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# pH regulates key players of nitrification in paddy soils

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ABSTRACT

Increasing lines of evidence have suggested the functional importance of ammonia-oxidizing archaea (AOA) rather than bacteria (AOB) for nitrification in upland soils with low pH. However, it remains unclear whether niche specialization of AOA and AOB occurs in rice paddy wetlands constrained by oxygen availability. Using DNA-based stable isotope probing, we conclude that AOA dominated nitrification activity in acidic paddy soils (pH 5.6) while AOB dominated in alkaline soils (pH 8.2). Nitrification activity was stimulated by urea fertilization and accompanied by a significant increase of AOA in acid soils and AOB in alkaline soils. DNA-based stable isotope probing indicated significant assimilation of $^{13}CO_2$ for AOA only in acidic paddy soil, while AOB was the solely responsible for ammonia oxidation in the alkaline paddy soil. Phylogenetic analysis further indicated that AOA members within the soil group 1.1b lineage dominated nitrification in acid soils. Ammonia oxidation in the alkaline soil was catalyzed by *Nitrosospira* cluster 3-like AOB, suggesting that the physiological diversity of AOA is more complicated than previously thought, and soil pH plays important roles in shaping the community structures of ammonia oxidizers in paddy field.



## 1. Introduction

Nitrification is the only biological process that links the reduced nitrogen and the oxidized nitrogen species to sustain global nitrogen cycling. Both ammonia-oxidizing bacteria (AOB) and archaea (AOA) possess the *amoA* genes encoding ammonia monooxygenase subunit A, which implies that both AOB and AOA are key players in nitrification (Francis et al., 2005; Leininger et al., 2006; Wuchter et al., 2006; Lam et al., 2007; Nugroho et al., 2009). Microbial regulating factors of nitrification have received increasing attention to help decipher the relative importance of AOA and AOB to nitrification. Tourna et al. (2008) and Offre et al. (2009) suggested that AOA plays a dominant role in soil nitrification, however, bacteria rather than archaea are more active ammonia oxidizers in an agricultural soil (Jia and Conrad, 2009) and in a N-rich grassland soil (Di et al., 2009). These contrasting results imply that the relative importance of AOB and AOA may vary depending on environmental conditions (Erguder et al., 2009; Wessén et al., 2010). Increasing lines of evidence have suggested that soil pH plays an important role in shaping the communities of active ammonia oxidizers in complex environments. For example, nitrification is driven by bacteria rather than archaea in N-rich grassland soils and agricultural soils with pH about 7.0 (Di et al., 2009; Jia and Conrad, 2009), whereas archaea control nitrification in acidic agricultural soils, low-nutrient or sulfide-containing environments (Erguder et al., 2009; Wessén et al., 2010).

It has indeed long been recognized that the nitrification process is highly sensitive to pH (Strayer et al., 1981; Paul and Clark, 1989; Curtin et al., 1998). Ammonia is generally thought to be the substrate for both AOA and AOB (Suzuki et al., 1974), and its concentration exponentially declines with decreasing pH due to the ionization of ammonia to ammonium (Allison and Prosser, 1993; Burton and Prosser, 2001). Soil pH thus most likely determines the chemical form, concentration and availability of substrates in association with nitrification (Kemmitt et al., 2006). A recent study has shown that AOA has by far the greatest substrate affinity identified for any autotrophic ammonia oxidizer, which is consistent with *in situ* nitrification kinetics measured in the low-nutrient open ocean (Martens-Habbena et al., 2009). The cultivation of the obligate acidophilic *Nitrosotalea devanaterra* provided further

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evidence for the extraordinary capability of AOA to thrive in low-pH soils (Lehtovirta-Morley et al., 2011). However, the ecological generalizations of AOA and AOB ecophysiology would be greatly substantiated in ly-contrasting environments (Prosser and Nicol, 2012).

The distribution and activity of archaeal and bacterial ammonia oxidizers are also influenced by soil pH (Prosser and Embley, 2002; Avrahami and Conrad, 2003; Nicol et al., 2008). Bacterial and archaeal amoA genes have been recovered from soils with pH values ranging from 3.7 (He et al., 2007) to 8.6 (Shen et al., 2008). Soil pH also appears to lead the selection for different bacterial and archaeal communities (Stephen et al., 1998; Nugroho et al., 2007; Nicol et al., 2008; Lehtovirta et al., 2009), suggesting adaptation and selection of particular phylogenetic groups in acidic soils.

Almost 155 million ha of land are used for rice cropping, and more than 50% of the world's population feeds on rice (FAOSTAT, 2010). Paddy soil development is driven by specific soil management practices that could lead to drastic changes in soil physiochemical properties (Kirk et al., 2004), and paddy soils usually face multiple drying–wetting cycles during the rice-growing season (Nishimura et al., 2004; Jia and Conrad, 2009). The periodic short-term redox cycles induced by this specific paddy management have effects on nitrification (Kikuchi et al., 2007; Xin et al., 2014). For example, a positive correlation between nitrification activity and the abundance of AOB in rice paddy soil was observed, and AOB was suggested more responsible than AOA for the ammonia oxidation (Li et al., 2014). However, it was also implied that both AOA and AOB are key players in nitrification for paddy soils (Alves et al., 2013). More studies are necessary to understand the relative contribution of AOA and AOB to nitrification in rice paddy soils. Therefore, DNA-based stable isotope probing method and sequencing analysis were used to reveal different groups of AOA and AOB responsible for ammonia oxidation in acid and alkaline paddy soils.

