Diversity and Abundance of Ammonia-Oxidizing Archaea and Bacteria in Diverse Chinese Paddy Soils

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Received December 2012, Accepted April 2013

Ammonia-oxidizing archaea (AOA) and bacteria (AOB) in three types of paddy soils of China before and after rice plantation were investigated by using an integrated approach including geochemistry, 454 pyrosequencing, and quantitative polymerase chain reaction (PCR). The abundances of AOA amoA gene were 1~2 orders of magnitude higher than AOB amoA gene. The types of paddy soils had important impacts on the diversities of both AOA and AOB via clay mineralogy (smectite or illite-rich) and bioavailability of ammonium. The Nitrososphaera subcluster 5 and Nitrosopumilus cluster of AOA, and Nitrosospira subcluster 5 and Nitrosospira subcluster 3 of AOB were well adapted to soils with high ammonium concentrations. AOA and AOB community structures were different before and after rice plantation, likely due to changes of pH and ammonium fertilization. The Nitrososphaera subclusters 2 and 9 were well adapted to acidic paddy soils. However, the sensitivity of AOA and AOB community structures to these factors may be complicated by other geochemical conditions. The results of this study collectively demonstrated that multiple environmental factors, such as clay mineralogy, ammonium content and total organic carbon as well as soil pH, shaped AOA and AOB community structure and abundance.

Keywords: abundance, AOA, AOB, Chinese paddy soil, diversity, flooding

Introduction

The application of fertilizer, such as nitrogen and phosphorus is an important means of maintaining soil fertility for sustainable agriculture. Ammonium salt is a commonly used nitrogen fertilizer. Because NH4+ ion can be easily bound by clays in soil and protected against leaching, ammonium-based nitrogen fertilizer is widely used in China (Nieder et al. 2011). However, a biogeochemical process, called nitrification, converts ammonia to nitrate through a two-step process: ammonia oxidation (NH3 + O2 → NO2−) and nitrite oxidation (NO2− + O2 → NO3−). The NO3− produced from nitrification is highly mobile and can be leached into groundwater (Kogel-Knabner et al. 2010; Luo et al. 2011). Thus, the conversion of ammonium (NH4+) into nitrate (NO3−) is an adverse process affecting soil fertility, and for this reason, the nitrification process has received much attention, especially as it is related to soil fertility in rice paddy soils (Briones et al. 2003; Kronzucker et al. 2000).

An alternative pathway for the loss of nitrate from agricultural soil is via anaerobic denitrification, where nitrate is reduced to various products such as nitrite and nitrous oxide (N2O), a greenhouse gas (Hayatsu et al. 2008; Kowalchuk and Stephen 2001; Shen et al. 2012). The denitrification process becomes particularly important when there is seasonal or artificial flooding of soils, where microaerophilic or anoxic condition may prevail to promote denitrification.

The first step of nitrification is rate-limiting and is mainly performed by two groups of microorganisms: ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Prosser and Nicol 2008; Shen et al. 2012). Recent molecular studies have revealed that the Nitrososphaera cluster (also referred to as soil or cluster 1.1b) and the Nitrosopumilus cluster (also referred to as marine or cluster 1.1a) are predominant AOA in paddy soils (Chen et al. 2011; Pester et al. 2012).

Because the physiology of AOA is still poorly known, the reasons for their predominance and relationship to geochemical conditions remain unclear. AOB in paddy soils are represented by Betaproteobacteria, which mainly consist of two genera: Nitrosospira and Nitrosomonas (Chen et al. 2011; Ke and Lu 2011). Overall, Nitrosospira-like AOB are predominant in most paddy soils, while Nitrosomonas-like AOB are prevalent in oxic soils such as plant rhizosphere and surface...
soil, and in soils with intense nitrogen fertilization (Nicolaïsen et al. 2004; Wang et al. 2009).

The compositions of AOA and AOB in paddy soils can be influenced by multiple physicochemical properties, such as contents of organic carbon and nitrogen, soil type, pH, and flooding-drying cycle. For example, AOB are well-adapted to eutrophic environments with high loads of organic carbon and flooding-drying cycle. For example, AOB are well-adapted to eutrophic environments with high loads of organic carbon and total ammonium (NH$_4^+$+NH$_3$), such as agricultural soils and wastewaters (Erguder et al. 2009; Shen et al. 2012). In contrast, AOA have competitive advantages over AOB in oligotrophic environments, apparently due to their higher affinity for ammonia (Martens-Habbema et al. 2009; Schleper and Nicol 2010; Zhalnina et al. 2012). However, in some paddy soils AOA are one to three orders of magnitude more abundant than AOB, although paddy soils usually have high contents of nitrogen and organic carbon, especially after long-term intense fertilization (Chen et al. 2010; Chen et al. 2011; Fujii et al. 2010; He et al. 2007; Leininger et al. 2006; Lv et al. 2011).

