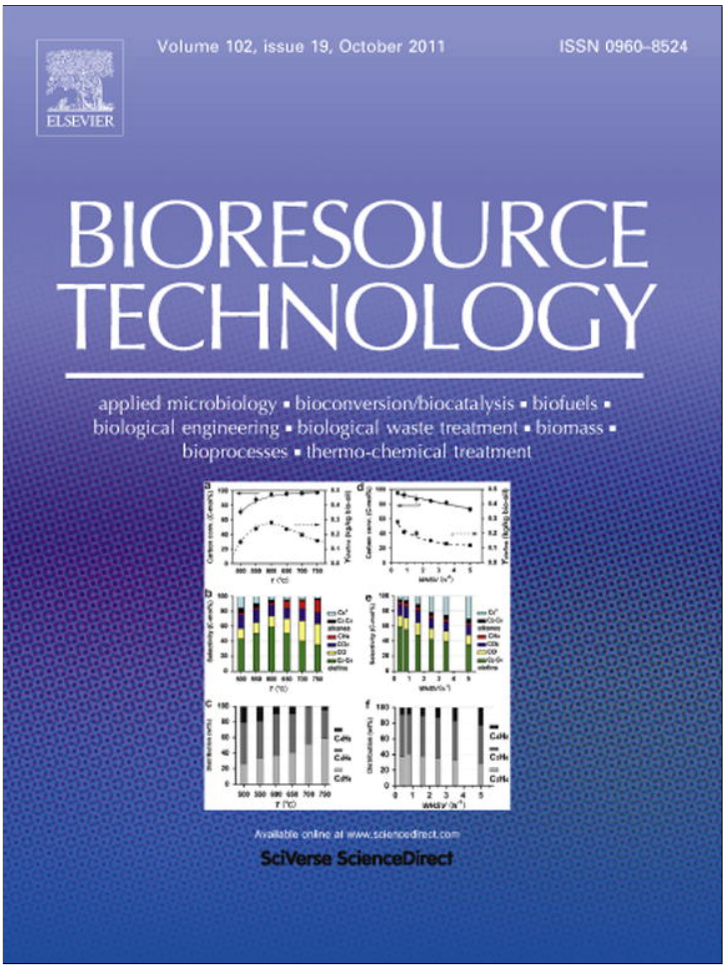

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Relative contributions of archaea and bacteria to microbial ammonia oxidation differ under different conditions during agricultural waste composting

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ABSTRACT

The aim of this study was to compare the relative contribution of ammonia-oxidizing archaea (AOA) and bacteria (AOB) to nitrification during agricultural waste composting. The AOA and AOB *amoA* gene abundance and composition were determined by quantitative PCR and denaturing gradient gel electrophoresis (DGGE), respectively. The results showed that the archaeal *amoA* gene was abundant throughout the composting process, while the bacterial *amoA* gene abundance decreased to undetectable level during the thermophilic and cooling stages. DGGE showed more diverse archaeal *amoA* gene composition when the potential ammonia oxidation (PAO) rate reached peak values. A significant positive relationship was observed between the PAO rate and the archaeal *amoA* gene abundance ($R^2 = 0.554$; $P < 0.001$), indicating that archaea dominated ammonia oxidation during the thermophilic and cooling stages. Bacteria were also related to ammonia oxidation activity ($R^2 = 0.503$; $P = 0.03$) especially during the mesophilic and maturation stages.

1. Introduction

Currently, composting is used as a major process of stabilizing agricultural organic wastes through the degradation of biodegradable components by microbial communities (Sánchez-Monedero et al., 2010; Yu et al., 2011; Zeng et al., 2010, 2011). It is of particular importance for providing a stable product that is high in nutrients easily accessible by plants. NH_3 and its unincorporated form NH_4^+ are the most important nitrogenous compounds available in the compost materials. An alkaline pH during the composting process may lead to substantial loss of nitrogen as gaseous NH_3. The emission of NH_3 results in a compost product of low nitrogen content, diminishing its value as a fertilizer (Beck-Friis et al., 2001). Nitrification, the transformation of NH_3 to NO_3^- via NO_2^- by ammonia oxidizing organisms (Bernal et al., 2009; Kowalchuk et al., 1999), can be regarded as the gatekeeper of nitrogen turnover during the composting process (Jarvis et al., 2009).

