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# Anaerobic Ammonium-Oxidizing Bacteria in Cow Manure Composting

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Composting is widely used to transform waste into valuable agricultural organic fertilizer. Anaerobic ammonium-oxidizing (anammox) bacteria play an important role in the global nitrogen cycle, but their role in composting remains poorly understood. In the present study, the community structure, diversity, and abundance of anammox bacteria were analyzed using cloning and sequencing methods by targeting the 16S rRNA gene and the hydrazine oxidase gene (*hzo*) in samples isolated from compost produced from cow manure and rice straw. A total of 25 operational taxonomic units were classified based on 16S rRNA gene clone libraries, and 14 operational taxonomic units were classified based on *hzo* gene clone libraries. The phylogenetic tree analysis of the 16S rRNA gene and deduced HZO protein sequences from the corresponding encoding genes indicated that the majority of the obtained clones were related to the known anammox bacteria *Candidatus* "Brocadia," *Candidatus* "Kuenenia," and *Candidatus* "Scalindua." The abundances of anammox bacteria were determined by quantitative PCR, and between $2.13 \times 10^5$ and $1.15 \times 10^6$ 16S rRNA gene copies per gram of compost were found. This study provides the first demonstration of the existence of anammox bacteria with limited diversity in cow manure composting.

**Keywords :** Anammox bacteria, compost, 16S rRNA gene, *hzo* gene, nitrogen cycle

## Introduction

Anaerobic ammonium-oxidizing bacteria participate in the biogeochemical nitrogen cycle and can convert ammonium and nitrite into dinitrogen gas ($N_2$) in an anoxic environment. Anaerobic ammonium oxidation (anammox) has been recently recognized as an alternative microbial metabolic pathway to the denitrification involved in $N_2$ emission [1]. Anammox was first discovered in samples isolated from a wastewater treatment plant [2]. Currently, five anammox bacteria genera have been described, namely *Candidatus* "Brocadia," [3] *Candidatus* "Kuenenia," [4] *Candidatus* "Scalindua," [5] *Candidatus* "Anammoxoglobus," [6] and *Candidatus* "Jettenia" [7]. Anaerobic ammonium-oxidizing bacteria have been identified in various ecosystems, including both aquatic [8–13] and soil [14–17] ecosystems.

Composting is a process of self-heating, in which organic waste is converted into humus, presenting a strategy to convert a large amount of agricultural waste to a form potentially useful in agriculture. During composting, N loss usually occurs through ammonium loss during ammonification and $N_2$ emission during nitrification and denitrification [18, 19]. Thus, ammonification, nitrification, and denitrification are the three major processes considered when measures are taken to prevent N loss in composting [19]. During composting, ammonium nitrogen and nitrite nitrogen are both present and there is an anaerobic environment, favorable conditions for the development of anammox bacteria. Thus, it is likely that there are anammox bacteria present in composting, but this has not been demonstrated. However, many studies have analyzed the composition and dynamics of nitrifier and denitrifier communities in compost [20, 21]. Transformations of nitrogen (N) are important in composting to support biodegradation processes, and anammox may contribute to $N_2$ emission, making it relevant to the resulting compost quality.

In this study, our objective was to examine the existence of anammox bacteria in compost of rice straw and cow

manure. To do this, we used molecular techniques, including PCR, quantitative PCR, and a cloning library, to analyze the diversity, community distribution, and abundance of anammox bacteria in compost.

## Materials and Methods

### Composting and Sampling

Three composting piles containing cow manure and rice straw were prepared at a ratio of 5:1 (dry weight) in a ventilated vessel (0.5 m length × 0.5 m width × 1.1 m height). The rice straw acts as a kind of bulking material. The raw materials had 67% moisture content and 30:1 C/N ratio. The vessel was insulated to retain the heat produced during composting, and air was periodically pumped into the vessel from the bottom. Temperature measurements were performed in three locations (surface, core, and bottom) daily, and the experiment was conducted for 30 days. Samples of compost were collected on days 0, 1, 4, 7, 13, 23, and 29, from the surface, core, and bottom of the compost pile and stored at −20°C for further analysis.

### Physicochemical Analysis

The temperature was measured at different locations within the stacks (surface, core, and bottom) throughout the process. The pH was measured in aqueous suspensions of the fresh compost samples (1:10 (w/v), compost/water ratio) using a digital pH meter (PHS-3C; Leici Shanghai, China). The water contents were determined by the weight loss of composting samples after drying at 105°C to a constant weight. The total organic carbon content was determined by the dry combustion method at 540°C for 4 h [22] and total Kjeldahl nitrogen was determined by the Kjeldahl method [23]. $NH_4^+$-N, $NO_3^-$-N, and $NO_2^-$-N were extracted with 2 M KCl and measured using a continuous flow analyzer (FIA QC8500; Lachat, USA). All samples were analyzed either in triplicates or duplicates and the results are presented as average values.

### DNA Extraction and PCR

DNA was extracted from the compost samples as described previously [24]. After DNA extraction, the crude DNA was purified using a DNA gel purification kit (Omega, USA) according to the manual. The purified DNA was stored at −20°C.

To identify anammox bacteria, four sets of primers were used (Table 1). The 16S rRNA genes were amplified according to two protocols. First, the initial PCR amplification was performed with forward primer Pla46f in combination with the reverse primer 630r, and then nested PCR was performed with the anammox-specific primers Amx368f and Amx820r using the PCR program described previously [5]. Second, PCR was performed with primer Pla46f in combination with Amx820R according to the previous study [25]. For the *hzo* gene, PCR was performed using primers hzocl1F1 and hzocl1R2 with the PCR program described by Schmid *et al.* [26]. All the PCRs were performed in a final volume of 25 μl, with 1 μl of DNA (30–50 ng/ml), 2.5 μl of 10× HG PCR buffer (1 ml), 2.5 μl of dNTPs (1 ml), 0.2 μl of each forward and reverse primer (20 μM), 0.3 μl of Super Taq polymerase (200 U), and 18.8 μl of $H_2O$.