However, high abundance of AOA does not necessarily suggest high activity: a study has shown that in nitrogen-rich soils AOB possess a higher ammonia oxidizing activity than AOA, although the latter may be more abundant (Di et al. 2009). In acidic soils, AOA have been shown to be more abundant and active than AOB, possibly due to low level of available ammonia, as ammonia tends to be ionized to ammonium in low pH environments (pK$_a$ of NH$_3$ : NH$_4^+$ = 9.25) (Pester et al. 2012; Zhang et al. 2011). Ammonia, not ammonium, is a direct substrate for ammonia monoxygenase as revealed by a study with *Nitrosomonas europaea* as a model organism (Suzuki et al. 1974).

Previous studies have observed that AOA and/or AOB community compositions differed in different types of paddy soils (Chen et al. 2010; Ke and Lu 2011), the underlying reasons for this difference, however, were not investigated. Clay composition is an important characteristic that directly impacts paddy soil type. Some clay minerals, smectite for example, have been shown to enhance AOA and AOB activity in acidic soils (Jiang et al. 2011). In addition, flooding (a routine management practice for rice planting) of paddy rice field is another important factor that controls microbial community structure. A successional study over a two-year period showed that flooding significantly decreased both AOA and AOB abundance due to depletion of oxygen in soil when flooded (Fujii et al. 2010), but this study was based on only one paddy soil, and thus extrapolation of this result to other kinds of paddy soils may be difficult. Overall, the above studies mostly focused on the effects of individual environmental conditions on AOA and AOB abundance and community structure in paddy soils, such as soil type, flooding, and pH, under well-controlled conditions. The combined effects of these environmental conditions remain unclear.

The purpose of this study was to assess the effects of multiple environmental factors (soil type, flooding, pH, and ammonium availability) on AOA and AOB abundance and community structure in paddy soils from three climatic zones of eastern China. An integrated approach was employed, including geochemistry, quantitative polymerase chain reaction, and bar-coded 454 pyrosequencing. The results show that multiple environmental factors as influenced by rice cultivation have important influence on AOA and AOB abundance and community structure.

**Methods**

**Site Description and Sampling**
Paddy soils were sampled from three different regions of eastern China: Suihua City in Heilongjiang Province (in abbrev. HLJ), Hefei City in Anhui Province (in abbrev. AH), and Dexing City in Jiangxi Province (in abbrev. JX) (Table 1). Heilongjiang is located in northeastern China and it is under cold temperate climate with a mean annual temperature (MAT) of 2.4°C and mean annual precipitation (MAP) of 531 mm. Black soil is the main type in HLJ. Anhui is located in central eastern China and is under a warm temperate climate with a MAT of 14.8°C and MAP of 796 mm. Yellow-brown soil is the main soil type in AH. Jiangxi, located in central southern China, is under a subtropical climate with a MAT of 17.2°C and a MAP of 1853 mm. Red soil is the main type in JX. Rice is one of the main crops in all these three regions.

Two samples of bulk paddy soils (away from the rhizosphere) were collected from each region: one was taken during the flooding period when rice was actively growing, and the other was taken at the same site during the dry period after rice was harvested (Table 1). Soil samples were collected from the top 10 cm with a pre-sterilized shovel. Each sample was a homogenous mixture of three separate soils collected from 0.5 m away from one another. All the collected samples were divided into two portions, which were immediately stored in 4°C and −80°C for geochemical and molecular analyses, respectively.

**Soil Geochemical Analyses**

Soil pH was determined in a 0.01 M CaCl$_2$ solution with a glass electrode (model 704 Mefrohm) (soil: CaCl$_2$ ratio 1:2) (Margesin and Schinner 2005). Total organic carbon (TOC), total nitrogen (TN), ammonium nitrogen (NH$_4^+$-N), nitrite nitrogen (NO$_2^-$-N), nitrate-nitrogen (NO$_3^-$-N), and nitrate-nitrogen (NO$_3^-$-N) were determined according to the established methods (Margesin and Schinner 2005). Microbial carbon (Micro-C), the microbial fraction of organic carbon, was determined by using the fumigation-incubation method as described previously (Martens 1995).