The oxidation of ammonia is the first and rate-limiting step of nitrification, and determines the transformation balance between oxidized and reduced forms of nitrogen (Jarvis et al., 2009; Wessén et al., 2010). It has been widely assumed that the first step of nitrification was performed almost exclusively by ammonia oxidizing bacteria (AOB) with all cultivated strains belonging to monophyletic groups within the *Beta-* and *Gammaproteobacteria* (Prosser and Nicol, 2008). Researchers have discovered the other group of ammonia oxidizers, i.e. the ammonia-oxidizing archaea (AOA) can also perform ammonia oxidation (Könneke et al., 2005; Venter et al., 2004). Both groups employ the same functional *amoA* gene, encoding a subunit of the ammonia monooxygenase enzyme responsible for the first step of the nitrification process.

The archaeal and bacterial ammonia oxidizers could theoretically respond differently to the micro-environmental conditions, as they belong to separate phylogenetic domains with different cell biochemical and metabolic process. AOB have been cultivated from the cattle manure compost sample (Shimaya and Hashimoto, 2008). Several clusters, such as the genera *Nitrosomonas* and *Nitrospira* have been detected in the household and animal waste composting (Jarvis et al., 2009; Kowalchuk et al., 1999; Maeda et al., 2010). AOB present were grouped in the *Nitrosomonas* lineage in the final cattle manure composting product (Innerebner et al., 2006). Several reports have shown that archaeal *amoA* gene have not been detected from any stages of the composting process (Maeda et al., 2010; Yamada et al., 2007). However, in another study, AOA were present in large numbers in cattle manure compost, where the archaeal *amoA* gene copies were significantly

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Table 1
The chemical characterization of composting materials.

	Moisture content (%)	TOC[a] (g kg^{-1})	TN[b] (g kg^{-1})	C/N[c] ratio	pH
Rice straw	11.7	428.0	8.8	48.8	n.d.
Vegetables	83.3	90.6	4.7	19.3	6.22
Bran	14.1	474.1	41.2	11.5	n.d.
Soil	30.2	121.1	7.4	16.4	6.45

n.d., not detected.
[a] TOC, total organic carbon.
[b] TN, total Kjeldahl nitrogen.
[c] C/N, TOC/TN.

higher than that of the bacterial *amoA* gene (Yamamoto et al., 2010). Those findings have raised the prospect of an important role for AOA in the nitrogen transformation during agricultural waste composting.

Both AOA and AOB were actively involved in nitrification of the cattle manure composting system (Yamamoto et al., 2010). AOA may be key community for ammonia oxidation in composting systems (Zhang et al., 2011). The roles of AOA and AOB, and to what extent these ammonia oxidizers might affect nitrogen transformation balance during the composting process are not well understood yet (Maeda et al., 2011; Yamamoto et al., 2010; Zhang et al., 2011). This research was conducted to determine the relative contributions of archaeal and bacterial ammonia oxidizers to nitrification under different conditions in the agricultural waste composting system. Potential ammonia oxidation (PAO) rate was investigated, and both AOA and AOB *amoA* gene copy numbers as well as the *amoA* gene composition were determined using quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE), respectively. Relationships between PAO rates and the *amoA* gene abundance were determined to assess the contribution of AOA and AOB to nitrification. It is critically important to determine such relationships for our understanding and management of the nitrogen cycle during agricultural waste composting.

2. Methods

2.1. Experimental set-up and sample collection

The typical agricultural wastes were collected from the suburb of Changsha, China. Air-dried rice straw was cut into 10–20 mm lengths, and used as organic materials difficult to be decomposed. Several kinds of air-dried vegetables chopped into 10–20 mm pieces were used as easy-degradable materials. Bran was used to adjust the initial C/N ratio of composting materials. Soil was collected from the Yuelu Mountain, Changsha, and sieved through a 40-mesh screen (0.38 mm) to remove coarse plant debris, and added to increase microbial population and offer some necessary nutrients. The chemical characterization of those materials is shown in Table 1.

