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Effects of dicyandiamide and acetylene on N_2O emissions and ammonia oxidizers in a fluvo-aquic soil applied with urea

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Abstract

Ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) are crucial for N_2O emission as they carry out the key step of nitrification. Dicyandiamide (DCD) and acetylene (C_2H_2) are typical nitrification inhibitors (NIs), while the comparative effects of these NIs on N_2O production and ammonia oxidizers (AOB and AOA) growth are unclear. Four treatments including a control, Urea, Urea + DCD, Urea + C_2H_2 were set up to investigate their effect of inhibiting soil nitrification, nitrification-related N_2O emission as well as the growth of ammonia oxidizers with a fluvo-aquic soil using microcosms for 28 days. N_2O emission and net nitrification rate increased after the application of urea, but were significantly restrained in Urea + NIs treatments, while C_2H_2 was more effective in reducing N_2O emission and nitrification rate than DCD. The abundance of AOB, which was significantly correlated with N_2O emission and net nitrification rate, was more inhibited by C_2H_2 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 *Nitrosopira* cluster 3, and the AOA community was clustered to group 1.1 b. Collectively, it indicated that application of urea combined with NIs (DCD or C_2H_2) could potentially alter N_2O emission, mainly through regulating the growth of AOB but not AOA in this fluvo-aquic soil.

Keywords Nitrification inhibitor; N_2O emission; *amoA* gene; Net nitrification rate; ammonia oxidizing bacteria; ammonia oxidizing archaea

Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (GHG) that confers a global warming potential (GWP) 300-fold greater than that of carbon dioxide (CO_2) and is involved in the depletion of ozone layer (Ravishankara et al., 2009; Butterbach-Bahl et al., 2013). The global N_2O 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 N_2O, accounting for nearly 65% of the atmospheric N_2O emissions (IPCC, 2007). N_2O 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 N_2O flux, depending on soil types, temperature and water content (Gödde 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 N_2O emission in agricultural soils.

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; Könneke 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., 2010; Zhang et al., 2012), suggesting that 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) are often used to suppress the nitrification process and N_2O emissions (Di et al., 2010b; Liu et al., 2013; Hinkle 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 monoxygenase (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 verse in nitrogen-rich soils (Di et al., 2009; Jia and Conrad et al., 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 NIs 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; 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 ^{o}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}; total N 2.0 g kg^{-1}, respectively. The freshly collected soil was then sieved through a 2-mm mesh and stored at 4 ^{o}C for one week.

Fresh soil (30 g dry weight basis) was placed in 250 ml serum bottles and pre-incubated for 5 days at 30 ^{o}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 day 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 (1996). Three bottles per treatment were destructively sampled at day 3, 7, 15 and 28 for soil samples. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were extracted with 2M KCl solution and analyzed colorimetrically using a flow analyzer. The soil samples for molecular analysis were immediately stored at -80 ^{o}C.

DNA extraction and quantitative PCR (qPCR)

DNA was extracted from 0.4 g frozen soil (dry weight) with MoBio Powersoil^{TM} 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), CrenamoA23F/CrenamoA616R (Tourna et al., 2008), respectively. All reactions were carried out on an iCycler iQ5 Thermocycler (Bio-Rad, USA). Each 25 µl reaction included 12.5 µl 2 x SuperMix (BioRad, USA), 0.5 µl DMSO, 0.5 µM of each primer, 2 µl 10-fold diluted DNA and 7 µl sterilized deionized water. The thermocycling conditions for AOA and AOB were: initial denaturation at 95 ^{o}C for 4 min, 40 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 56 ^{o}C for 40 s, extension at 72 ^{o}C for 50 s and data collection at 83 ^{o}C for 15 s. Products 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 qPCR. The efficiencies for AOB and AOA *amoA* amplifications ranged from 95.0% to 97% with correlation coefficients (R^2) of 0.997 for both genes.

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

For T-RFLP analysis, PCR amplification was conducted using the fluorescently labeled primers FAM-amoA-1F/amoA-2R and FAM-CrenamoA23F/CrenamoA616R, respectively. The 50 µl PCR reactions contained 25 µl Premix EX Taq™ (Takara Biotechnology, Dalian, China), 1 µl DMSO, 1 µM of each primer, 2 µl tenfold diluted DNA and 21 µl sterilized deionized water. The PCR reaction 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 *HyCH4V* for AOA at 37 °C, respectively. The size and relative abundance of terminal restriction fragments (TRFs) were determined by 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).

Sequences 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 (OTU) 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 N_2O flux, NH_4^+ and NO_3^- concentrations among the treatments; $P < 0.05$ level was considered significant. Principle component analysis (PCA) was performed to investigate the effects of urea and NIs treatments on ammonia oxidizers community structure within CANOCO 5.0 (Microcomputer Power). Correlations of *amoA* gene copies with N_2O emission rate and net nitrification rate were assessed by Pearson's correlation analysis.