Quantitative powder X-ray diffraction was performed to identify the mineralogy of the paddy soil samples using a previously described method (Eberl 2003). Prior to analysis, soil samples (1 g) were well mixed with 0.25 g of an internal standard (corundum). Samples were X-ray scanned from 2 to 70 degrees 2θ with Cu K-alpha radiation (40 kV, 35mA), a 0.02 degree step size and a count time of 5 seconds per step. The XRD data were analyzed quantitatively and converted into weight percent using the RockJock computer program (Eberl 2003).

**DNA Extraction and Polymerase Chain Reaction (PCR) Amplification**

DNA was extracted from soils (~0.5 g per sample) with the use of FastDNA SPIN Kit for Soil (MP Biomedicals, Ohio,
Table 1. Physicochemical and mineralogical characteristics of the studied paddy soils.a

<table>
<thead>
<tr>
<th>Province</th>
<th>Heilongjiang</th>
<th>Anhui</th>
<th>Jiangxi</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT (°C)</td>
<td>2.4</td>
<td>14.8</td>
<td>17.2</td>
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<tr>
<td>MAP (mm)</td>
<td>531</td>
<td>796</td>
<td>1853</td>
</tr>
<tr>
<td>Sample ID</td>
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<td>AH1</td>
<td>JX1</td>
</tr>
<tr>
<td>L &amp; L (N, E)</td>
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<td>31.92169, 117.64561</td>
<td>29.12647, 117.9363</td>
</tr>
<tr>
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<td>102</td>
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<tr>
<td>pH</td>
<td>5.64</td>
<td>5.29</td>
<td>5.33</td>
</tr>
<tr>
<td>TOC (g kg⁻¹)</td>
<td>39.7</td>
<td>9.6</td>
<td>21.8</td>
</tr>
<tr>
<td>Micro-C (g kg⁻¹)</td>
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<td>0.5</td>
<td>2.86</td>
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<td>Micro-C/TOC (%)</td>
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<td>0.20</td>
<td>13.10</td>
</tr>
<tr>
<td>TN (mg kg⁻¹)</td>
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<td>1028.2</td>
<td>1580.8</td>
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<tr>
<td>NH₄⁺-N (mg kg⁻¹)</td>
<td>32.1</td>
<td>9.6</td>
<td>97.9</td>
</tr>
<tr>
<td>NO₃⁻-N (mg kg⁻¹)</td>
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<td>0.3</td>
<td>0.1</td>
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<td>46.6</td>
<td>44.1</td>
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<td>K-feldspar</td>
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<td>10.7</td>
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<td>1.0</td>
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<td>Dolomite</td>
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<td>0.4</td>
<td>0.9</td>
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<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
<tr>
<td>Pyrite</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
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<td>Total nonclay</td>
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<td>75.0</td>
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<td>25.9</td>
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<tr>
<td>Illite</td>
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<td>0.0</td>
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<tr>
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<tr>
<td>Muscovite</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
<tr>
<td>Total clay</td>
<td>36.0</td>
<td>25.0</td>
<td>47.6</td>
</tr>
</tbody>
</table>

aThe shaded samples were collected from flooded paddy soils with growing rice, and the nonshaded ones were collected after rice was harvested and water was drained.

Notations: amean average temperature, bmean average precipitation, clongitude and latitude, dtotal organic carbon, emicrobial carbon, and ftotal nitrogen.

UDL: under detection limit 0.1%.

USA) according to the manufacturer’s protocol. DNA quantity and quality were assessed by using a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The AOA and AOB amoA genes were amplified with the primer sets of Arch-amoAF (5'-STA ATG GTC TGG CTG AGA CG-3')/Arch-amoAR (5'-GGG GTT TCT ACT GGT GTG-3')/amoA2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') (Rotthauwe et al. 1997), respectively. To pool multiple samples for one run of 454 sequencing, a sample tagging method was used (Hamady et al. 2008). Special 5'-end tagged primers for each sample were designed by combining primers (Hamady et al. 2008) with adaptors ('TC' and 'CA' for forward and reverse primers, respectively) and a unique 8-mer tag (i.e., barcodes).

The AOA amoA gene PCR conditions were as follows: an initial denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The AOB amoA gene PCR conditions were as follows: an initial denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min.

