10^6 copies g⁻¹ soil, reduced to 4.13×10^5 copies g⁻¹ soil at the end of incubation. AOB abundance strongly increased with the application of urea, but this effect was inhibited when NIs were added ($P < 0.05$). AOB abundance increased by 3.7- and 2.2-fold in the urea and urea + DCD treatments than in the control treatment at day 7, respectively, and then decreased to the end of the incubation period. No significant difference of AOB abundance was observed between the urea + C₂H₂ and control treatments (Fig. 3a). The abundance of AOA genes in the soil under different treatments is shown in Fig. 3b. AOA *amoA* gene copies in all treatments ranged from 5.5 to 25.2×10^7 copies g⁻¹ soil. No significant differences were observed among the treatments.

Positive correlations were observed between AOB abundance and net nitrification rate ($P < 0.01$) and N₂O emission rate ($P < 0.01$) across all treatments (Table 2). Net nitrification rates and N₂O emission rates were also positively correlated ($P < 0.01$). Contrary to AOB, no quantitative relationships were observed between AOA abundances and N₂O emission rates and net nitrification rates (Table 2).

Community analysis of AOB and AOA by T-RFLP

Six TRFs of AOB were obtained from soil by using the enzyme *RsaI* and TRFs of 211, 251, 255, and 269 bp that had the highest relative abundance among all the treatments, ranging

Fig. 3 Dynamics of *amoA* gene copies of AOB (a) and AOA (b) in the fluvo-aquic soil without urea and NIs (DCD or C₂H₂) (CK), with urea addition only (Urea), with urea plus dicyandiamide (Urea + DCD), and with urea plus acetylene (Urea + C₂H₂). Data are presented as mean values with standard deviations ($n = 3$)

from 12.4 to 30 % (Fig. 4a). The relative abundance of TRFs 255 and 269 bp showed no significant differences between the urea + NIs and control treatments during the incubation period. At day 7, TRF 255 bp had a higher relative abundance (ca. 30 %) in the urea treatment compared to the CK treatment. The relative abundance of TRFs 211 and 269 bp obviously decreased after urea application (ca. 12.4 and 14.8 %, respectively), compared with the control treatment during incubation. PCA analysis showed that urea and NIs had obvious

Table 2 Correlations between log numbers of *amoA* abundance, N₂O emission, and net nitrification rate

	AOB	AOA
Net-Nit	0.523**	0.256
N ₂ O	0.555**	0.288

Net-Nit net nitrification rate

**Correlation significant at the 0.01 level (two-tailed)

Fig. 4 T-RFLP profile (a) and principal component analysis (PCA) (b) of AOB TRFs in different treatments (CK, Urea, Urea + DCD, Urea + C₂H₂) at three sampling times. Data are presented as mean values with standard deviations ($n = 3$)

impacts on AOB community compositions (Fig. 4b). The first two PCA axes explained 91.53 % of the total variance in bacterial community structure. Urea + NIs treatments were clearly separated from the urea treatment throughout the incubation, which were all separated from the controls. However, no significant differences of the composition of AOB TRFs were found between the urea + DCD and urea + C₂H₂ treatments.

Six AOA TRFs were observed using the enzymes *HpyCH4V*, and TRFs 204 and 249 bp were the dominant groups (Fig. 5a). PCA analysis indicated that the first two axes explained 91.1 % of the total variance (Fig. 5b). At day 7, the urea treatment was clearly separated from other treatments owing to the decrease of TRF 204 bp and the increase of TRF 249 bp. However, at days 15 and 28, no obvious differences were found in the composition of AOA among all the treatments.

Phylogeny of AOB and AOA

A total of 50 AOB clones and 49 AOA clones were selected for the phylogenetic analysis. All AOB sequences were affiliated within *Nitrosospira* cluster 3 (Fig. 6). The TRFs of (bp) 251, 255, 273, and 380 were assigned to cluster 3a.2, while the

Fig. 5 T-RFLP profile (a) and principal component analysis (PCA) (b) of AOA TRFs in different treatments (CK, Urea, Urea + DCD, Urea + C₂H₂) at three sampling times. Data are presented as mean values with standard deviations ($n = 3$)

TRF of 211 belonged to clusters 3a.1 and 3b. The TRF of 269 bp was assigned to clusters 3a.2 and 3b.