### Generation Clone Libraries

Purified PCR products were ligated into the pMD18-T Vector (Takara, Japan) according to the manufacturer's protocol, and ligated products were then transformed into *E. coli* DH5α competent cells. The clone libraries were subsequently created by randomly picking white colonies from each clone library. Positive clones were verified by PCR amplification (with primers M13/RV-M) and then sequenced using an ABI 3730xl analyzer (Applied Biosystems, USA).

### Phylogenetic Analysis

Phylogenetic analyses were conducted using the MEGA 6.06 program [27]. Before constructing phylogenetic trees, the 16S rRNA gene sequences with greater than 97% sequence similarity were grouped into one representative sequence, the *hzo* gene

**Table 1.** Primers used in this study.

| Targets | Primers | Sequence 5′-3′[a] | Specificity | Target site | References |
|---|---|---|---|---|---|
| 16S rRNA gene | 338F | ACTCCTACGGGAGGCAGCAG | Total bacterial | 338–359[b] | Muyzer *et al*. 1993 |
| | 518R | ATTACCGCGGCTGCTGG | Total bacterial | 518–534[b] | Muyzer *et al*. 1993 |
| | Pla46f | GACTTGCATGCCTAATCC | Planctomycetales | 46–63[b] | Neef *et al*. 1998 |
| | Amx368f | CCTTTCGGGCATTGCGAA | All anammox organisms | 368–385[b] | Schmid *et al*. 2003 |
| | Amx820r | AAAACCCCTCTACTTAGTGCCC | Brocadia, Kuenenia | 820–841[b] | Schmid *et al*. 2000 |
| | 630r | CAKAAAGGAGGTGATCC | Universal bacteria | 1529-1545[b] | Juretschko *et al*. 1998 |
| Functional gene | hzocl1F1 | TGYAAGACYTGYCAYTGG | *hzo* | 739–757[c] | Schmid *et al*. 2008 |
| | hzocl1R2 | ACTCCAGATRTGCTGACC | *hzo* | 1192–1209[c] | Schmid *et al*. 2008 |

[a]Modified bases: K = GT, Y = CT, R = AG.

[b]16S rRNA position, *E. coli* numbering (Brosius *et al*. 1978).

[c]Positions refer to the Ca. Kuenenia stuttgartiensis *hzo* gene (CAJ72085).

sequences were translated to amino acid sequences, and then identical amino acid sequences were grouped and representative sequences were selected. All representative sequences from each clone library were compared for homology with closest relatives in GenBank using BLAST. The most closely related affinities and additional reference sequences were retrieved and then aligned with representative clones using ClustalW. Phylogenetic trees were constructed using the neighbor-joining algorithm and the Jukes-Cantor distance model, and bootstrap values were obtained from data resampling of 1,000 replicates.

### Quantification of Anammox Bacteria

Anammox bacterial and total bacterial 16S rRNA genes from each sample were quantified by quantitative PCR (qPCR) analysis. Real-time PCR was performed by using SYBR *Premix Ex Taq* II (Takara) in an CFX96 Real-Time PCR Detection System. qPCR was performed with the primers AMX808F and AMX1040R under conditions previously described [28]. A standard plasmid carrying the anammox 16S rRNA genes was generated by cloning 16S rRNA genes from compost material as described above. The plasmid DNA (NZ_JRYM00000000.1) concentration was determined on a SmartSpec Plus spectrophotometer (Bio-Rad, USA), and the copy number of the target gene was calculated directly based on the concentration of the extracted plasmid DNA. Cycle thresholds were determined by comparison with standard curves constructed after serial dilution of the quantified standard plasmids. The $R^2$ values were greater than 0.98 for the curves. Samples and standards were prepared in triplicates. The specificity of the PCR amplification was determined by melting curve analysis and gel electrophoresis.

### Data Analysis

The determinations of operational taxonomic unit (OTU), Shannon and Simpson indices, and Chao were done with Mothur (http://www.mothur.org, ver. 1.35.1) [29]. Library coverage (*C*) was calculated using the equation [1 − (*n*/*N*)], where *n* is the number of phylotypes represented by a single clone and *N* is the total number of clones retrieved [30]. The sequence alignment files used in Mothur analyses were created with ClustalW. Correlations between the anammox bacterial communities and environmental factors were determined by performing canonical correspondence analysis (CCA) using the software CANOCO (ver. 4.5; Microcomputer Power).

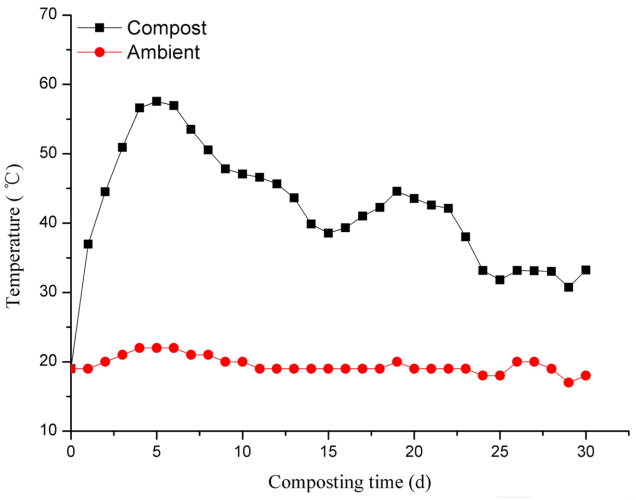


**Fig. 1.** Temperature in the compost pile.

### Sequence Accession Number

The obtained 16S rRNA gene and *hzo* gene sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers KR780762–KR780765, KT003627–KT003638, KT321662–KT321672, and KT070851–KT070863.