To obtain enough amplicons for pyrosequencing, four PCRs were run in parallel for each sample and the products were assessed by using a Nanodrop® ND-1000 UV-Vis spectrophotometer. Finally, the purified PCR amplicons were pooled from all the samples in an equimolar concentration for 454 pyrosequencing.
Pyrosequencing and Data Analysis

The pyrosequencing work was commercially performed on a 454 GS FLX platform (Chinese National Human Genome Center in Shanghai). The obtained sequences were extracted, trimmed, quality-screened, and aligned with the use of the Mothur 2.25.0 pipeline (Schloss et al. 2009). Sequencing reads were assigned to each sample according to their unique barcodes, and low quality sequences (quality score < 25, length < 150 bp, ambiguous bases ≥1, homopolymer ≥6) were removed. The sequences from the reverse primer end of the amplicons (Arch-amoAR and amoA2R for AOA and AOB, respectively) were used for downstream data analysis. A total of 18638 and 4594 high-quality raw sequence reads were obtained for AOA and AOB, respectively.

The obtained high-quality raw sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity via the Qiime (quantitative insights into microbial ecology) pipeline (Caporaso et al. 2010; Kunin et al. 2010). To minimize potential deviant genotypes, OTUs with <0.5% relative abundance were treated as rare sequences and removed from further analysis. The remaining OTUs (relative abundance > 0.5%) were checked against a local amoA gene database (downloaded from the Ribosomal Database Project FunGene: http://fungene.cme.msu.edu/index.spr on November 27th 2011) and the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Chimeric sequences were identified and eliminated. A total of 102 (representing 16,642 sequences) and 86 (representing 4207 sequences) OTUs were obtained for AOA and AOB, respectively, and they were used for further downstream analyses. The raw sequence reads were deposited into the NCBI sequencing read archive (SRA056884).

Phylogenetic Analyses

One sequence was randomly selected from each OTU as a representative. To determine the phylogenetic affinities of the representative amoA gene sequences, previously published reference sequences and cluster nomenclatures of AOA (Pester et al. 2012) and AOB (Avrahami and Conrad 2003) were used. The representative amoA and reference sequences were combined and aligned by ClustalW. The alignments were manually screened for correct protein coding and DNA sequenced -based phylogenetic trees were constructed with the Neighbor-joining algorithms based on Jukes-Cantor distance matrix in Geneious V5.5.4 (http://www.geneious.com) (Drummond et al. 2011). These trees, although not shown, formed a basis for classification of all AOA and AOB community structure into various subclusters (see below).

Quantitative PCR

Abundances of archaeal and bacterial amoA genes and 16S rRNA genes in the investigated samples were determined according to a previously published procedure (Jiang et al. 2009). Briefly, the same primer sets (Arch-amoAF/Arch-amoAR for AOA and amoA1F/amoA2R for AOB, respectively) as those used for pyrosequencing but without tags were used for quantitative PCR (qPCR) of amoA genes. The archaeal and bacterial 16S rRNA gene copy numbers were determined with the primer sets of Arch349F/Arch806R and Bac331F/Bac797R, respectively. qPCRs were performed in triplicate on an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Amplification conditions for the 16S rRNA gene were 95°C for 15 min, followed by 40 cycles (15 s at 94°C, 30 s for annealing at 52°C and 55°C for bacteria and archaea, respectively, and 30 s at 72°C). qPCR amplifications were performed in a 20 μL reaction volume, containing 10 μL of 2× SYBR® Premix Ex Taq™ (Takara, Japan), 0.2 μM of each primer, 0.4 μL of ROX Reference Dye II (50×), 20 ng of Bovine Serum Albumin (Takara, Japan), and 1 μL of soil DNA.

PCR products (using the primer sets of Arch-amoAF/Arch-amoAR and amoA1F/amoA2R for AOA and AOB, respectively) of archaeal and bacterial amoA gene fragments from two soil samples were used for clone library construction. Purified amoA gene plasmids of two randomly selected clones (one each from AOA and AOB clone libraries) served as standards for AOA and AOB qPCRs, respectively.

A similar procedure was employed to prepare the bacterial and archaeal 16S rRNA standards. Serial dilutions of the standards were made and used to yield reliable exponential patterns with the amount of template in the range of 10^1 to 10^8 amoA gene copies and 10^1 to 10^8 16S rRNA gene copies, respectively. The data were used to create standard curves correlating the Ct values with gene copy numbers. Linear plots (not shown) between the Ct value and log (copy numbers/reaction) for amoA and 16S rRNA genes were obtained with correlation coefficients of R^2 >0.99. The qPCR amplification efficiencies were in the range of 95–101%. Blank controls (with double distilled H2O as template) were performed.

Statistical Analyses

Alpha diversity estimates for AOA and AOB including taxonomic richness (Chao1), Shannon diversity and Equitability evenness were calculated at the 97% cutoff level in the Qiime pipeline (Caporaso et al. 2010) after normalizing all remaining libraries to the one with the smallest number of reads (477 reads for AOA and 200 for AOB). Sequence coverage was calculated as follows: C = 1-n/N, where n is the number of phytophotypes that occurred only once in the clone library and N is the total number of clones analyzed (Jiang et al. 2009).