An experimental composting system with a wet weight of about 25 kg materials was set up indoors in the present study. Rice straw, vegetables, bran and soil were homogenized at a ratio of 11:3:2:8 (wet weight) and packed loosely in an open box with dimension of 0.5 m × 0.4 m × 0.45 m (length × width × height). The initial organic matter content and C/N ratio of the mixture were about 60% (dry weight) and 30:1, respectively. The mixture had good heat preservation. The experiment was conducted for 50 days. At each sampling occasion, three subsamples for parameter analysis were taken from different places of the composting material. Samples for total DNA extraction were pooled, mixed and stored immediately at −20 °C before used. After sampling, moisture content was monitored and adjusted to about 55–60% during the first fermentation phase and about 45–50% during the second fermentation phase by the addition of sterile deionized water. To provide some aeration, the mixture was turned twice a week during the first 2 weeks and once a week afterwards.

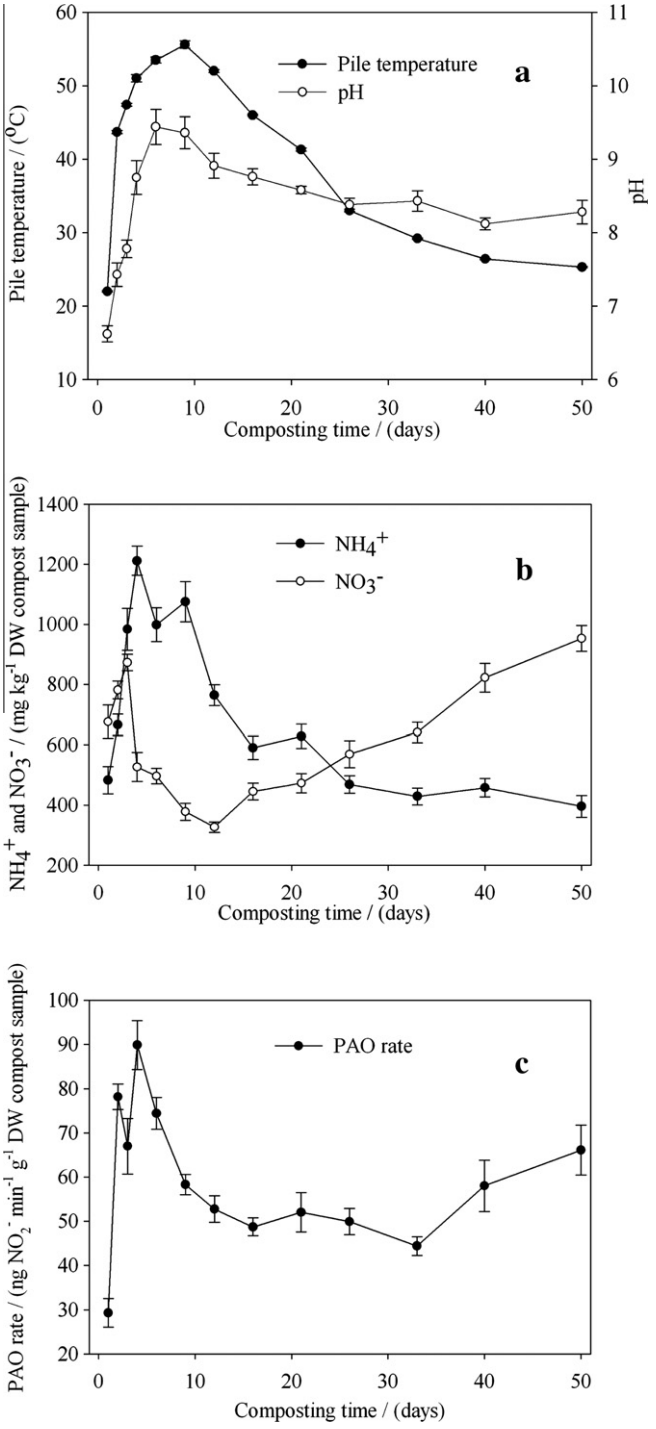

Fig. 1. Changes of (a) pile temperature and pH, (b) NH_4^+ and NO_3^-, and (c) PAO rate during agricultural waste composting process. The bars represent the standard deviations of the mean values (n = 3).