## Results

### Physicochemical Parameters

The compost reached 50°C in the first 3 days of composting, and began to peak by day 5; the average temperature change of the compost is shown in Fig. 1. The thermophilic stage persisted for 10 days. After reaching the peak, the temperature began to decline. Based on the changes of temperature, the following stages of composting were identified: the mesophilic stage (days 1–2), the thermophilic stage (days 3–12), the cooling stage (days 13–20), and the maturation stage (days 21–30).

**Table 2.** Changes in chemical and physical properties during composting.

| Properties | CMC0 (day 0) | CMC1 (day 1) | CMC2 (day 4) | CMC3 (day 7) | CMC4 (day 13) | CMC5 (day 23) | CMC6 (day 29) |
|---|---|---|---|---|---|---|---|
| pH | 8.86 | 8.87 | 8.98 | 8.81 | 8.75 | 8.65 | 8.60 |
| Moisture content (%) | 67.0 | 65.3 | 67.4 | 64.6 | 60.7 | 54.7 | 50.6 |
| C/N | 30.0 | 29.3 | 28.1 | 25.8 | 21.1 | 19.0 | 18.3 |
| $NH_4^+$-N (mg/kg) | 1,410 | 1,320 | 1,503 | 1,664 | 1,350 | 780 | 531 |
| $NO_3^-$-N (mg/kg) | 183 | 210 | 232 | 360 | 762 | 1,090 | 1,430 |
| $NO_2^-$-N (mg/kg) | 0.87 | 2.05 | 2.76 | 8.07 | 388 | 207 | 112 |

Changes in the chemical and physical composition of the compost mixtures throughout the composting process were detected and are shown in Table 2. The moisture content continuously declined from 67.0% to 50.6% during the composting process. The C/N ratio decreased continuously, reaching the lowest value of 18.3 at the end of the composting process. A C/N ratio below 20 is considered a marker of compost maturity [31]. The pH value and $NH_4^+$-N increased initially and then decreased, with the maximum pH value and $NH_4^+$-N occurring on the fourth day and the seventh day, respectively. The amounts of $NO_3^-$-N and $NO_2^-$-N increased during the composting process.

**Diversity of Anammox Bacteria during Composting**

Using the products of the PCR targeting the 16S rRNA gene, a total of 337 clones were randomly selected from each clone library and then sequenced (Table 3). The number of species as a function of the number of samples was plotted to generate the rarefaction curve for each library, as shown in Fig. S1A. The results of the sequencing indicated that 282 clones were related to Planctomycetes, of which 179 clones belonged to anammox bacteria. From each of the 16S rRNA gene clone libraries, 3 to 23 positive clones were obtained. The proportion of Planctomycetes in the clone libraries was different for each of the seven compost samples and also varied between the two sets of primers, ranging from 60.0% in the sample of CMC1 to 90.9% in the sample of CMC4 from primer set Pla46f/Amx820r. The percentage of anammox bacteria also varied, from 21.4% to 66.7%. When using primer set Amx368f/Amx820r, the fraction of Planctomycetes varied from 78.6% to 96% among each clone library, and the proportion of anammox varied from 36.7% to 100.0%. Comparing the percentage of Planctomycetes and anammox bacteria in different compost samples, the samples with the higher percentage of Planctomycetes did not necessarily have a

**Table 3.** Diversity of anammox bacteria 16S rRNA and *hzo* genes.

| Samples | | CMC0 day 0 | CMC1 day 1 | CMC2 day 4 | CMC3 day 7 | CMC4 day 13 | CMC5 day 23 | CMC6 day 29 |
|---|---|---|---|---|---|---|---|---|
| Pla46f/Amx820r | No. of clones | 20 | 15 | 18 | 20 | 22 | 20 | 23 |
| | No. of Planctomycetes | 18 | 9 | 12 | 18 | 20 | 17 | 19 |
| | Percentage of Planctomycetes (%) | 90.0 | 60.0 | 66.7 | 90.0 | 90.9 | 65.0 | 82.6 |
| | No. of anammox bacteria | 8 | 6 | 8 | 10 | 11 | 3 | 10 |
| | Percentage of anammox bacteria (%) | 44.4 | 66.7 | 66.7 | 55.6 | 55.0 | 21.4 | 52.6 |
| | No. of OTUs (1%) | 3 | 3 | 3 | 4 | 3 | 4 | 4 |
| | Shannon index | 0.97 | 0.85 | 0.87 | 1.28 | 0.93 | 1.15 | 1.19 |
| | Chao | 3 | 3 | 3 | 4 | 4 | 4 | 4 |
| | Coverage (%) | 100 | 93.3 | 100 | 100 | 100 | 95.0 | 100 |
| Amx368/Amx820r | No. of clones | 23 | 25 | 25 | 28 | 30 | 33 | 35 |
| | No. of Planctomycetes | 20 | 21 | 24 | 20 | 27 | 27 | 30 |
| | Percentage of Planctomycetes (%) | 87.0 | 84.0 | 96.0 | 78.6 | 90.0 | 81.8 | 85.7 |
| | No. of anammox bacteria | 17 | 13 | 21 | 20 | 23 | 18 | 11 |
| | Percentage of anammox bacteria (%) | 85.0 | 61.9 | 87.5 | 100.0 | 85.2 | 66.7 | 36.7 |
| | No. of OTUs (1%) | 5 | 5 | 4 | 4 | 4 | 3 | 4 |
| | Shannon index | 1.38 | 1.30 | 1.06 | 1.36 | 1.19 | 0.89 | 1.05 |
| | Chao | 5 | 5 | 4 | 4 | 4 | 3 | 4 |
| | Coverage (%) | 100 | 90.0 | 100 | 100 | 100 | 100 | 96.6 |
| *hzo* | No. of clones | 18 | 20 | 18 | 26 | 21 | 23 | 24 |
| | Percentage of anammox bacteria (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | No. of OTUs (1% ) | 3 | 3 | 3 | 4 | 4 | 3 | 3 |
| | Shannon index | 1.02 | 0.95 | 0.85 | 1.21 | 0.96 | 0.84 | 0.83 |
| | Chao | 3 | 3 | 3 | 4 | 3 | 3 | 3 |
| | Coverage (%) | 100 | 100 | 100 | 96.2 | 95.2 | 95.7 | 95.8 |