Results

Soil N_2O emissions

The dynamics of N_2O varied significantly, with large differences among the treatments ($P < 0.05$, Fig. 1a). N_2O emission was very low throughout the incubation period in the control treatment, while only application of urea substantially increased N_2O emission ($P < 0.01$), reaching a peak of 110.0 ng N g^{-1} soil d^{-1} at day 3, and then declined sharply to a low level. When applying urea combined with DCD or C_2H_2, N_2O emissions declined dramatically, with the peaks being 4.5 and 2.4 ng N g^{-1} soil d^{-1}, respectively (Fig. 1a). After 7 days' incubation, the N_2O emissions were not significantly different among treatments. The cumulative N_2O emission from the Urea treatment was 22.7 times higher than that from the control (Fig. 1b). Compared to the Urea treatment, the cumulative N_2O emissions were reduced by 86.7% and 93.0% with Urea + DCD and Urea + C_2H_2 treatments, respectively, and the inhibitory effect of C_2H_2 was much stronger than DCD.

NH_4^+-N and NO_3^--N concentrations

In the control treatment, NH_4^+-N concentration remained low during the incubation period, indicating the slow nitrification process (Fig. 2a). With the hydrolysis of urea, the NH_4^+-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 NH_4^+-N concentration of the Urea treatment was almost completely consumed in the first 7 days. In the Urea + DCD treatment, the NH_4^+-N concentration gradually decreased to 25.0 mg N kg^{-1} soil at the end of incubation. On the other hand, the NH_4^+-N

concentration remained at a higher level 3 days after applying urea, indicating that C_2H_2 completely inhibited the nitrification process.

The NO_3^--N concentration changed conversely to that of the NH_4^+-N concentration (Fig. 2b). The NO_3^--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 NO_3^- reached the peak at day 7 and then stabilized at about 297.5 mg N kg^{-1} soil. In the Urea + DCD treatment, the NO_3^- concentration gradually increased during the incubation, which was much higher than that in the control treatment ($P < 0.05$). In the Urea + C_2H_2 treatment, the NO_3^--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 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 + C_2H_2 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 at between 2.15 and 2.58 × 10^6 copies g^{-1} soil while it 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 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 + C_2H_2 and control treatments (Fig. 3a). The abundance of AOA genes in the soil under different treatments is shown in Figure 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 N_2O emission rate ($P < 0.01$) across all treatments (Table 2). Net nitrification rates and N_2O emission rates were also positively

correlated ($P < 0.01$). Contrary to AOB, no quantitative relationships were observed between AOA abundances and N_2O 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 bp, 251 bp, 255 bp and 269 bp that had the highest relative abundance among all the treatments, ranging from 12.4% to 30% (Fig. 4a). The relative abundance of TRFs 255 bp 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 bp 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 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 T-RFs were found between the Urea + DCD and Urea + C_2H_2 treatments.

Six AOA TRFs were observed using the enzymes *HpyCH4V*, and TRFs 204 bp 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 day 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 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 the Group1.1b (Fig. 7).

Discussion

Nitrification and denitrification are the two major microbial pathways producing N_2O in arable soils (Bremner, 1997; Wrage et al., 2001; Nakajima et al., 2005). Several studies have reported that nitrification was the major N_2O 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 N_2O emissions in the tested soil for the following reasons: i) only urea addition resulted in the greatest increase in N_2O emission, while DCD and C_2H_2 reduced total N_2O 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 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); 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 microbes, and microbiological decomposition often occurred at above 25 ^{o}C (Hauser and Haselwandter, 1990). In our study, the incubation temperature was 30 ^{o}C, so we concluded that biological decomposition of DCD might be one of the reasons for the lower inhibitory effect compared to C_2H_2 (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 N_2O 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 C_2H_2 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 C_2H_2 than DCD was observed, suggesting a coincidence of dynamics between the AOB population and N_2O 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 N_2O 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 *Nitrospira* 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 *Nitrosopira* 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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Table 1 Net nitrification rate (mean±SE) among the four treatments during incubation

Interval	Net nitrification rate (mg N kg^{-1} day^{-1})			
	CK	Urea	Urea + DCD	Urea + C_2H_2
d0-d3	0.72±0.29b	28.56±2.7a	6.94±0.82a	undetected
d3-d7	1.92±0.64a	20.36±0.89b	7.8±0.78a	undetected
d7-d15	0.27±0.07c	0.22±0.08c	4.37±0.73b	undetected
d15-d28	0.6±0.05b	0.56±0.19c	4.4±0.21b	undetected

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

Table 2 Correlations between log numbers of *amoA* abundance, N_2O emission and net nitrification rate

	AOB	AOA
Net-Nit	0.523**	0.256
N_2O	0.555**	0.288

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

Net-Nit means net nitrification rate.

