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# Archaea are the predominant and responsive ammonia oxidizing prokaryotes in a red paddy soil receiving green manures

Song-juan Gao[a,b,c,1], Dan-na Chang[b,d,1], Chun-qin Zou[c,*], Wei-dong Cao[b,e,**], Ju-sheng Gao[b,f], Jing Huang[b,f], Jin-shun Bai[b], Nao-hua Zeng[b], Robert M. Rees[g], Kristian Thorup-Kristensen[h]

[a] College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, 210095, China
[b] Key Laboratory of Plant Nutrition and Fertilizer, Ministry of Agriculture / Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, 100081, China
[c] College of Resources and Environmental Sciences, Center for Resources, Environment and Food Security, Key Laboratory of Plant-Soil Interactions, Ministry of Education, China Agricultural University, Beijing, 100193, China
[d] Institute of Plant Nutrition, Agricultural Resources and Environment Sciences, Henan Academy of Agricultural Sciences, Zhengzhou, 450002, China
[e] Qinghai Academy of Agriculture and Forestry, Qinghai University, Xining, 810016, China
[f] Red Soil Experimental Station in Hengyang, Chinese Academy of Agricultural Sciences, Qiyang, 426182, China
[g] Scotland's Rural College (SRUC), West Mains Road, Edinburgh, EH9 3JG, UK
[h] Department of Plant and Environmental Sciences, University of Copenhagen, 2630, Denmark

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ABSTRACT

Application of green manures is an effective approach to optimizing N management in paddy soils. Nitrification is a key process in the N cycle and ammonia oxidization is the first and typically limiting step in nitrification. In this study, we investigated the changes of ammonium oxidizing prokaryotes after the application of green manure in a red paddy soil using pot experiments. The experiment included four treatments; milk vetch-rice, radish-rice, ryegrass-rice and winter fallow-rice. The nitrification potential was measured, and the abundance and community of *amoA* genes from ammonia-oxidizing archaea (AOA) and bacteria (AOB) were quantified. The results showed that the AOA to AOB ratios ranged from 7 to 80, and that the milk vetch treatment increased the abundances of AOA and AOB. The abundance of AOA showed negative correlations with nitrification potential and $NH_4^+$-N, and positive correlation with soil pH in the acidic red paddy soil. DNA sequence analyses revealed that the *Nitrososphaera* and *Nitrosospira* were the dominant clusters of AOA and AOB, respectively. The dominant clusters of AOA were significantly changed by utilization of green manures, especially radish. Partial least squares path modeling analysis showed that green manures exerted larger effects on the abundances of AOA than on AOB, and the community structure of AOA had the strongest effect on nitrification potential. The high abundance of AOA found in this study and their responsiveness to green manuring suggests that AOA are critically important for soil ammonia oxidation in these soils and more sensitive to green manuring than AOB.

## 1. Introduction

Nitrification is the conversion of inorganic nitrogen from a reduced form to an oxidized state [1]. Ammonia oxidization is the first and typically the rate-limiting step, carried out by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The *amoA* gene, which can occur in both AOA and AOB is a good indicator of the abundance and diversity of ammonia oxidizing prokaryotes, and is commonly used as molecular biomarker in the studies of nitrification [2]. The abundance and communities of AOA and AOB are influenced by soil properties in agricultural soil. Many reports have revealed that pH is the main factor driving the community changes of AOA and AOB [2–6]. Substrate availability such as the amount of ammonium is also an important driver of both AOA and AOB species richness [2,7,8]. Other field management practices and soil properties such as fertility regime [9,10], manure input [11,12], soil moisture and temperature [13] may also affect the nitrification process and the distribution and activity of ammonia oxidizers. The relative contribution of AOA and

* Corresponding author.
** Corresponding author. Key Laboratory of Plant Nutrition and Fertilizer, Ministry of Agriculture / Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, 100081, China.
*E-mail addresses:* zcq0206@cau.edu.cn (C.-q. Zou), caoweidong@caas.cn (W.-d. Cao).
[1] These authors contributed equally to this work.

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AOB in different types of soils are still under debate [10]. Numerous studies have confirmed that AOA are predominate ammonia-oxidizing prokaryotes when compared to their bacterial counterparts in multiple terrestrial environments [14], such as those with alternating oxygenated/hypoxic conditions [15], and acidic soils [16], suggesting that AOA could have a potentially greater role in the overall nitrification than their bacterial counterparts [17].

The utilization of green manures in crop rotations is a management practice designed to improve soil fertility and reduce chemical fertilizer applications [18–20]. In southern China, the planting of winter green manures is used as an effective means of improving the productivity and sustainability of paddy fields under double-rice cropping systems [21,22], and milk vetch (*Leguminosae*), radish (*Cruciferae*) and ryegrass (*Gramineae*) are popular green manure species in this area. The production practices have proved that all of these three kinds of green manure improved the grain yield and soil fertility, and the effects of these three green manures have differences, but the mechanisms are unclear. The characteristics of the plant residues differ between plant families, and the release of nutrients during decomposition may have various influences on the abundance and diversity of AOA and AOB. Many studies have investigated the ammonia oxidizers in different kinds of paddy soils with various management practices [4,9,23,24]. However, the effects of different green manures on the abundance and community structure of ammonia oxidizers is not well understood. It is possible for us to find out the driving factors in nitrification by evaluating the responses of ammonia oxidizer on different green manures. After incorporation of green manures, the decomposition of plant residues and the priming effects may have more profound influences on soil conditions than the stage after rice harvest. In the period after rice cultivation, the interactive effects of green manure decomposition and rice growth may also change the nitrification process and activity of ammonia oxidizers. So, it is reasonable to compare the variations before and after rice cultivation.