OTU, operational taxonomic unit.

higher percentage of anammox bacteria. For example, using primers Amx368f/Amx820r, sample CMC6 showed a higher fraction of Planctomycetes (85.7%), but the anammox bacteria made up only 36.7%. Anammox bacteria were detected in all samples, but their proportion varied dramatically, especially using the primers Amx368f/Amx820r. The results indicated that anammox bacteria are present in the initial materials and remain present in all stages of the composting process, indicating their ability to survive the thermophilic stage of composting. The diversity of anammox bacteria community, as indicated by the Shannon-Wiener index, was different in different samples, and the highest diversity index was observed in sample CMC3. As the composting progressed, the diversity declined during the first few days, then increased to a peak on day 7 during the theromophilic stage, and finally declined during the maturation stage. The coverage value of primer set Pla46f/Amx820r was between 93.3% and 100%, and for primer set Amx368f/Amx820r was between 90% and 100%, indicating that the majority of the phylotypes in the clone libraries were detected.

A total of 150 *hzo* gene clones were sequenced from the seven *hzo* gene clone libraries. The results of the sequencing showed that all the clones were anammox bacteria (Table 3), indicating that anammox bacteria were present both in the initial materials and throughout all stages of composting. The determined rarefaction curve for each library is shown in Fig. S1B. This finding of anammox bacteria throughout composting was consistent with the above findings from the analysis of the 16S rRNA clone libraries. The coverage value of all libraries ranged between 95.2% and 100%. The analysis of the *hzo* gene clone libraries suggested that the diversity of anammox bacteria (1.02) was highest in the initial materials compared with later in the composting process. During composting, the pattern of change in the *hzo* gene was similar to that observed for the 16S rRNA clone libraries, which declined initially during composting, increased to a peak on day 7 (1.21) during the theromophilic stage, and finally declined during the maturation stage. The lowest Shannon index (0.83) was observed in the sample taken on day 29 during the maturation stage.

**Phylogenetic Analysis of the 16S rRNA and *hzo* Genes**

The reference sequences in NCBI and closely related sequences with more than 83% similarity to the representative sequences obtained here were selected for phylogenetic analysis. Each single phylogenetic tree was separately constructed from the clone libraries generated by different primer sets and the results are shown in Figs. 2 and 3.

The phylogenetic tree was constructed based on the sequences of the 16S rRNA gene (Fig. 2). A total of 282 clones of Planctomycetes were classified into 25 OTUs using a cutoff value of 99% sequence similarity with the furthest neighbor algorithm in the Mothur program. The OTUs of 16S rRNA clones were tentatively categorized into nine phylotypes as shown in Fig. 2. The branch included phylotypes K1, B1, B2, S1, S2, U1, and U2, and the phylotype composition was compared between the samples (Fig. 4A). Of the 282 clones of Planctomycetes, 179 clones (63.5%) belonged to anammox bacteria, and appeared closely related to *Candidatus* Brocadia, *Candidatus* Kuenenia, and *Candidatus* Scalindua. The lineage related to the genus "Brocadia" comprised seven OTUs (B1 and B2) and constituted 36.9% of all the clones sequenced in this study. The sequence similarity between OTU4 and the closest matching *Candidatus* Brocadia fulgida (JX243652) was 96%. Between OTU7 and the closest matching *Candidatus* Brocadia fulgida (JQ864321), there was 99% sequence similarity. Three OTUs (OTU11, OTU6, and OTU20) formed two small clusters (S1 and S2) with *Candidatus* "Scalindua" and constituted 10.6% of all the clone sequences. OTU20 shared 95% similarity with *Candidatus* Scalindua wagneri (KT229792) and OTU6 shared 96% similarity with *Candidatus* Scalindua brodae. Two OTUs (OTU2 and OTU3) formed a cluster K1, and constituted 16.0% of all the clones sequenced. OTU2 is closely related to *Candidatus* Kuenenia stuttgartiensis (HM769656, similarity 95%). In addition, 36.5% of the clones (included U1 and U2) in Planctomycetes were outside the anammox bacterial cluster. This was largely caused by the low specificity of the used primers, and these isolated sequences show no close similarity to cultivated organisms [3–5]. It was also interesting that no closest reference sequence was found for U2, suggesting U2 might be a novel group.

In order to clearly identify diverse *hzo* gene sequences, a total of 150 *hzo* gene clones were sequenced and 14 OTUs were classified using a cutoff value of 97% sequence similarity with the furthest neighbor algorithm (Fig. 3). After phylogram analysis, the phylogenetic tree of anammox bacteria with three clusters was drawn based on the anammox bacteria *hzo* gene sequences. All protein sequences coded by the *hzo* gene were included in the three clusters, as the *hzo* gene (BAF98478, CAJ71806) of *Candidatus* "Jettenia" and *Candidatus* "Kuenenia" were put into an outgroup. Similar to 16S rRNA analysis, as shown in the phylogenetic tree (Fig. 2), the HZO protein sequences were also closely related to *Candidatus* "Brocadia," *Candidatus* "Scalindua,"