Pairwise correlation analysis between the abundance of AOA and AOB and geochemical variables was performed with the PAST version 2.16 software (Hammer et al. 2001). To compare similarity of AOA (or AOB) community structure among the six samples, UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering analysis was performed based on the Bray-Curtis matrix of OTU composition at the 97% sequences identity. Analysis of similarity (ANOSIM) was performed to test for any significant difference in microbial community composition among the defined groups according to UPGMA clustering. SIMPER (similarity percentage) analysis was applied to rank the contributions of AOA/AOB clusters or subclusters to the differences observed in the UPGMA clustering pattern.
The metaMDS and envfit functions (‘vegan’ package) in the R software were used to display the correlations between AOA and AOB diversity and soil geochemical variables. By plotting individual environmental parameters onto a given nonmetric multidimensional scaling (NMDS) ordination of Bray Curtis similarities at the subcluster level, the correlations between the major subclusters of AOA and AOB and soil variables were made. Spearman test was performed to confirm the correlations between microbial groups and environmental variables. Other than coverage index, all statistical analyses were performed in the PAST software version 2.16 (Hammer et al. 2001).

Results

Paddy Soil Physiochemical Characteristics

There were large ranges in pH, TOC and TN, micro-C, NH\textsubscript{4}+, NO\textsubscript{3}–, and NO\textsubscript{2}– (Table 1). Differences in the physical-chemical characteristics were observed between nonflooded and flooded soils. For example, as paddy soils underwent the dry (no rice grown) to wet (rice grown) cycle, pH changed from nearly neutral (6.46–7.66) to acidic (5.29–5.64), TOC content decreased from 11.2–43.1 to 9.6–39.7 mg/kg, and TN content decreased from 1188.8–3778.5 to 1028.2–2501.1 mg/kg. In contrast, the NH\textsubscript{4}+ increased as a result of application of ammonium fertilizer.

XRD data showed that soil minerals could be divided into nonclay and clay fractions. Nonclay minerals were dominated by quartz (20.6%–50.8%), feldspar (K-feldspar and plagioclase together 8.6%–9.5%), and minor carbonates (calcite and dolomite). For clay minerals, smectite (21.1%–25.9%) was the most abundant in the four soils from HLJ and AH provinces, but kaolinite, smectite, and illite dominated the JX soil samples. As expected, there were no major differences in the mineralogy between flooded and nonflooded soils. Some small differences were probably caused by soil inhomogeneities.

Abundance of Archaeal and Bacterial amo\textsubscript{A} Genes

The numbers of archaeal and bacterial amo\textsubscript{A} gene were 2.07×10\textsuperscript{6}–8.47×10\textsuperscript{7} and 2.18×10\textsuperscript{5}–4.27×10\textsuperscript{6} copies per gram dry soil, respectively. AOA were more abundant than AOB except for HLJ2 (Figure 1A). The ratio of AOA amo\textsubscript{A} gene to total archaeal 16S rRNA gene ranged from 0.5% to 24.1%.

![Fig. 1. The abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in the paddy soils. A: abundances of AOA and AOB amo\textsubscript{A} genes (represented by black and grey bars, respectively). Error bars indicate standard deviations (n = 3); and B: The ratio of AOA/total amo\textsubscript{A} gene abundances to AOB abundance (represented by black and grey bars, respectively).](https://example.com/fig1.png)
and this ratio was the highest for the AH samples. In contrast, the ratio of AOB \textit{amoA} gene to total bacteria 16S rRNA gene was lower than \(10^{-6}\) for all the investigated samples (Figure 1B). Flooding promoted AOB for all three types of soils, but inhibited (for JX soils) or did not change AOA (AH soils). For HLJ soil, flooding also promoted AOA.

**AOA and AOB Diversity**

A total of 16642 AOA and 4207 AOB \textit{amoA} gene sequences were used for phylogenetic analysis. These sequences clustered into 188 OTUs (102 and 86 for AOA and AOB, respectively) at the 97% similarity level. Coverage index was higher than 98% except AOB of HLJ1 (84.5%), indicating that sequencing depth was sufficient to cover AOA and AOB diversity in the samples.

All the three alpha diversity indices (Chao1, Shannon, and Equitability) for AOB were higher than those for AOA in the paddy soils from HLJ Province, but this trend was reversed for AH soils and varied for JX soils (Figure 2). Although the effects of flooding on the estimated OTU numbers (i.e., Chao 1) varied among different soils (Figure 2A), Shannon diversity and Equitability evenness indices for AOA in all three types of paddy soils decreased as a result of flooding. The same trend was observed for AOB in HLJ and AH soils (Figures 2B and 2C), but the AOB diversity pattern in JX soils was reversed after flooding.