List of figures

Fig.1

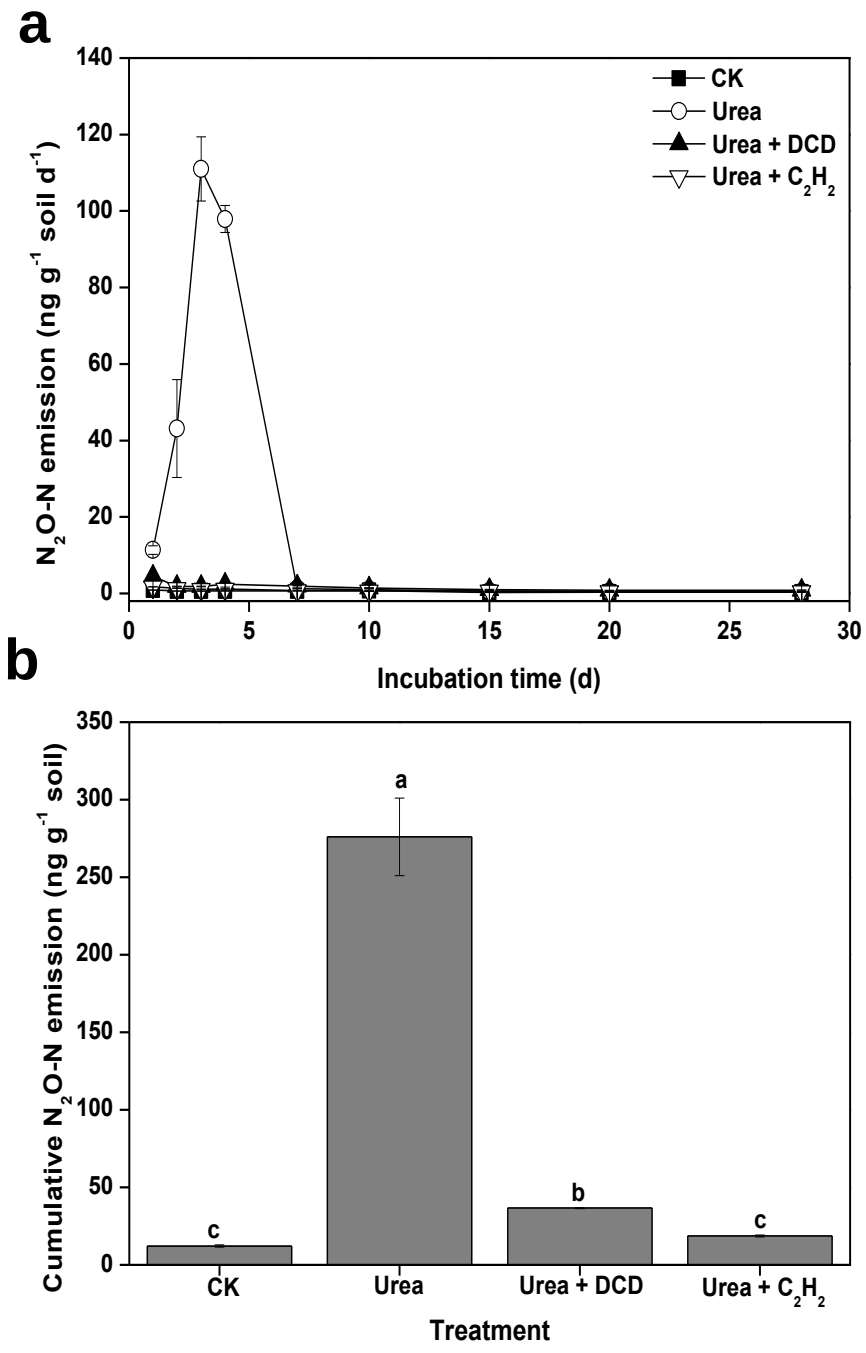

a
CK
Urea
Urea + DCD
Urea + C2H2
N2O-N emission (ng g-1 soil d-1)
Incubation time (d)
b
Cumulative N2O-N emission (ng g-1 soil)
a
b
c
c
CK
Urea
Urea + DCD
Urea + C2H2
Treatment