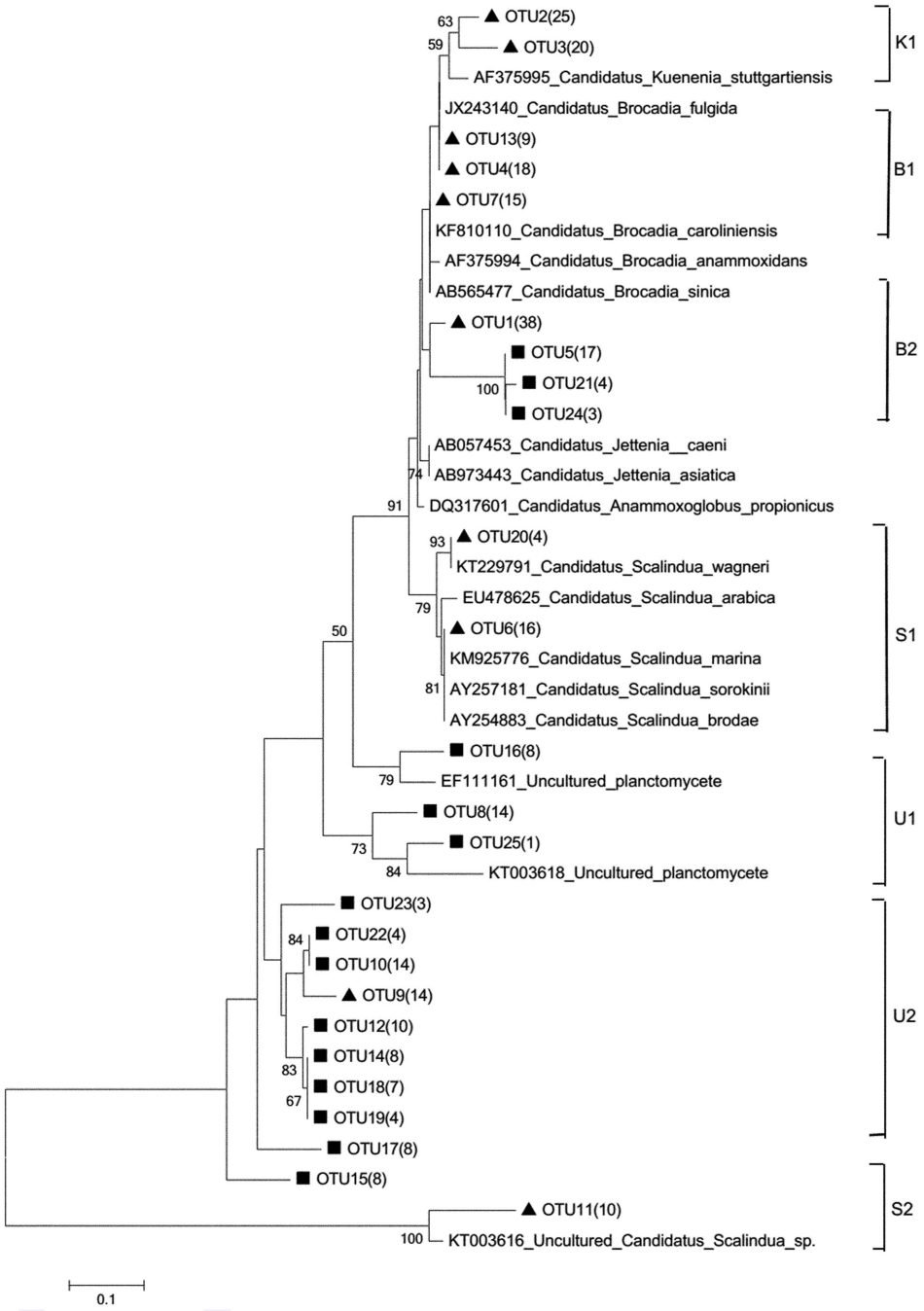


**Fig. 2.** Phylogenetic tree constructed after alignment of experimental and retrieved 16S rRNA gene sequences for neighbor-joining analysis.

Numbers in the brackets show the number of times a sequence was detected among all the tested clones. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1,000 resampled data sets (only values >50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar. Twenty-five phylotypes (B1–B2, K1, S1–S2, U1-U2) of 16S rRNA gene clones (OTUs) from cow manure compost are shown; squares indicate sequences from primers Amx368f-Amx820r, triangles for primers Pla46f-Amx820r.

and *Candidatus* "Kuenenia". However, the proportion of the three genera was different, with 12%, 26% and 62% of the clones closely related to *Candidatus* "Brocadia," *Candidatus* "Kuenenia," and *Candidatus* "Scalindua", respectively. The average sequence similarity between the HZO sequences and the closest matching Scalindua sequence was more

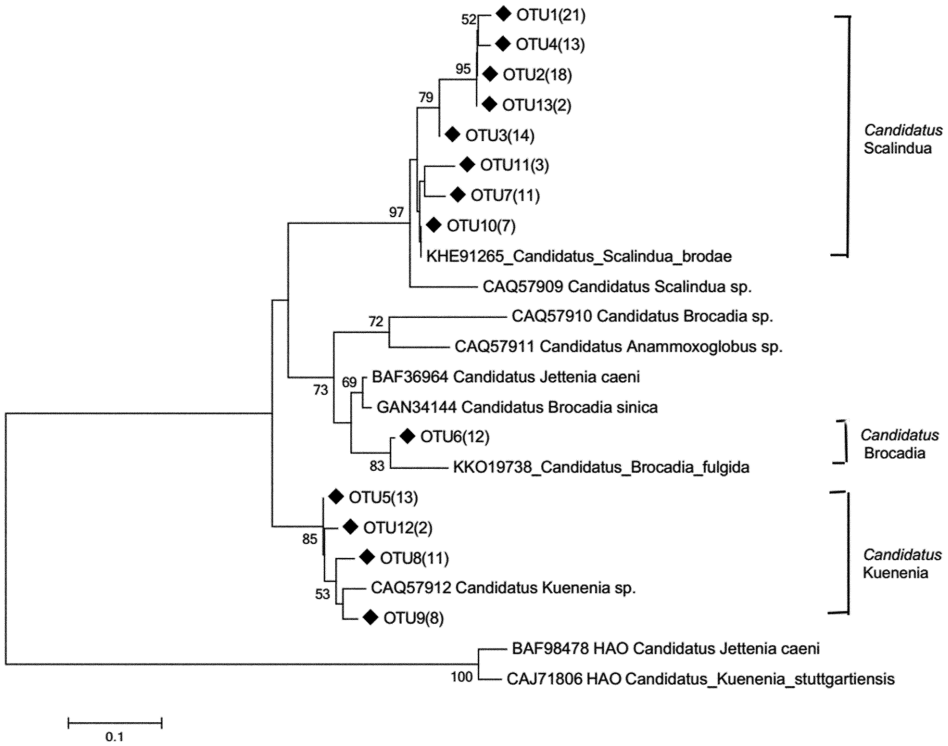


**Fig. 3.** Phylogenetic tree constructed after alignment of deduced experimental and retrieved HZO protein sequences for neighbor-joining analysis.