2.2. Physico-chemical parameters and PAO rate analysis

The pile temperature was monitored by inserting a temperature meter into three different locations in the centre of the composting material. The pH was measured with a digital pH meter by

mechanically shaking the fresh sample in water suspension at a ratio of 1:10 (w/v) at 200 rpm for 40 min. NH_4^+ and NO_3^- were extracted with 2 M KCl and determined by flow injection analysis (FIAstar 5000, FOSS, Denmark).

PAO rates were measured as accumulated NO_2^- using the chlorate inhibition method according to Jarvis et al. (2009) and Kurola et al. (2005) with some modification. In brief, each fresh compost sample (2 g) was transferred to 100 mL Erlenmeyer flask containing 40 mL of phosphate buffer solution (g L^{-1}: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 0.2; NaH_2PO_4, 0.2; pH 7.1) with 1 mM $(NH_4)_2SO_4$. Potassium chlorate with a final concentration of 10 mM was added to the flask to inhibit NO_2^- oxidation. The suspension was subsequently incubated at 25 °C, 100 rpm on a rotary shaker for 24 h in darkness. Aliquot of 2 mL was taken periodically after 0, 4, 8, 16 and 24 h of incubation, respectively. The NO_2^- concentration was determined by flow injection analysis (FIAstar 5000, FOSS, Denmark). The PAO rate was calculated by linear regression of the accumulated NO_2^- with time.

2.3. Genomic DNA extraction and qPCR

The total genomic DNA was extracted from 200 mg of freeze-dried compost samples according to the method described by Yang et al. (2007). The DNA extracts were 10-fold diluted before qPCR to overcome the possible inhibition effect of humic acids. Primers CrenamoA-23f and CrenamoA-616r (Tourna et al., 2008) and amoA-1f and amoA-2r (Rotthauwe et al., 1997) were used for the quantification of archaeal and bacterial *amoA* gene copy numbers, respectively. The qPCR was performed in triplicate on an iCycler IQ5 Thermocycler (Bio-Rad, USA). Each reaction was performed in a 20 μL volume containing 10 μL of 2× SYBR real-time PCR premixture (Bioteke, Beijing), 0.2 μM of each AOA or AOB primer, 1 μL of DNA template and adjusted with sterile water. The *amoA* gene fragments were amplified using an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, 40 s at 55 °C for AOA or 30 s at 55 °C for AOB, 30 s at 72 °C and 20 s at 83 °C. Data were retrieved at 83 °C. Ten-fold serial dilutions of linearized plasmids containing cloned *amoA* genes were used to produce the standard curves for qPCR. The orders of magnitude were 2.0×10^3 to 2.0×10^8 copies of template for archaeal *amoA* gene and 1.0×10^3 to 1.0×10^8 copies of template for bacterial *amoA* gene, respectively. All reactions were finished with a melting curve to verify amplicon specificity. Inhibitory effects on qPCR performance were tested for all samples as described previously (Wessén et al., 2010). PCR efficiencies and linearity (R^2) for each standard curve were 90.0–97.2% and 0.990–0.992, respectively.

2.4. DGGE analysis

Because of the difficulties in obtaining suitable archaeal *amoA* gene PCR products with the GC clamp on primers for DGGE analysis, the archaeal *amoA* genes were amplified using primers CrenamoA-23f and CrenamoA-616r without the requirement of a GC clamp (Tourna et al., 2008). Primers amoA-1f-GC and amoA-2r (Rotthauwe et al., 1997) were used for the bacterial *amoA* gene amplification. The PCR mixture was prepared with 25 μL of 2× Power Taq PCR MasterMix (Bioteke, Beijing), 1 μL of each primer (10 μM), 1 μL of DNA extract, 1 μL of BSA (20 mg mL^{-1}) and adjusted to a final volume of 50 μL with sterile water. PCR amplification was run on a MyCycler thermal cycle (Bio-Rad, USA) using the following cycling conditions: 95 °C for 4 min; 35 cycles of 20 s at 95 °C, 40 s at 55 °C for AOA or 30 s at 55 °C for AOB, and 30 s at 72 °C; followed by a final extension step at 72 °C for 10 min, and end at 4 °C.