Fig.2

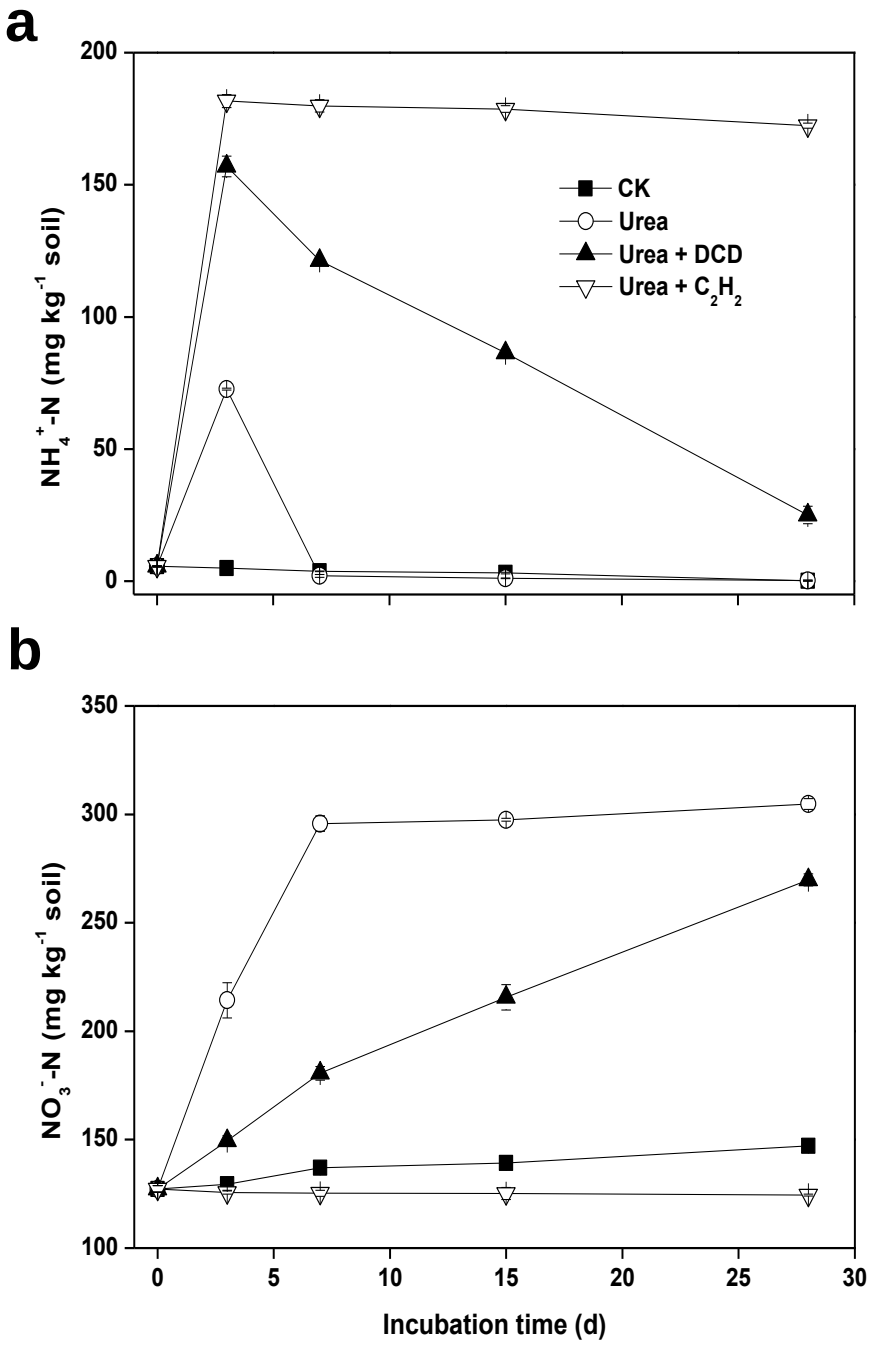

a
200
150
100
50
0
NH_4^+-N (mg kg^{-1} soil)
CK
Urea
Urea + DCD
Urea + C_2H_2
b
350
300
250
200
150
100
NO_3^--N (mg kg^{-1} soil)
0
5
10
15
20
25
30
Incubation time (d)