Numbers in the brackets show the number of times a sequence was detected among all the tested clones. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1,000 resampled data sets (only values >50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar. The sequences from cow manure compost are depicted by diamonds.

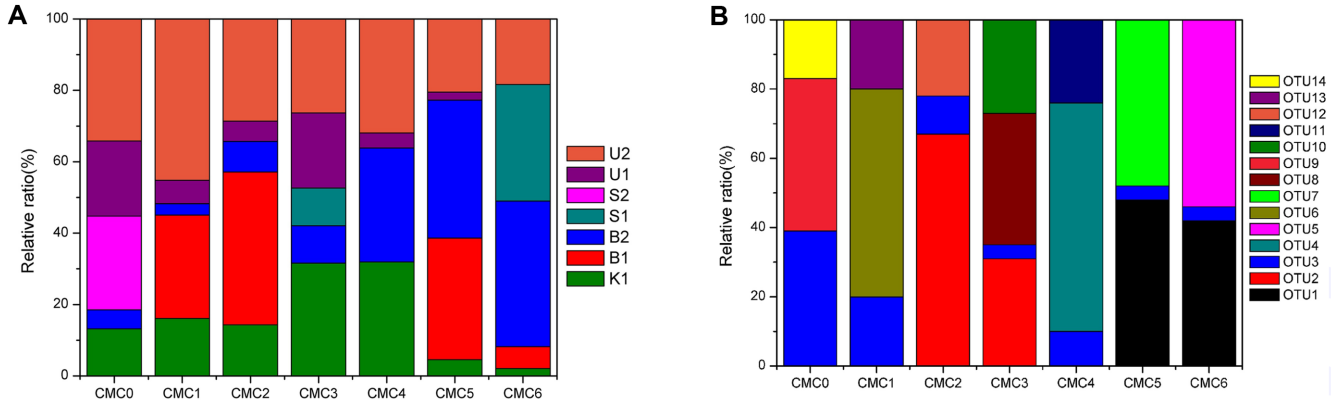


**Fig. 4.** Phylotype composition of anammox-related 16S rRNA gene (**A**) clones and *hzo* gene (**B**) clones in cow manure composting.

The phylotypes are shown in Figs. 2 and 3, respectively.

than 90%. The sequences in the cluster *Candidatus* "Kuenenia" shared an average of 95% similarity with *Candidatus* Kuenenia stuttgartiensis. OTU3 was related to *Candidatus* "Brocadia" with 96% similarity. Compared with the OTUs in the phylogenetic tree of the 16S rRNA gene, more OTUs were observed in *Candidatus* "Scalindua" in the phylogenetic tree of HZO protein sequences, typical of a variety of aquatic environments [32]. This result could be explained by the fact that the 16S rRNA gene primer Amx820r is biased towards *Candidatus* "Brocadia" and *Candidatus*

"Kuenenia" [25].

Based on the percentage of OTUs for the 16S rRNA and *hzo* gene clone libraries, different classifications were constructed in the phylotype composition analysis (Figs. 4A and 4B). As revealed by the 16S rRNA gene analysis presented in Fig. 4A, each phylotype consists of the OTUs related to each other with 99% sequence similarity. Phylotypes K1, B2, and U2 were prevalent communities in the whole composting process, with K1 related to *Candidatus* "Kuenenia" and B2 related to *Candidatus* "Brocadia" (Fig. 2). Phylotype K1 included OTU2 and OTU3, and was relatively abundant in samples taken from CMC3 and CMC4, whereas B1 was relatively abundant in samples CMC1, CMC2, and CMC5. Phylotypes S1 and S2 were closely related to *Candidatus* "Scalindua," and were relatively abundant in samples CMC6 and CMC0, suggesting that they might be more adapted to the currently used composting conditions. Phylotypes U1 and U2 were related to unknown Planctomycetes, with a percentage of 36.5% (Table 3). The anammox bacteria revealed by *hzo* gene analysis in Fig. 4B were detected in all stages of composting, but only OTU3 was observed in all of the seven samples. A specific community was found for each compost sample. OTU9, OTU6, OTU8, OTU4, and OTU5 were both predominant and specific in samples CMC0, CMC1, CMC3, CMC4, and CMC6, respectively, which were samples from the initial material, the mesophilic stage, the thermophilic stage, the cooling stage, and the maturation stage. OTU2 (95% similarity with *Kuenenia*) mainly occurred in the thermophilic stage, and OTU1 (93% similarity with *Scalindua*) mainly occurred in the maturation stage.