DGGE was carried out using the Dcode™ Universal Mutation Detection System according to the manufacturer's instructions (Bio-Rad, USA). The purified PCR products (30 μL) were loaded onto the 0.75-mm-thick 8% (w/v) polyacrylamide gels with a denaturing gradient of 15–50% for archaeal and 30–50% for bacterial *amoA* gene products. The electrophoresis was performed in 1× TAE buffer at 60 °C, 90 V for 12 h. After staining with SYBR Green I, the gel was scanned with the Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

2.5. Data analysis

Three replicates were used in all analysis. One-way ANOVA for PAO rates, as well as archaeal and bacterial *amoA* gene copy numbers, was performed using the software package SPSS for Windows (version 11.5, SPSS, Chicago, IL, USA). Comparisons of mean values for different sampling time were made using least significant difference test. DGGE profiles were digitized after average background subtraction for the entire gel. Band numbers and relative intensity (within lane) were quantified with the QuantityOne software (version 4.5, Bio-Rad, Hercules, CA, USA) as described previously (Zhang et al., 2011). The Shannon diversity index (H) was calculated as:

$$H = \sum (N_i/N) \ln(N_i/N)$$

where N_i is the relative intensity of each band i, i is the number of bands in each DGGE profile, and N is the sum of all relative intensity in a given DGGE profile. To determine the relative contribution of archaea and bacteria to nitrification, regression was performed between the PAO rates and AOA and AOB *amoA* gene abundances, respectively.

3. Results and discussion

3.1. Physico-chemical parameters and PAO rate

The changes of pile temperature during the composting process are shown in Fig. 1a. According to the changes of pile temperature, the composting process could be divided into two phases: (i) the first fermentation phase (day 1 to day 12) which included the mesophilic stage (day 1 to day 3) and the thermophilic stage (day 4 to day 12, exceeding 50 °C), and (ii) the second fermentation phase (day 13 to day 50) which included the cooling stage (day 13 to day 26) and the maturation stage (day 27 to day 50). The pH in-

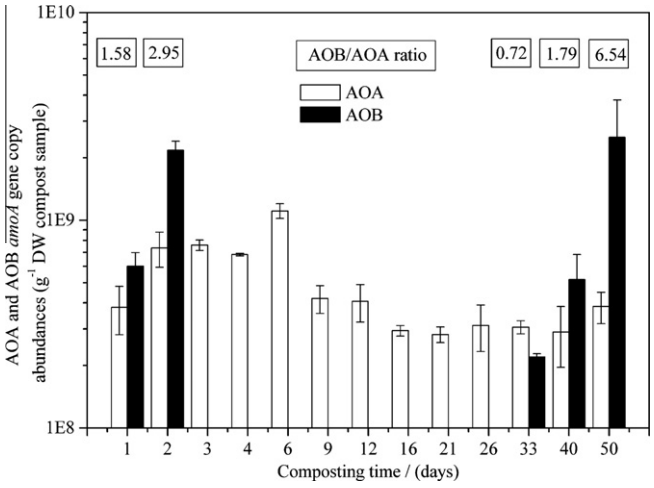

Fig. 2. AOA and AOB *amoA* gene copy abundances during agricultural waste composting process. The numbers on the *x*-axis refer to the sampling days. The AOB/AOA ratios are shown in the boxes in the upper part of the chart. The bars represent the standard deviations of the mean values ($n = 3$).

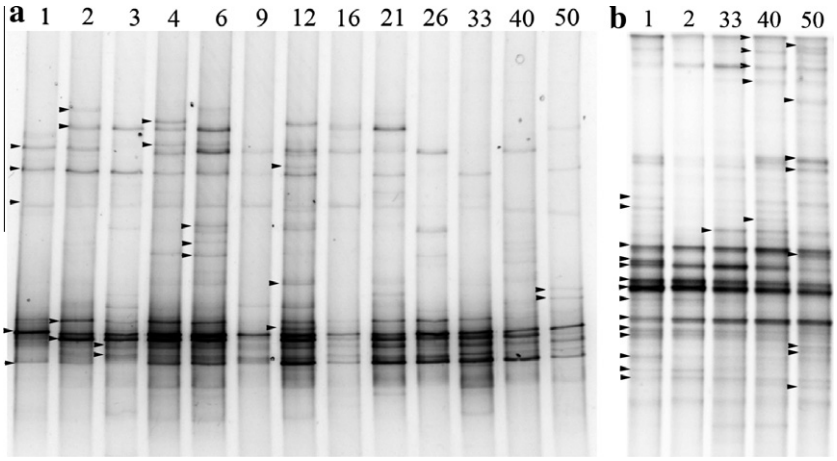

Fig. 3. DGGE profiles of the (a) archaeal *amoA* gene and (b) bacterial *amoA* gene composition during agricultural waste composting process. The numbers refer to the sampling days. The arrows indicate the archaeal and bacterial *amoA* DGGE bands.