**AOA and AOB Community Structures in Correlation with Geochemistry**

The obtained AOA \textit{amoA} gene sequences were mainly affiliated with \textit{Nitrososphaera}, \textit{Nitrosostalea}, and \textit{Nitrosospumilus}, which accounted for 70.0%, 17.6%, and 12.4% of all sequence reads, respectively. The distribution of these clusters and their subclusters varied across the samples (Figure 3A). Bootstrapped UPGMA clustering, based on the Bray–Curtis similarity at the 97% OTU level similarity, revealed specific groupings of AOA and AOB communities in the studied soil samples (Figure 3). AOA showed four groups, approximately in accordance with the geographical location (i.e., provinces) (cophenetic coefficient 0.96), which was also supported by the NMDS ordination analysis (Figure 3).

The difference among the UPGMA-defined four groups was confirmed by ANOSIM analysis (ANOSIM, R-statistic = 0.78, \(p = 0.09\)), although this difference may not be statistically significant. The two soils from HLJ grouped together. The two soils from JX were different from the HLJ and AH soils as well as from each other (Figure 3A). SIMPER analysis was used to rank the contributions of different taxonomic groups to the dissimilarities among the four AOA groups (Table 2). As expected, dominant clusters or subclusters contributed to the dissimilarities among these four groups. For example, the \textit{Nitrosostalea} cluster, the \textit{Nitrososphaera} subcluster 9 and subcluster 1.1 were the top three responsible for the differences between the HLJ and AH groups.

\textit{Nitrosostalea} was the first and second most abundant group in AH1 (73.2%) and AH2 (32.6%), respectively, yet no \textit{Nitroso-

![Fig. 2. Alpha diversity of ammonia-oxidizing archaea (AOA, black symbols) and ammonia-oxidizing bacteria (AOB, grey symbols) in the Chinese paddy soils, including A) Chao1, B) Equitability evenness and C) Shannon diversity.](image-url)
Table 2. Contribution of clusters/subclusters to the dissimilarity between the different AOA groups defined in the UPGMA cluster analysis

<table>
<thead>
<tr>
<th>AOA taxon</th>
<th>HLJ vs. AH</th>
<th>HLJ vs. JX1</th>
<th>HLJ vs. JX2</th>
<th>AH vs. JX1</th>
<th>AH vs. JX2</th>
<th>JX1 vs. JX2</th>
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<tbody>
<tr>
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AOB taxon

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For AOA, four groups were identified: HLJ group (HLJ1 and HLJ2); AH group (AH1 and AH2); JX1; and JX2. The numbers in the table are percentage of each subcluster. The top three clusters/subclusters are highlighted. For AOB, three groups were defined. Group 1 contains HLJ1, AH1 and AH2; Group 2 contains HLJ2 and JX1 and Group 3 contains JX2 only.

![UPGMA cluster tree based on the Bray-Curtis matrix obtained at the 97% cutoff value, and bar graphs showing phylogenetic composition of AOA and AOB in each sample (subcluster level) in relative percentages. A bootstrap test with 1000 iterations was performed.](image-url)
AOA and AOB in Chinese Paddy Soils

Fig. 4. Nonmetric multidimensional scaling (NMDS) ordination plots of AOA (A) and AOB (B) in the six studied paddy soils (black symbols) and subclusters (grey diamonds). A biplot was overlaid on each ordination to the displayed soil geochemical variables that were correlated with the AOA/AOB community structure, only significant variables were displayed (Spearman's p < 0.05). Certain subclusters are displayed to show their correlations with geochemical variables.

3a.2 and Nitrosomonas subcluster 8 accounted for the dissimilarities between these three groups (Table 2).

The Nitrosospira subcluster 2 was the most abundant group in HLJ1 (54.9%), AH1 (95.6%) and AH2 (90.0%). The Nitrosospira subcluster 3a.2 was abundant in HLJ2 (46.5%) and JX1 (90.6%), but the Nitrosomonas subcluster 8 constituted the largest group in JX2 (52.8%). Despite its close geographic proximity to HLJ1, HLJ2 was different in AOB composition. Likewise, although JX1 and JX2 were of the same soil type and from the same province, they had different AOB compositions (Figure 3B).