**Quantification Analysis of Anammox Bacteria**

The abundance of anammox bacteria and total bacteria in the cow manure compost was estimated based on the quantification of anammox bacteria and the total bacterial 16S rRNA genes as measured by qPCR. Melting-curve analysis of qPCR for the anammox bacteria and total bacteria confirmed that the fluorescent signals were obtained from specific PCR products. The copy numbers of anammox bacteria ranged from $2.13 \times 10^5$ to $1.16 \times 10^6$ copies per gram of dry composting material among the samples, with the highest abundance in sample CMC5 followed by CMC4, CMC0, CMC1, CMC6, CMC3, and CMC2 (Fig. 5). The qPCR results further confirmed the occurrence of anammox bacteria samples. The copy numbers of total bacteria ranged from $1.18 \times 10^7$ to $2.83 \times 10^7$ copies per gram of dry composting material. A higher copy number of total bacteria was found in samples CMC5 and CMC6,

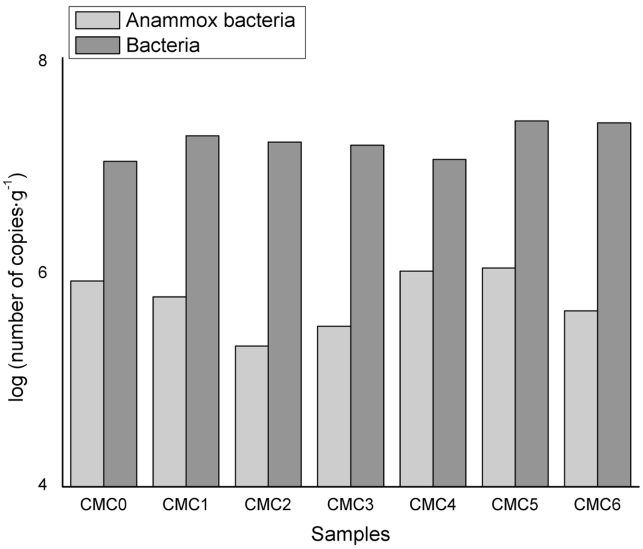


**Fig. 5.** Abundance of anammox bacteria and total bacteria 16S rRNA gene copy numbers in cow manure compost.

obtained during the maturation stage (Fig. 5). During the thermophilic stage of composting, the copy number of the anammox bacteria and the total bacteria were reduced by high temperature. During the maturation stage, the compost pile temperature gradually decreased, making it more suitable for the growth of mesophilic bacteria, which may have contributed to the increase in the copy number of the total bacteria. Generally the proportion of anaerobic ammonium oxide bacteria (relative to total bacteria) was quite low, ranging from 1.19% in CMC2 to 8.84% in CMC4, indicating that the role of anammox bacteria was limited to the total metabolism of the composting process.

**Anammox Bacterial Community Distribution and Correlation with Environmental Factors**

CCA was conducted to identify correlations between the distribution of anammox bacterial community and the environmental variables in the composting process, based on the OTU matrix for anammox sequences. The results showed that the CCA axes in 16S rRNA gene explained 48.0% but the CCA axes in HZO sequences explained 35.4% of the total cumulative variance of the correlation between the anammox bacterial community distribution and environmental factors (Fig. 6). CCA analysis showed that all of the anammox bacteria were divided into three groups; the first group was CMC1, CMC0, and CMC4, the second group was CMC2 and CMC3, and the third group included CMC5 and CMC6. The distribution of anammox bacteria was significantly correlated ($p$ = 0.004, 1,000 Monte

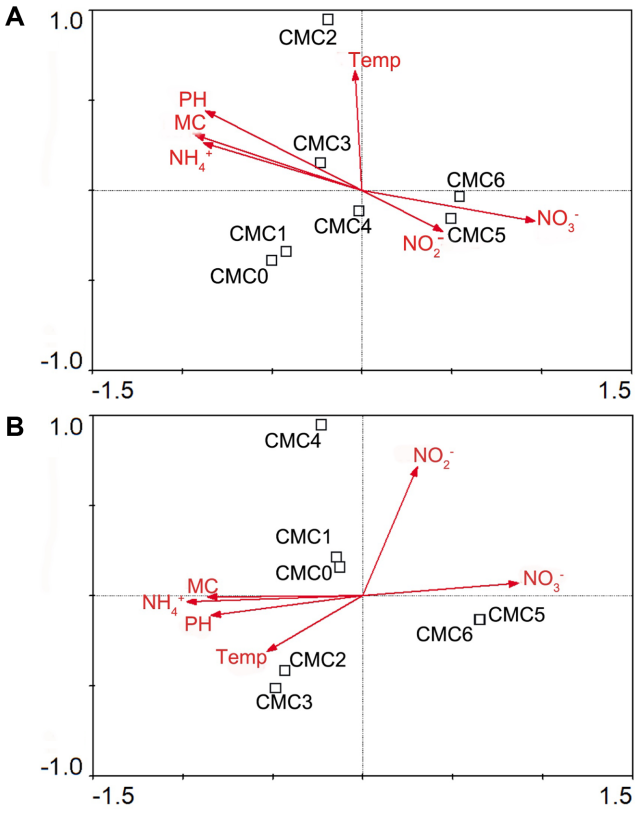


**Fig. 6.** Canonical correspondence analysis (CCA) ordination plots for the first two dimensions to show the relationship between the distribution of anammox bacterial community with environmental parameters, analyzed using 16S rRNA gene sequences (**A**) and HZO protein sequences (**B**) in the cow manure compost.

Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors). Temp, temperature; MC, moisture content.

Carlo permutations) with the concentration of $NH_4^+$-N. In the early stage of composting (CMC0, CMC1, CMC2, and CMC3), the concentration of $NH_4^+$-N was positively correlated with the anammox bacteria community. Other environmental factors did not contribute significantly ($p > 0.500$) to the anammox bacterial community, but the pH and concentration of nitrate were significantly correlated ($p < 0.01$) with the concentration of $NH_4^+$-N .

## Discussion

Anammox bacteria have been observed in various natural ecosystems [33] since their first discovery in a wastewater treatment plant [2], indicating that anammox is probably a common part in the nitrogen cycle in nature. Composting is a complex process in which the favorable prerequisites for the occurrence of anammox are available, namely the presence of ammonium and nitrite and an anaerobic environment. Our results show that anammox is present in compost. The presence of anammox bacteria in cow manure compost was determined based on the detection of 16S rRNA and *hzo* genes in this study. This is the first report of anammox bacteria in compost. Anammox bacteria were detected at each stage of the composting process in this study, indicating that N loss resulted from anammox may occur throughout composting. Considering that anammox plays a crucial role in the transformation of nitrogen, understanding this novel pathway is of significance for increased understanding of the process of nitrogen transformation in composting.