All the AOA TRFs in T-RFLP profiles were detected in AOA clones. The AOA phylogenetic analysis showed that all *amoA* gene sequences exclusively belonged to group 1.1b (Fig. 7).

Discussion

Nitrification and denitrification are the two major microbial pathways producing N₂O in arable soils (Bremner 1997; Wrage et al. 2001; Nakajima et al. 2005). Several studies have reported that nitrification was the major N₂O production process in fluvo-aquic soils (Wan et al. 2009; Cui et al. 2012; Zhang et al. 2016). We obtained similar results that nitrification is the main contributor to N₂O emissions in the tested soil for the following reasons: (i) only urea addition resulted in the greatest increase in N₂O emission, while DCD and C₂H₂ reduced total N₂O emissions by 86.7 and 93.0 %, respectively, due to their inhibitory effects on ammonia oxidation (McCarty 1999); (ii) the significant correlation between net nitrification

Fig. 6 Neighbor-joining phylogenetic tree of bacterial *amoA* sequences retrieved from a fluvo-aquic soil. Sequences from this study are described as clone name and TRF size in **bold**. Reference sequences are described as clone name (accession number and environment). Bootstrap values (50 %) are indicated at branch points. The scale bar represents 2 % estimated sequence divergence

rate and N_2O emission indicated that nitrification was a key regulating process of N_2O production in the fluvo-aquic soil; (iii) DOC content in the soil was low, which also supported the hypothesis that the weak contribution of denitrification to N_2O emission in the fluvo-aquic soil might be primarily attributed to the low content and availability of soil organic carbon for denitrifiers (Wan et al., 2009); and (iv) all samples were conducted aerobically with the moisture content maintained at 55 % WFPS, which was more favorable to nitrification than denitrification (Bateman and Baggs 2005). Therefore, it was inferred that N_2O was mainly produced via nitrification rather than denitrification in the fluvo-aquic soil, which can further be supported by Cui et al. (2013) and Huang et al. (2014).

Our study showed that the application of DCD and C_2H_2 inhibited nitrification and reduced N_2O emission for the fluvo-aquic soil, but to different extents. The latter showed a stronger inhibitory effect than DCD. It could be ascribed to several following reasons. Firstly, C_2H_2 is a suicide substrate for the ammonia monooxygenase (AMO), i.e., the oxidation of C_2H_2 by AMO results in permanent inhibition of the enzyme. It is reported that low C_2H_2 concentrations (10 Pa) are enough for 100 % inhibition of autotrophic nitrification (Bateman and Baggs 2005; Offre et al. 2009). Therefore, C_2H_2 is always used to eliminate N_2O production from nitrification-related processes (Zhu et al. 2013). Secondly, DCD is highly water soluble and mobile. As a nitrogen source, it could be used by

Fig. 7 Neighbor-joining phylogenetic tree of archaeal *amoA* sequences retrieved from a fluvo-aquic soil. Sequences from this study are described as clone name and TRF size in **bold**. Reference sequences are described as clone name (accession number and environment). Bootstrap values (50 %) are indicated at branch points. The scale bar represents 2 % estimated sequence divergence

microbes, and microbiological decomposition often occurred above 25 °C (Hauser and Haselwandter 1990). In our study, the incubation temperature was 30 °C, so we concluded that biological decomposition of DCD might be one of the reasons for the lower inhibitory effect compared to C₂H₂ (Chen et al. 2015). Additionally, several studies suggested that soil texture, water content, temperature, and pH might be the key factors influencing the effectiveness of NIs on nitrification and nitrification-related N₂O emission (Hatch et al. 2005; Chen et al. 2010; Di and Cameron 2011; Cui et al. 2013; Liu et al. 2015). Further research is required to improve our understanding of the inhibitory effects of DCD and C₂H₂ under different soil conditions.