The AOA and AOB community structure was strongly shaped by the geochemical (i.e., TOC, TN, and ammonium) properties of the soils as revealed by NMDS ordination (Spearman p < 0.05, Figure 4). Clay mineralogy of the soil samples showed a strong influence on both AOA and AOB: AOA and AOB community structures in soils from HLJ and AH provinces with higher contents of smectite were very different from those in JX soils (Figures 3 and 4), which were dominated by kaolinite and illite.

In addition, high loads of TOC and TN exhibited a profound effect on the AOA composition in HLJ1 and HLJ2 (Figure 4A). In particular, these high TOC and TN soils favored Nitrososphaerae subcluster 9. High NH$_4^+$-N concentration in JX soils appeared to be important in shaping both AOA and AOB composition as well. In particular, Nitrosospira subcluster 3a.2 and Nitrosomonas cluster appeared to be abundant in JX soils with high loads of ammonium. Although NMDS did not identify pH as an important factor in controlling AOA and AOB community structure (Figure 4), the Nitrosospira subcluster 2 was associated with lower pH of AH1 and AH2 soils. Specifically, acidic AH1 and AH2 soils favored the Nitrosospira subcluster 2. The AOB community structure shifted from the Nitrosospira subcluster 3 in slightly alkaline HLJ2 to the Nitrosospira subclusters 2 and 9 in acidic HLJ1.

Discussion

Effects of Paddy Soil Type on AOA and AOB

Clay minerals in soils play important roles such as nutrient cycling and plant growth (Dong 2012). For example, smectite can slow down organic matter degradation by microorganisms (Curry et al. 2007; Yu et al. 2009 and references) and fix NH$_4^+$ by intercalation into the smectite interlayer (Kyle and Schroeder 2007). A previous study (Jiang et al. 2011) has shown that montmorillonite (a particular type of smectite) amendment increased pH, which resulted in a decrease of the NH$_4^+$ activity and production of NH$_3$, supposedly through the reaction NH$_4^+$ + OH$^- \rightarrow$ NH$_3$ + H$_2$. The liberated NH$_3$ enhanced AOA and AOB abundance and nitrification activity. Although it was not possible to assess the effect of smectite on nitrification activity because of lack of bulk nitrification activity measurement in our study, we did observe different community structure in the soil samples with different clay mineralogy (Figure 3).

Because of high abundance of smectite in these soils, some ammonium is likely intercalated into the smectite structure interlayer. Therefore, despite one order of magnitude difference in total ammonium concentration among the samples (for example, between AH and JX soils), the difference in the amount of bioavailable ammonium may not be as large, because the release of ammonium from the smectite interlayer may be a slow process and can be kinetically controlled.
Despite the fact that some ammonium may be associated with clay minerals and may not be available, AOB community structure still displayed a distinct response to ammonium-N (Table 1, Figure 3B). Previous studies have observed change of AOB community structure in response to ammonium fertilization (Baolan et al. 2012; Verhamme et al. 2011). Nitrosomonas-related AOB are traditionally considered to become dominant in soils with high ammonium contents (Kowalchuk and Stephen 2001; Wang et al. 2009), and the Nitrosospira subcluster 3 was also found to be ubiquitous in soils with high nitrogen fertilizer supply (Shen et al. 2011; Wang et al. 2009). These two subclusters of AOB (Nitrosomonas and Nitrosospira subcluster 3) were indeed observed in JX1 and JX2 soils with high loads of ammonium. In contrast, in HLJ and AH paddy soils where ammonium concentrations were comparatively low, AOB communities were largely dominated by other subclusters of Nitrosospira.

Because of a general lack of physiological data for AOA (Stahl and de la Torre 2012), it was not possible to assess the role of ammonium in shaping AOA community structure. One interesting observation was that Nitrosopumilus maritimus strain SCM1 apparently tolerates a low level of ammonia (half-saturation constant of total ammonia \( K_m = 132 \) nM) (Martens-Habbena et al. 2009), yet the Nitrosopumilus-related sequences were abundant in JX2 soil (with high ammonium). Again this observation suggests that not all measured ammonia may be available to Nitrosopumilus because of ammonium intercalation into the smectite interlayer.

In contrast to smectite, illite and kaolinite clay minerals have lower cation exchange capacity (CEC) than smectite (SAWI-INEY 1972; Knaebel et al. 1994; Nieder et al. 2011). A recent study has reported presence of ammonium in illite and other clays such as vermiculite (Bobos 2012), but this ammonium is not expected to be bioavailable and is not expected to have any effects on AOA and AOB activity, likely because of the nonexpandable nature of illite and vermiculite.