## 2. Materials and methods

### 2.1. Experimental location and soil sampling

The field experiment was carried out at the Purple Soil Ecology Experimental Station of Southwest University, located in the southwest of China, since 1990 (30° 26′ N, 106° 26′ E). The climate is subtropical with a mean annual temperature of 18.2 °C and a mean annual total rainfall of 1080 mm. A alkaline (pH 8.2) and an acidic flooded paddy soil (pH 5.6) were selected for this study. Rice (*Oryza sativa* L.) was planted in flooded fields in summer and the land remained fallow and flooded in winter. Fertilization was applied to field plots at 270 kg $ha^{-1}$ urea, 500 kg $ha^{-1}$ calcium superphosphate and 150 kg $ha^{-1}$ potassium chloride annually over the last two decades. The size of the plots was $4 \times 5$ $m^2$, and the experiment was organized in a randomized complete block design (RCBD) with four replications.

Both the soils were classified as a hydargric Anthrosol (FAO, 1988), but the alkaline soil developed from purple mudstone and the acidic soil developed from purple sandstone. The properties for the acid soil are: soil pH 5.6 ($H_2O$), 11.3 g organic matter $kg^{-1}$, 0.83 g total N $kg^{-1}$, 11.9 mg $NH_4^+$-N $kg^{-1}$, 9.1 mg $NO_3^-$–N $kg^{-1}$, 0.7 g $kg^{-1}$ total P, 19.7 g $kg^{-1}$ total K, 4.4 mg $kg^{-1}$ available P, and 49.4 mg $kg^{-1}$ available K. The properties for the alkaline soil are as follows: pH ($H_2O$) 8.2, 21.7 g $kg^{-1}$ organic matter, 1.7 g $kg^{-1}$ total N, 12.1 mg $NH_4^+$-N $kg^{-1}$, 6.9 mg $NO_3^-$–N $kg^{-1}$, 0.8 g $kg^{-1}$ total P, 22.7 g $kg^{-1}$ total K, 6.2 mg $kg^{-1}$ available P, and 121 mg $kg^{-1}$ available K. Theoretical calculations showed that the acid soil had an ammonia concentrations of 0.642 μM in the field, while up to 238 μM occurred in the alkaline soil.

Four field replicates were collected from each plot at a 0–20 cm depth in October 2012, with each replicate being composed of five individual soil cores (diameter was 5.5 cm). The composite samples from each plot were homogenized, air-dried to a water content about 15% (m/m), passed through a 2.0-mm sieve, and stored at −20 °C before use.

### 2.2. Chemical analysis

The soil pH was determined using a Mettler Toledo 320-S pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) with a water-to-soil ratio of 2.5. The soil organic matter content was determined using the dichromate oxidation method, while total N was determined using the Kjeldahl method. Ammonium and nitrate were extracted from soil samples with 2 M KCl, and concentrations measured by a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands). Available P was extracted with sodium bicarbonate, and concentrations determined using the molybdenum blue method. Available K in the soil was extracted using ammonium acetate, and concentrations were measured by flame photometry.

### 2.3. DNA-SIP microcosms

Soil DNA-SIP microcosms were constructed to investigate the active soil-nitrifying community as described previously (Jia and Conrad, 2009). It includes two treatments of $^{13}CO_2$-labeled microcosms and $^{12}CO_2$-control microcosms. Pairwise comparison between the $^{13}CO_2$-labeled and $^{12}CO_2$-control treatment was used to assess whether ammonia oxidizers assimilated $^{13}CO_2$ for autotrophic growth. For each treatment, 10 g of sieved fresh soil (equivalent to 8.0 g dry weight gram soil, *d.w.s.*) was placed in a 120 ml serum bottle tightly capped with a butyl rubber stopper, and the headspace of the bottle contained 5% (vol/vol) $^{13}CO_2$ or $^{12}CO_2$. Microcosms were incubated at 60% of the soil maximum water-holding capacity at 28 °C in the dark. The $^{13}CO_2$ treatments received 100 μg $^{13}$C-urea-N weekly, and the $^{12}CO_2$ treatments received 100 μg $^{12}$C-urea-N $g^{-1}$ *d.w.s.* weekly throughout the eight-week incubation period. The headspace of the each bottle was flushed weekly with pressurized synthetic air (20% $O_2$, 80% $N_2$) for 1 min to maintain oxic conditions. Water loss was replaced by adding sterilized water, and the $^{13}CO_2$ and $^{12}CO_2$ were also renewed immediately after the headspace air exchange.

The $^{13}$C-urea and $^{12}$C-urea (99 atom% carbon) was purchased from the Shanghai Engineering Research Center of Stable Isotopes (Shanghai, China), and $^{13}CO_2$ (99 atom% carbon) was purchased from Sigma–Aldrich Co. (St Louis, MO, USA).$^{12}CO_2$ was produced by acidifying sodium carbonate. Destructive sampling was performed in triplicate from each treatment during the incubation period, and the soil samples were transferred immediately to a −80 °C freezer for subsequent molecular analysis. The rest of the samples were used for determination of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N concentrations.

### 2.4. SIP gradient fractionation

Soil DNA was extracted using a FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions. The concentration of extracted DNA was determined with a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the total DNA extract was then subjected to isopycnic centrifugation to separate $^{13}$C-DNA from native $^{12}$C-DNA in the labeled and control microcosms (Xia et al., 2011). Three micrograms of total DNA extract with an initial CsCl buoyant density of 1.725 g $ml^{-1}$ was placed in a 5.1 ml