Phylogenetic analysis showed that all the detected anammox bacteria in compost were closely associated with *Candidatus* "Scalindua," *Candidatus* "Brocadia," and *Candidatus* "Kuenenia" (Figs. 2 and 3). Compared with anammox bacteria communities in marine sediments where only one genus was observed [9], our results revealed that anammox bacteria were very diverse in cow manure compost, reflecting the larger variety of anammox niches offered in cow manure compost. This is in agreement with recent studies in various soil ecosystems showing a high diversity of anammox bacteria [14]. *Candidatus* "Brocadia" and *Candidatus* "Kuenenia" are the most common of these bacteria in freshwater and terrestrial ecosystems with human influences [11, 14]. These two genera are also the dominant species in many high-temperature habitats such as hot springs (52°C) [34], in petroleum reservoirs (72°C) [35], and even in hydrothermal vents (85°C) [36]. These findings indicate that *Candidatus* "Brocadia" and *Candidatus* "Kuenenia" can live under a broad range of temperatures. In this study, *Candidatus* "Brocadia" and *Candidatus* "Kuenenia" were also detected in compost samples isolated during the thermophilic stage (>45°C), as well as other stages of composting (Fig. 4A phylotype K1). In previous studies, *Candidatus* "Scalindua" organisms were dominant in aquatic environments with moderate temperature, such as the marine ecosystem [9]. In this study, however, anammox bacteria of *Candidatus* "Scalindua" occurred in all stages of composting, especially in the initial material and the maturation stage (Fig. 4B, OTU1 and OTU9).

In this study, although anammox bacteria were detected throughout composting, higher anammox bacteria diversity was found during the thermophilic stage. It is reasonable to assume that anammox bacteria may be active and N loss may be intensive during the thermophilic stage of composting.

The coexistence of ammonium nitrogen and nitrite nitrogen in an anaerobic environment is a prerequisite for the occurrence of anammox bacteria. During the thermophilic stage of composting, sufficient ammonium was available since the concentration of ammonium usually peaks during this period of composting (Table 2). A number of studies have suggested that aerobic ammonium oxidation (nitrification), rather than nitrate reduction (denitrification), may be the main source of nitrite for anammox [12, 37]. Large numbers of ammonia-oxidizing bacteria are consistently detected in composting piles, and nitrification is active even at temperatures approaching 70°C [20, 39], which probably provides the nitrite needed for anammox. A large amount of oxygen ($O_2$) is consumed during the thermophilic stage owing to the highly active aerobic microbial reactions. Therefore, some anaerobic niches probably exist in a compost pile, which is a highly complex ecosystem. Based on the above-mentioned analysis, the conditions suitable for anammox bacteria can be well satisfied during the thermophilic stage of composting, suggesting the potential for a higher diversity of anammox bacteria during the thermophilic stage.

Owing to the slow growth rate, the detection of anammox bacteria in environment has long been limited mostly to molecular techniques [37, 39]. Ever since the first enrichment of anammox bacteria, 16S rRNA gene sequences have been available in GenBank and could be used for PCR primer design. Several primer sets targeting the 16S rRNA gene of anammox bacteria were designed and applied in a wide range of environments [40]. In this study, two sets of PCR primers were used to detect the signals of the anammox bacterial 16S rRNA gene. The results showed that the expected fragment length of 450 bp (primer set Amx368f-Amx820r) and 750 bp (primer set Pla46f-Amx820r) were successfully obtained from all samples (Table 3). The results of the present study showed that 55% and 75% of the 16S rRNA gene sequences obtained by amplification with primer sets Pla46f-Amx820r and Amx368f-Amx820r, respectively, belonged to the anammox group. As shown in Fig. 2, more 16S rRNA gene sequences obtained by using primer set Amx368f-Amx820r were similar to known anammox bacteria than those produced using primer set Pla46f-Amx820r. HZO is a key enzyme involved in anammox, as it oxidizes the unique anammox transformation of intermediate hydrazine to dinitrogen gas ($N_2$) [41]. Therefore, primers that could amplify the *hzo* gene are often used to study anammox bacteria [24]. In this study, one published primer set was used to analyze the *hzo* gene [26] and sequences closely related to anammox bacteria were amplified in samples taken from different phases of composting.

The specificity of the PCR primer set is critical for the detection of anammox bacteria in samples [39]. The primers targeting the functional *hzo* gene are more accurate and specific for classification and phylogeny, since the sequences of the 16S rRNA gene are highly conserved [26]. Therefore, the *hzo* gene seems to be a better genetic marker to estimate the anammox bacterial community in various ecosystems [24]. However, the application of primers targeting the 16S rRNA gene is also necessary because the information obtained from both specific PCRs are often complementary to each other. Primers of the 16S rRNA gene can also be used as an alternative when primers that should target the *hzo* gene fail to detect anammox under certain circumstances. In this study, our results were consistent with previous works [37, 39] that showed that the primers targeting the *hzo* gene were more specific than the primers targeting the 16S rRNA gene, with only 65% of 16S rRNA gene clones, but 100% of *hzo* gene clones, belonging to known anammox bacteria.

To explain the activity of anammox bacteria, it is necessary to measure the anammox bacterial growth coupled with the rates of ammonia oxidation and nitrite reduction. In addition, the dynamics of functional genes also should be monitored to explain the activity of the bacteria. Therefore, in this study, it is difficult to explain the role of anammox bacteria in the transformation of nitrogen that occurs during composting, although anammox bacteria were detected. Thus, further efforts are required to obtain a more complete understanding of the role of anammox bacteria and anammox in composting.

In conclusion, our results provide molecular evidence that anammox bacteria are present during composting of cow manure, which is an important consideration for the study of nitrogen transformation in compost systems.

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