Table 2
The *amoA* gene band number and Shannon diversity index (*H*) of DGGE profiles for each compost sample.

Index	Days of composting												
	1	2	3	4	6	9	12	16	21	26	33	40	50
Archaeal *amoA* gene DGGE													
Band number	7	9	9	13	14	8	16	8	12	10	8	7	8
Shannon diversity index (*H*)	1.303	1.783	1.560	2.194	2.181	1.614	2.343	1.438	2.053	1.638	1.748	1.520	1.314
Bacterial *amoA* gene DGGE													
Band number	19	14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14	21	23
Shannon diversity index (*H*)	2.692	2.503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.591	2.798	2.905

n.d., not detected.

creased from 6.75 to 9.33 during the first week as the production of NH_3 and mineralization of organic nitrogen, and decreased to 9.02 at the later stage.

With an increase in pile temperature, as well as the mineralization of organic nitrogen, the NH_4^+ content increased significantly and reached peak values on day 4, and decreased to low level afterwards (Fig. 1b). The NO_3^- concentration showed a significant increase in the first 3 days. As the high pile temperature and excessive amount of ammonia inhibited the activity and growth of nitrifying organisms (Zhang et al., 2011), the NO_3^- content decreased sharply to 326.6 mg kg^{-1} DW compost sample on day 12, and gradually increased to 953.0 mg kg^{-1} DW compost sample by the end of the composting process (Fig. 1b).

The PAO rate changes during the composting process are given in Fig. 1c. The values varied between 29.3 and 89.9 ng NO_2^- min^{-1} g^{-1} DW compost sample. Samples collected on day 2 to day 6 displayed the highest activities, which is in agreement with the earlier study showing that thermophilic conditions favour high PAO rates during organic household waste composting (Jarvis et al., 2009). The PAO rate increased further during the maturation stage and reached 66.1 ng NO_2^- min^{-1} g^{-1} DW compost sample by the end of the process.

3.2. Archaeal and bacterial amoA gene abundance

The archaeal *amoA* gene was abundant throughout the entire composting process. The abundance of archaeal *amoA* gene did not vary much between samples and was in the range of 2.8×10^8 to 1.1×10^9 gene copies g^{-1} DW compost sample (Fig. 2). The abundance during the first fermentation phase was relatively higher than that of the second fermentation phase. In contrast, the bacterial *amoA* gene abundance showed more variation between samples. The content increased significantly in the first 2 days, and then decreased to undetectable level (1×10^4 gene copies g^{-1} DW compost sample) from day 3 to day 26, suggesting niche differentiation between the two groups. Until in the samples collected during the maturation stage, the bacterial *amoA* gene re-established again and the gene abundance increased as high as 2.5×10^9 copies g^{-1} DW compost sample (Fig. 2). The AOB/AOA ratio ranged from 0.72 to 6.54 (when AOB were present) (Fig. 2).

Higher archaeal *amoA* gene copy numbers have been detected during the thermophilic stage, indicating the existence of active archaeal oxidizers with tolerance to high temperature and NH_4^+ concentration contents. Previous research has revealed that the AOA community might be ammonia-dependent (Limpiyakorn et al., 2011; Wells et al., 2009). AOA are proposed to be the possible major ammonia oxidizer in environments low in ammonium (Erguder et al., 2009). While in the composting system, as a high-organic and -ammonium content environment, AOA still dominate. In the cattle manure composting system, Yamamoto et al. (2010) reported that the AOA sequences, which were classified into a soil/sediment cluster, were present after the pile temperature decreased, and the archaeal *amoA* gene abundance was also significantly higher than that of bacterial *amoA* gene.