In the present study, the abundance of AOB rather than AOA significantly increased in the urea treatment (Fig. 3a), which was in agreement with previous studies in a large

number of agricultural soils (Wu et al. 2011; Habteselassie et al. 2013; Chen et al. 2015). It has been reported that AOB growth is favored by high-N soil conditions (Jia and Conrad 2009; Verhamme et al. 2011; Taylor et al. 2012), whereas AOA growth is not affected (Di et al. 2009, 2010a). The stronger suppression of the AOB population size by C₂H₂ than DCD was observed, suggesting a coincidence of dynamics between the AOB population and N₂O emission. In contrast to AOB, AOA did not respond to urea and NIs (Fig. 3b), which was consistent with previous conclusions (Jia and Conrad 2009; O'Callaghan et al. 2010; Gong et al. 2013). Moreover, Shen et al. (2013) concluded that AOA might be less sensitive than AOB to different NIs due to their fundamental cellular and metabolic differences. Moreover, our study showed that the abundance of AOB was positively correlated with net nitrification rates and N₂O emissions in the

soils ($P < 0.01$) (Table 2), indicating the more important role of AOB than AOA in nitrification and N_2O emissions. Similar findings have been reported in New Zealand grassland soils (Di and Cameron 2011; Dai et al. 2013), thus confirming our findings that C_2H_2 resulted in a greater inhibitory effect on N_2O emission and AOB growth than DCD.

The AOB phylogenetic analysis showed that all the bacterial *amoA* sequences in the fluvo-aquic soil belonged to *Nitrosospira* cluster 3 (Fig. 6), confirming similar results obtained from various agricultural soils (Phillips et al. 2000; Chu et al. 2007; Ouyang et al. 2016). It has been suggested that *Nitrosospira* cluster 3 often outcompeted other *Nitrosospira* under fertile soil conditions (Tourna et al. 2010). T-RFLP analysis showed that urea addition had a significant impact on the community structure of AOB compared to other treatments (Fig. 4a), which was consistent with previous findings (Jia and Conrad 2009; Chen et al. 2015). However, the AOB community composition between the urea + DCD and urea + C_2H_2 treatments was similar in our study. Likewise, no obvious differences of the AOB community response to NIs (DCD and DMPP) were found in a Chinese arable soil (Gong et al. 2013).

The AOA phylogenetic analysis showed all archaeal *amoA* gene sequences were exclusively clustered with group 1.1b (Fig. 7), which was in line with previous studies that group 1.1b might be more abundant in neutral and alkaline soils (Jia and Conrad 2009; Hu et al. 2015), whereas group 1.1a had a higher ammonia affinity and preferred to live in acidic red soil (Gubry-Rangin et al. 2010). T-RFLP and PCA analysis showed the AOA community structure remained relatively stable among the treatments (Fig. 5). Similarly, some studies indicated NIs and N fertilization had little effects in AOA communities in high-pH soils (Jia and Conrad 2009; Gong et al. 2013). However, others observed shifts in AOA community structure under NIs and N fertilization in low-pH soils (He et al. 2007; Ying et al. 2010; Zhang et al. 2012). These reports suggested that the effectiveness of N fertilization and NIs on AOA community structure may be determined by pH and soil types (Nicol et al. 2008; Liu et al. 2015).

Conclusions

Our results showed that urea application stimulated nitrification and N_2O emission in a fluvo-aquic soil. C_2H_2 exerted a greater effect than DCD in inhibiting N_2O emission and nitrification, in accordance with the variation of AOB abundance. Only AOB abundances were found to be correlated to net nitrification rates and N_2O emissions in the soil. The abundance and community structure of AOB were more sensitive to urea and NIs than AOA in our study. These results suggested that C_2H_2 was more effective than DCD in N_2O mitigation by inhibiting ammonia-oxidizing bacteria growth in a

high N level agricultural soil. The comparative analysis of C_2H_2 and DCD in nitrification could shed new light on the physiological properties of AOA as well as AOB. Further research is needed to understand the relative importance of AOB and AOA in nitrification and nitrification-related N_2O emission in different terrestrial ecosystems.

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