In addition to clay mineralogy and ammonium availability, TOC and TN also appeared to be important in influencing AOA and AOB. The low alpha diversity and distinct AOA community structure in high TOC and TN soils (HLJ) supported the view that AOA may be more sensitive to organic matter than AOB. AOA used to be believed to be autotrophic (Schleper and Nicol 2010), but this view was challenged by the cultivation results of AOA whose growth was dramatically enhanced from soils (Tourn et al. 2011) and by culture-independent surveys of AOA in organic matter rich soils (Schleper and Nicol 2010; Shen et al. 2012). Therefore, AOA may be mixotrophic or heterotrophic in soils. The predominance of recently nominated Nitrosoaphala subcluster 8.1 and 9 in HLJ black soils is consistent with this speculation.

Effect of Flooding on AOA and AOB Community Structure

The different AOA and AOB community structures under flooded and dry conditions were likely due to changes of soil pH and ammonium-N (Table 1). Because the oxidation of ammonia to nitrite produces acidity, nitrification by AOB is highly sensitive to changes in pH, likely due to the exponential decrease of free ammonia at acidic pH (De Boer and Kowalchuk 2001). Free ammonia is thought to be the substrate for the enzyme ammonia mono-oxygenase (Suzuki et al. 1974), and the transport into the cells is by diffusion (Woods 1986).

However, transport of ammonium (the dominant form at acidic pH) into the cells is not by passive diffusion but requires energy. Despite this fact, autotrophic nitrification has been reported in acidic soils at pH values as low as 3.3. Among the reasons for nitrification in such acidic soils are growth and/or activity of AOB on soil particle surfaces or in soil aggregates and ureolytic activity (Prosser and Embley 2002). Clearly, abundant clay minerals in the studied paddy soils could have served this role by providing large surface areas and soil aggregates.

The predominance of the Nitrosospira subcluster 2 and 9 in acidic paddy soils was consistent with previous studies. For example, the Nitrosospira subcluster 2 has been previously observed to be dominant in acidic environments and contains putatively acidophilic AOB (Kowalchuk and Stephen 2001), and the Nitrosospira subclusters 9, 10, 11 and 12 are major groups in acidic paddy soils (Chen et al. 2011; Ying et al. 2010). However, the pH effect may be complicated by additional factors such as ammonium, as a previous study (Bruns et al. 1999) has shown an association between the Nitrosospira cluster 3 subgroup and soils amended with inorganic N. Similarly, the observed shift from a mixture of the Nitrosomonas subcluster 8 and the Nitrosospira subcluster 3.2 of AOB in slightly alkaline JX2 soil to the Nitrosospira subcluster 3a.2 in acidic JX1 soil was likely due to a combination of both pH change and ammonium fertilization.

Previous studies have shown the importance of pH in affecting the structures of AOA as well (Gubry-Rangin et al. 2011; Nicol et al. 2008; Zhang et al. 2011). The Nitrososphaera (1.1b) and Nitrospumilis (1.1a) clusters are referred as alkaliphilic and acido-neutrophic, respectively (Zhalnina et al. 2012), whereas the Nitrosotalea cluster (1.1a associated) is referred as acidophilic (Gubry-Rangin et al. 2011; Ying et al. 2010).

However, more recent studies (Baolan et al. 2012; Gubry-Rangin et al. 2011; Pester et al. 2012) do not support these observations and have shown that some subclusters within Nitrososphaera can be acido-neutral, and Nitrosotalea can be important in nonacidic soils. Our study appeared to support these more recent studies in showing that there was no specific dependence of AOA on pH. In fact, Nitrosotalea and Nitrososphaera clusters were ubiquitous in acidic soils (HLJ1 and JX1), and the Nitrosotalea cluster was one of major AOA components in a nonacidic soil (AH2). The underlying reasons for responses (or lack) of AOA composition to pH still remain unclear, likely because of lack of AOA cultures.

In summary, AOA and AOB community structures were different under flooded and dry conditions, likely because of changes of pH and ammonium fertilization. However, the sensitivity of AOA and AOB community structures to these factors may be complicated by other geochemical conditions such as soil mineralogy.
Acknowledgments

This work was supported by the National Program on Key Basic Research Project (973 Program) (2012CB822000) and the Scientific Research Funds for the 1000 “Talents” Program Plan from China University of Geosciences-Beijing. The authors are grateful to Mr. Joseph P. Peacock from the University of Nevada, Dr. Marco J. L. Coolen from Woods Hole Oceanographic Institute and Dr. James T. Hollibaugh from the University of Georgia for their suggestions on 454 pyrosequencing data analysis. We are grateful to two anonymous reviewers whose comments improved the quality of this manuscript.

References