Various salts and organic matter can influence the growth and activity of AOB (Yan et al., 2010). In this experiment, bacterial *amoA* gene abundance decreased to undetectable level during the thermophilic and cooling stages. It is possible that the high temperature and low oxygen concentration during these stages might have provided an unfavourable condition for the AOB populations. AOB require oxygen concentrations in the range between 1 and 10% (Béline et al., 1999). Due to the heterogeneous characteristic of agricultural waste materials, partially anaerobic condition within the compost matrix might exist. Denitrification was enhanced

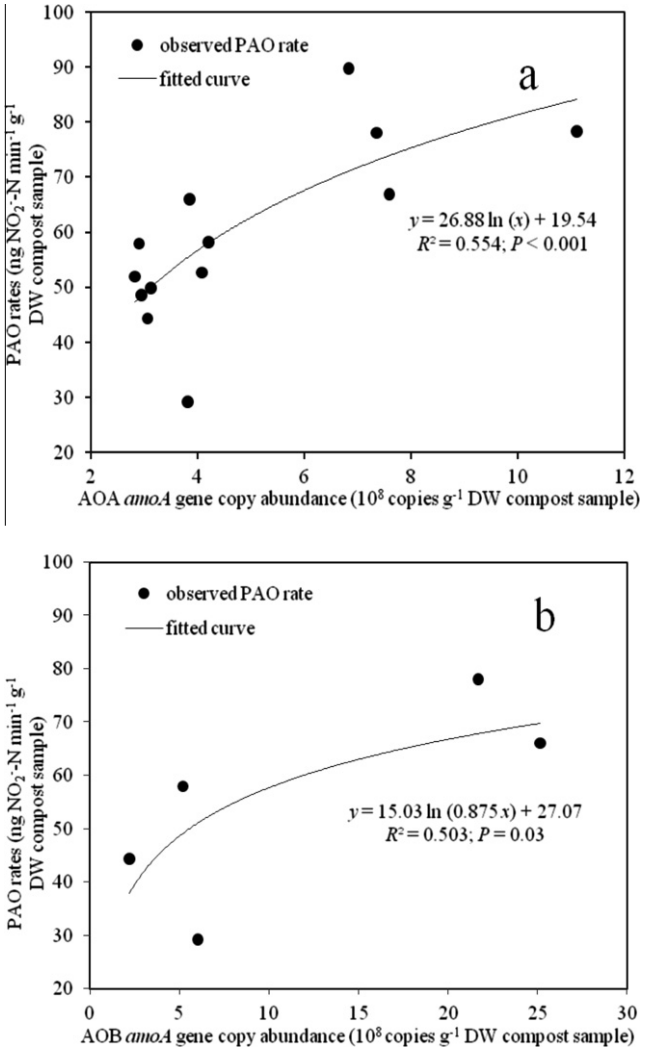

Fig. 4. Relationships between PAO rate and (a) archaeal and (b) bacterial *amoA* gene abundance during agricultural waste composting process. Each trend line indicates the fitted curve, where y is the PAO rate (ng NO_2^- min^{-1} g^{-1} DW compost sample) and x is the AOA or AOB *amoA* gene abundance (10^8 copies g^{-1} DW compost sample).

by the reduced oxygen solubility at high temperature and high moisture content of the composting materials.

3.3. Archaeal and bacterial amoA gene composition

The archaeal and bacterial *amoA* gene composition were obtained in the resulting gene band profiles (Fig. 3). Twenty-one different archaeal *amoA* gene bands and 28 different bacterial *amoA* gene bands were detected in the DGGE profiles. DGGE profiles varied between samples, indicating that the gene composition was dynamic during the composting process. Most of those bands were ubiquitous but differed in relative abundance in different samples. Higher archaeal *amoA* band numbers and more diverse gene composition were present during the thermophilic stage (Fig. 3a and Table 2) when the PAO rate reached peak values.

The large amounts of soluble NH_4^+ and easily available organic compounds (data not shown) in the materials stimulated and allowed the action of AOA that transformed the NH_4^+ into NO_2^- and finally to NO_3^- (Sánchez-Monedero et al., 2010). The archaeal ammonia oxidizers are more abundant in soils than their well-known bacterial counterparts (AOB) (Leininger et al., 2006). Other study has also shown that AOA community is less sensitive than that of AOB to operational conditions, i.e., ammonium concentration and dissolved oxygen in the biological nitrogen removal reactor (Jin et al., 2010). Based on these reports and our results, we suspect that AOA potentially have a greater ecophysiological diversity, and have the potential to cover a broader range of habitats during agricultural waste composting.