Fig.3

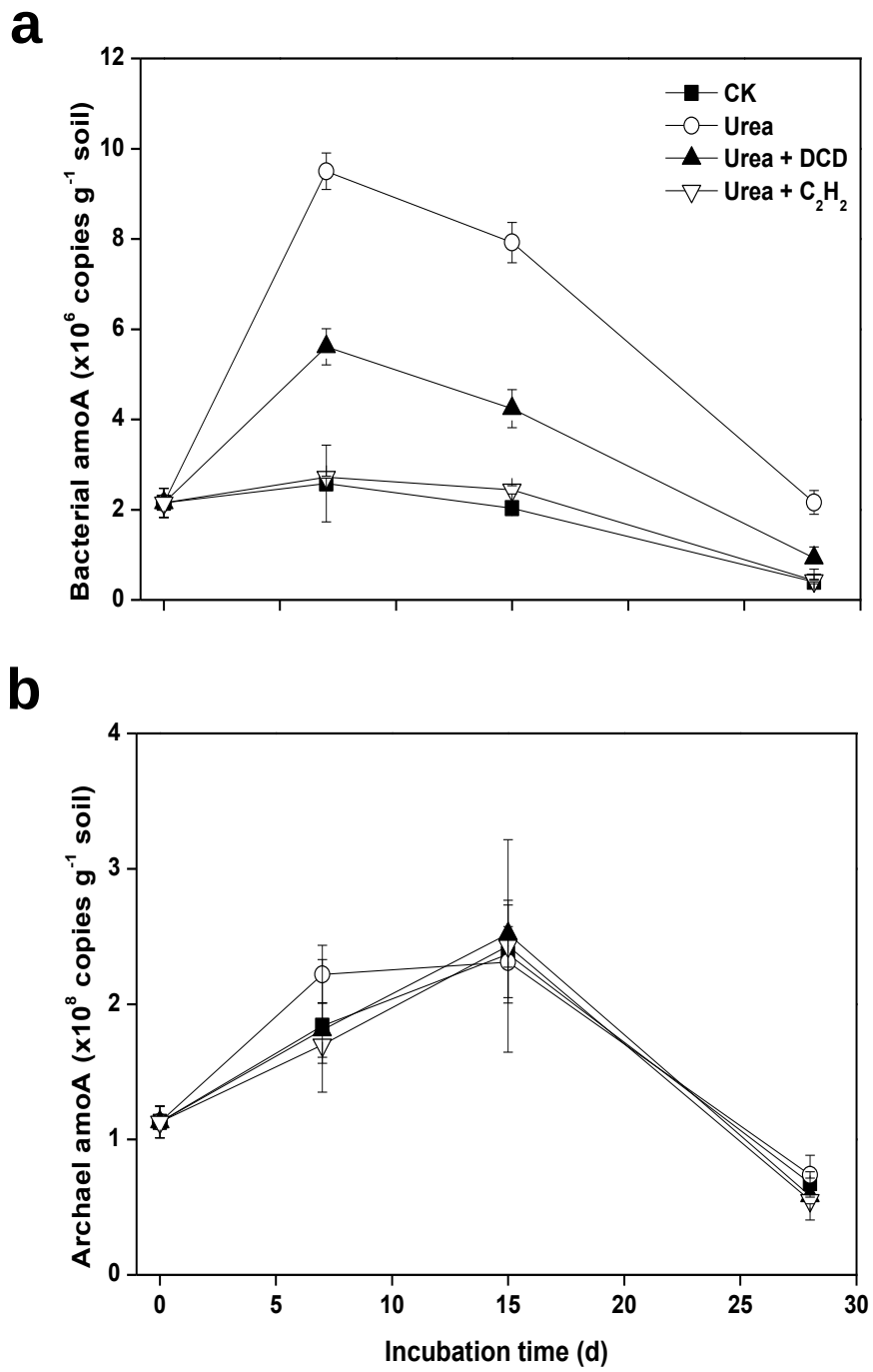

a
b
CK
Urea
Urea + DCD
Urea + C2H2
Bacterial amoA (x10^6 copies g^-1 soil)
Archael amoA (x10^8 copies g^-1 soil)
Incubation time (d)
0
2
4
6
8
10
12
1
3
5
15
20
25
30

Fig.4

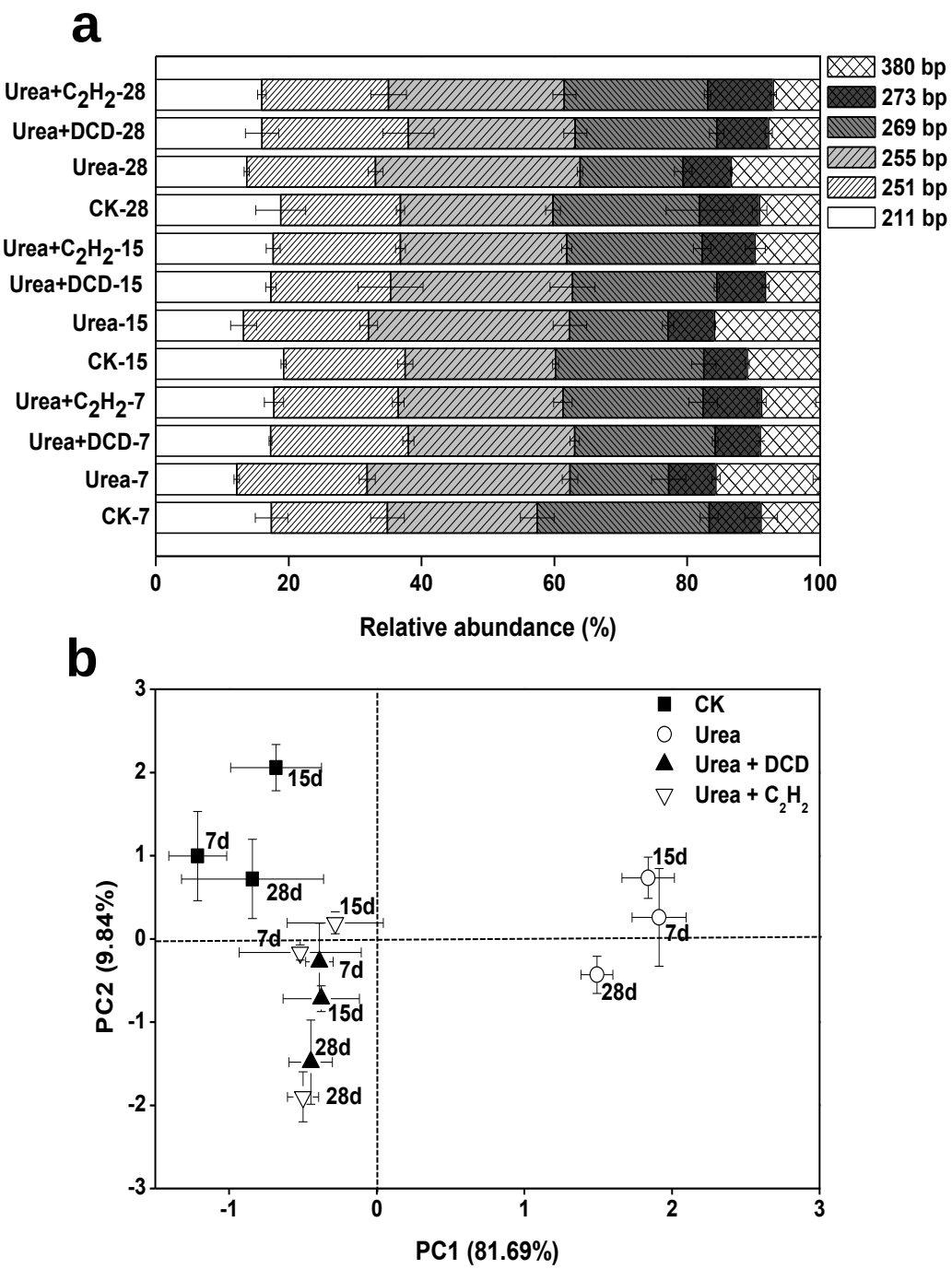

a
Urea+C2H2-28
Urea+DCD-28
Urea-28
CK-28
Urea+C2H2-15
Urea+DCD-15
Urea-15
CK-15
Urea+C2H2-7
Urea+DCD-7
Urea-7
CK-7
380 bp
273 bp
269 bp
255 bp
251 bp
211 bp
0
20
40
60
80
100
Relative abundance (%)
b
CK
Urea
Urea + DCD
Urea + C2H2
15d
7d
28d
PC2 (9.84%)
PC1 (81.69%)
3
2
1
0
-1
-2
-3