In this study, we investigated the abundance and community diversity of ammonia oxidizers at the stages before rice transplantation and after rice cultivation in the winter green manure – double rice system. We hypothesized that (i) AOA *amoA* gene would be more abundant in this acidic paddy soil thus indicate the predominant of AOA compared with AOB, (ii) the different green manures had various effects on AOA and AOB, (iii) sampling stage may be one of the driving factors that led to the changes of ammonia oxidizers.

## 2. Materials and methods

### 2.1. Plant materials and soils

Pot experiments were conducted in the Red Soil Experimental Station (26°45′ N, 111°52′ E; elev. 150 m) of Chinese Academy of Agricultural Sciences, Qiyang, Hunan Province, China. The paddy soil is derived from Quaternary red clay, and classified as a Ferralic Cambisol [25]. On October 20, 2013, milk vetch (*Astraglus sinicus* L.), radish (*Raphanus sativus* L.) and ryegrass (*Lolium multiflorum*) were planted in three field plots, with a winter fallow plot as a control (weeds in the winter fallow plot were removed by hand and the plot was kept plant free). Soils and plants were collected at the full-bloom stage of milk vetch and radish (March 25, 2014). The above-ground parts of milk vetch, radish, ryegrass and their corresponding soils, as well as the soil from the winter fallow plots were sampled for the use of following pot experiments. Soils was collected to a depth of 0.2 m and sieved through a 2 mm mesh prior to use in the pot experiment. The properties of green manures and soils are shown in Tables S1 and S2.

### 2.2. Experimental design and sampling

Four treatments were designed including milk vetch-rice (the milk vetch soil was incorporated with the corresponding milk vetch, MV), radish-rice (the radish soil was incorporated with the corresponding radish, RD), ryegrass-rice (the ryegrass soil was incorporated with the corresponding milk ryegrass, RG) and winter fallow-rice (the milk vetch soil without plant residues was incorporated, WF). Each treatment was conducted in triplicate with a completely randomized design. The size of pots used in the experiments was 275 mm in height and 270 mm in diameter, and a total of 10 kg soil was packed into each pot. The green manures were collected at their full bloom stage, then the plants were chopped and weighed. The amount of green manure incorporated in each pot was 200 g fresh biomass. Each pot was received 0.6 g N, 0.6 g $P_2O_5$ and 0.6 g $K_2O$ (4 g 15-15-15 NPK compound fertilizer) as a basal fertilizer. All chemical fertilizer and green manures were applied on March 25, 2014. Rice plants were transplanted 30 days after the application of green manures. All pots were flooded after fertilization and during the growth of the rice.

Soils were sampled at two stages: 1) 30 days after the incorporation of green manure (before the rice was transplanted, on 25 April, Stage 1), and 2) after the rice was harvested (on 11 July, Stage 2). After being mixed well and sieved (< 2 mm), soil samples were divided, one batch was then stored at −20 °C for DNA extraction, and a second batch was stored at 4 °C prior to chemical analyses. After the rice harvest, the grain, shoots and roots were sampled separately, dried and milled to measure the yields and nutrient contents.

### 2.3. Chemical analysis

All soil analyses were conducted according to Lu [26]. Soil pH was measured using a pH electrode with a soil to water ratio of 1:2.5. Soil total nitrogen (TN) was determined using the Kjeldahl method. Soil organic matter (SOM) and total C of the plants were determined with the potassium dichromate oxidation method. Soil available phosphorus (AP) and available potassium (AK) were extracted by 0.5 mol $L^{-1}$ $NaHCO_3$ and 1 mol $L^{-1}$ $CH_3COONH_4$ respectively. Soil $NH_4^+$-N and $NO_3^-$-N were extracted by 2 M KCl, and measured on a continuous flow analyzer (AA3, SEAL, Germany).

### 2.4. Nitrification potential

Nitrification potential (NP) was measured using the chlorate slurry inhibition assay with slight modifications [1]. Briefly, each soil sample (5.0 g) was transferred to a 25 ml centrifuge tube including the 20 ml liquid medium (the concentration of $NH_4^+$-N was 100 mg $L^{-1}$ and the pH of the medium was adjusted to 7.5 using $H_2SO_4$ or NaOH solution). The slurry with soil and liquid medium was incubated for 5 h at 25 °C, and 0.2 ml sodium chlorate (1 M) was added to inhibit the oxidization of nitrite to nitrate during the incubation. After incubation, 5 ml of 2 M KCl was added to the extract and the nitrite released during the incubation period was then determined on a continuous flow analyzer (AA3, SEAL, Germany).

### 2.5. DNA extraction and real time quantitative PCR

Three DNA extractions per sample were performed using a FastDNA Spin Kit for Soil (MP Bio, Santa Ana, CA, USA) following the manufacturer's procedures. Three extractions from each soil sample were pooled and DNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). The DNA samples were stored at −80 °C for further analysis.

Real-time quantitative PCR of archaeal and bacterial *amoA* genes were performed on 7500 Real time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, US) in triplicate. The 25 μL reactions contained the following ingredients: 12.5 μL of Power SybrGreen qPCR Master Mix (Thermo Fisher Scientific Inc.), 0.5 μL each of 10 μM forward and reverse primers for ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), 9.5 μL of $ddH_2O$ and 2 μL of 10-fold diluted extracted DNA. Cycling protocols were 5 min at 95 °C followed