3.4. Relationship between nitrification and ammonia oxidizer abundance

Although numerically abundant, it remains to be shown whether archaea in compost also dominate with respect to their nitrification activities. Significant positive relationships were observed between PAO rate and AOA and AOB *amoA* gene abundance (Fig. 4). The regression equations were obtained and tested against the null hypothesis. The PAO rate increased as the ammonia oxidizer's population abundance increased exponentially. The AOA and AOB population can account for 55.4% and 50.3% of the variation in nitrification rate as indicated by the R^2 value, respectively (Fig. 4). These results indicated that archaea dominated microbial ammonia oxidation during the thermophilic and cooling stages; while bacteria were also related to ammonia oxidation activity especially during the mesophilic and maturation stages.

There have been ambiguities concerning the relative importance of those different ammonia oxidizers in compost (Maeda et al., 2011; Zhang et al., 2011). The gene abundance and AOB/AOA ratio alone are not sufficient to determine the relative contribution of either group towards the ammonia oxidation process (Wessén et al., 2010). Therefore, we assessed the PAO rate, and correlated this evidence of function to the AOA and AOB *amoA* gene abundance to compare their relative contribution to microbial ammonia oxidation under different conditions during agricultural waste composting.

The difference in the relative contributions of bacterial and archaeal ammonia oxidizers may vary depending on the environmental conditions, with one or the other being more competitive under a given set of conditions. Recent findings have shown that AOA played a more important role than AOB in nitrification under many conditions, such as agricultural soil (Leininger et al., 2006; Wessén et al., 2010). During the animal manure composting system, both AOA and AOB were actively involved in the nitrification process (Maeda et al., 2010; Yamamoto et al., 2010). Zhang et al. (2011) commented that AOA may be key community for ammonia oxidation in the agricultural waste composting system. The archaea may gain the competitive advantage in conditions of high temperature stress and low oxygen supply during agricultural waste composting. Several studies have reported that AOA may be particularly adapted to unfavourable environmental conditions, such as high temperature or low oxygen supply environments (Hatzenpichler et al., 2008; Herndl et al., 2005). Additionally, genomic analysis indicates that AOA have the potential to function as a mixotroph, utilizing both carbon dioxide and organic material as carbon sources (Hallam et al., 2006a,b).

Notwithstanding, it did not imply that AOB were of no importance in affecting nitrogen transformation balance during agricultural waste composting process. When present, the *amoA* gene abundance of bacteria was relatively more abundant than that of archaea, except in sample collected on day 33 (Fig. 2). In addition, significant relationship has also been obtained between the bacterial *amoA* gene abundance and PAO rate (Fig. 4b), indicating that bacterial *amoA* gene was also related to ammonia oxidation activity, especially in samples collected during the mesophilic and maturation stages in this experiment.

The community that appears relatively more important in a given study seems to be somewhat site-specific, depending on the

special composting materials conditions and different composting regimes. There will be a continuous debate of the relative contribution of archaeal and bacterial ammonia-oxidizing organisms to nitrification in the composting system. It is necessary for researchers to determine and better interpret how the changes of complex microbial community and population dynamics will affect nitrification activities in future. Additionally, numerous factors such as oxygen concentration, pH and pile temperature could affect their distribution and activity. How AOA and AOB communities respond to the fluctuations of different physico-chemical parameters and ultimately affect the compost end product quality are also poorly understood and remain an area of continuing scientific interest.

4. Conclusions

This study discussed the relative contribution of AOA and AOB to nitrification under different conditions during agricultural waste composting. It was shown that archaeal *amoA* gene was abundant throughout the composting process, while the bacterial *amoA* gene abundance decreased to undetectable level during the thermophilic and cooling stages. Both AOA and AOB *amoA* gene abundance correlated to the difference in the PAO rate. These results indicate that archaea dominated microbial ammonia oxidation during the thermophilic and cooling stages; while bacteria were also related to ammonia oxidation activity especially during the mesophilic and maturation stages.

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