Fig.5

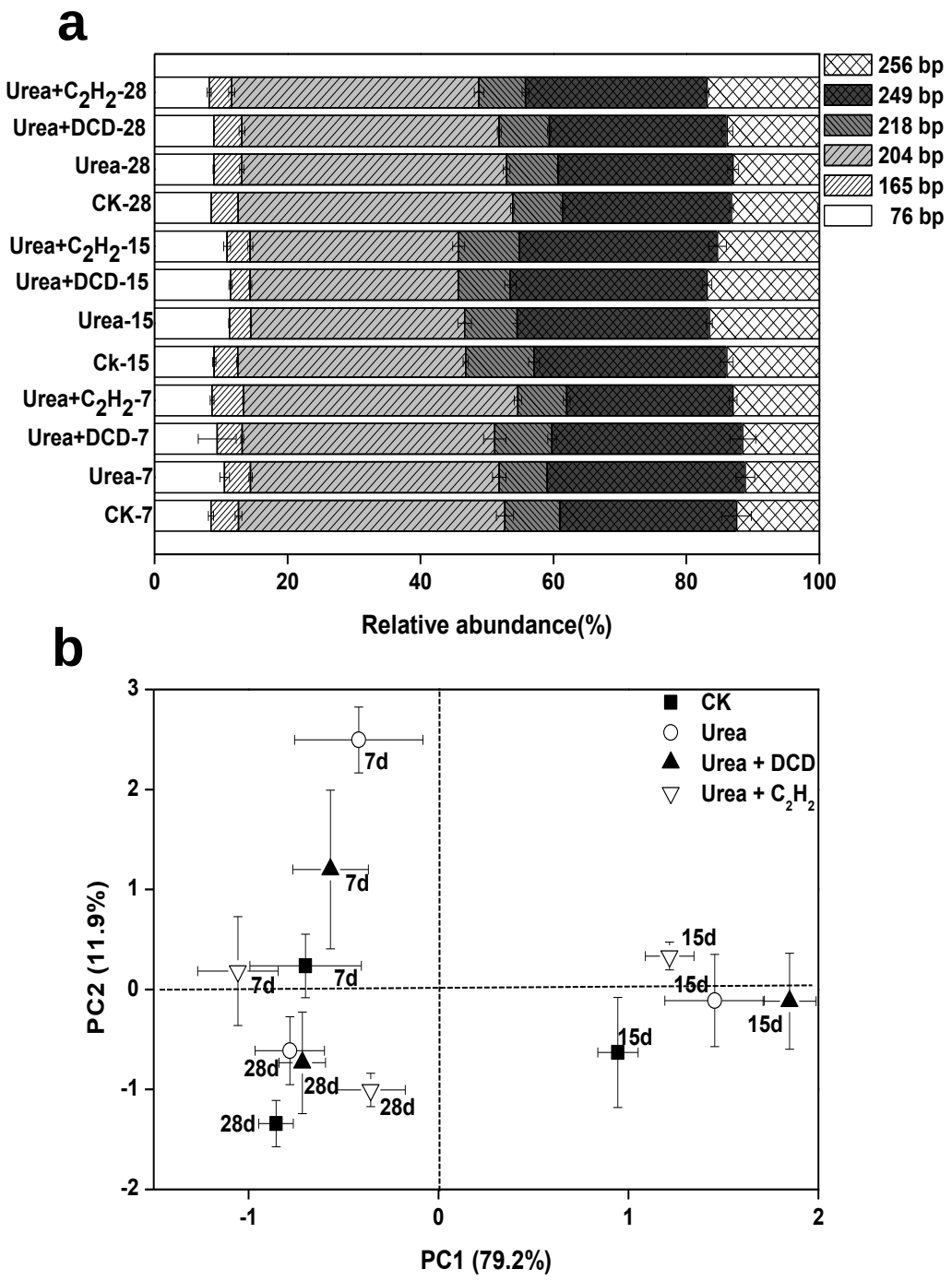
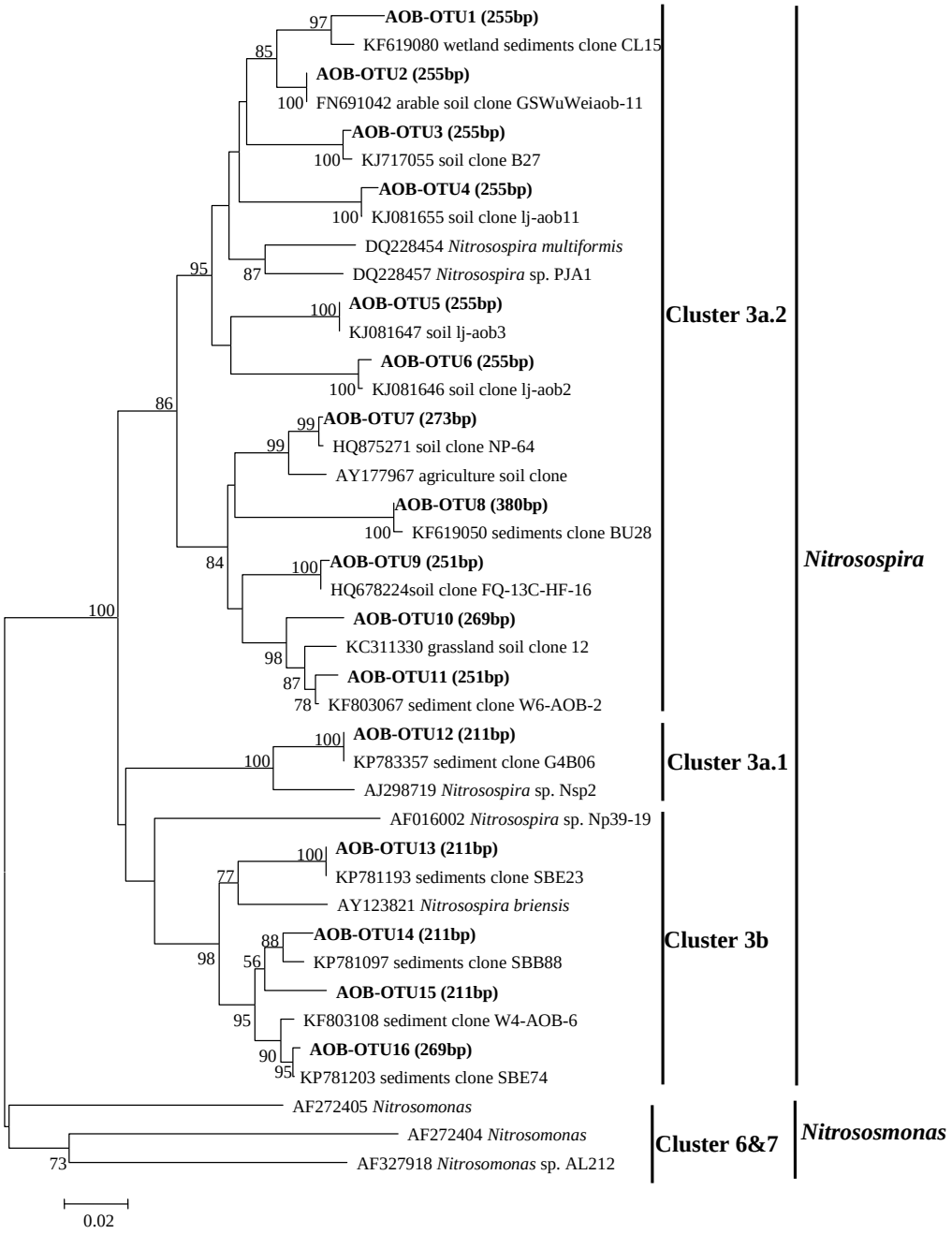

a
Urea+C2H2-28
Urea+DCD-28
Urea-28
CK-28
Urea+C2H2-15
Urea+DCD-15
Urea-15
Ck-15
Urea+C2H2-7
Urea+DCD-7
Urea-7
CK-7
256 bp
249 bp
218 bp
204 bp
165 bp
76 bp
0
20
40
60
80
100
Relative abundance(%)
b
CK
Urea
Urea + DCD
Urea + C2H2
7d
15d
28d
PC2 (11.9%)
PC1 (79.2%)
3
2
1
0
-1
-2
-1
0
1
2

Fig.6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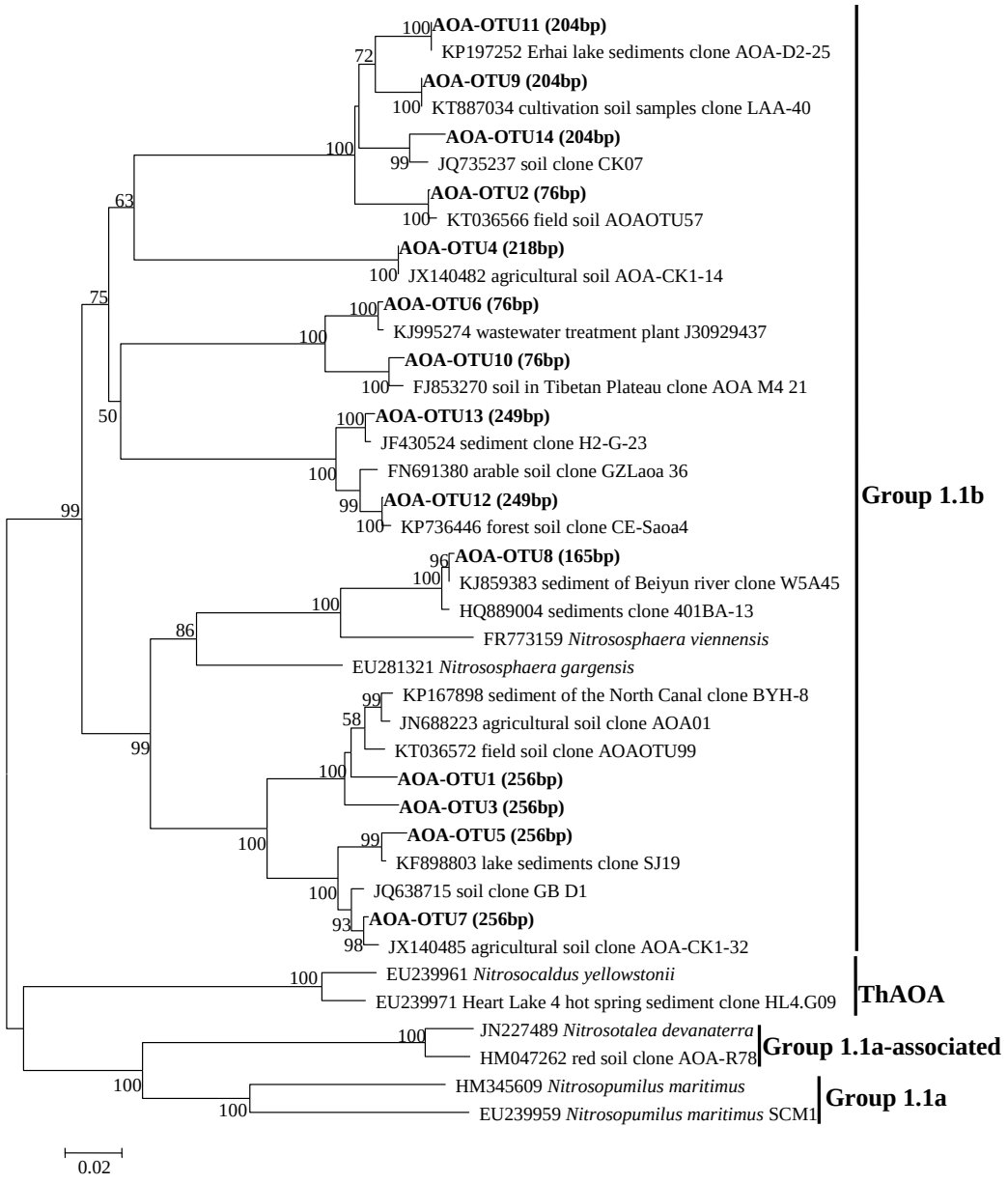
KF619080 wetland sediments clone CL15
AOB-OTU2 (255bp)
FN691042 arable soil clone GSWuWeiaob-11
AOB-OTU3 (255bp)
KJ717055 soil clone B27
AOB-OTU4 (255bp)
KJ081655 soil clone lj-aob11
DQ228454 Nitrosospira multiformis
DQ228457 Nitrosospira sp. PJA1
AOB-OTU5 (255bp)
KJ081647 soil lj-aob3
AOB-OTU6 (255bp)
KJ081646 soil clone lj-aob2
AOB-OTU7 (273bp)
HQ875271 soil clone NP-64
AY177967 agriculture soil clone
AOB-OTU8 (380bp)
KF619050 sediments clone BU28
AOB-OTU9 (251bp)
HQ678224soil clone FQ-13C-HF-16
AOB-OTU10 (269bp)
KC311330 grassland soil clone 12
AOB-OTU11 (251bp)
KF803067 sediment clone W6-AOB-2
AOB-OTU12 (211bp)
KP783357 sediment clone G4B06
AJ298719 Nitrosospira sp. Nsp2
AF016002 Nitrosospira sp. Np39-19
AOB-OTU13 (211bp)
KP781193 sediments clone SBE23
AY123821 Nitrosospira briensis
AOB-OTU14 (211bp)
KP781097 sediments clone SBB88
AOB-OTU15 (211bp)
KF803108 sediment clone W4-AOB-6
AOB-OTU16 (269bp)
KP781203 sediments clone SBE74
AF272405 Nitrosomonas
AF272404 Nitrosomonas
AF327918 Nitrosomonas sp. AL212
Cluster 3a.2
Cluster 3a.1
Cluster 3b
Cluster 6&7
Nitrosospira
Nitrososmonas
0.02

Fig.7


AOA-OTU11 (204bp)
KP197252 Erhai lake sediments clone AOA-D2-25
AOA-OTU9 (204bp)
KT887034 cultivation soil samples clone LAA-40
AOA-OTU14 (204bp)
JQ735237 soil clone CK07
AOA-OTU2 (76bp)
KT036566 field soil AOAOTU57
AOA-OTU4 (218bp)
JX140482 agricultural soil AOA-CK1-14
AOA-OTU6 (76bp)
KJ995274 wastewater treatment plant J30929437
AOA-OTU10 (76bp)
FJ853270 soil in Tibetan Plateau clone AOA M4 21
AOA-OTU13 (249bp)
JF430524 sediment clone H2-G-23
FN691380 arable soil clone GZLaoa 36
AOA-OTU12 (249bp)
KP736446 forest soil clone CE-Saoa4
AOA-OTU8 (165bp)
KJ859383 sediment of Beiyun river clone W5A45
HQ889004 sediments clone 401BA-13
FR773159 Nitrososphaera viennensis
EU281321 Nitrososphaera gargensis
KP167898 sediment of the North Canal clone BYH-8
JN688223 agricultural soil clone AOA01
KT036572 field soil clone AOAOTU99
AOA-OTU1 (256bp)
AOA-OTU3 (256bp)
AOA-OTU5 (256bp)
KF898803 lake sediments clone SJ19
JQ638715 soil clone GB D1
AOA-OTU7 (256bp)
JX140485 agricultural soil clone AOA-CK1-32
EU239961 Nitrosocaldus yellowstonii
EU239971 Heart Lake 4 hot spring sediment clone HL4.G09
JN227489 Nitrosotalea devanaterra
HM047262 red soil clone AOA-R78
HM345609 Nitrosopumilus maritimus
EU239959 Nitrosopumilus maritimus SCM1
Group 1.1b
ThAOA
Group 1.1a-associated
Group 1.1a
0.02