# Study on performance of granular ANAMMOX process and characterization of the microbial community in sludge

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**Abstract** Anaerobic Ammonium Oxidation (ANAMMOX) is a novel biological nitrogen removal process, which is regarded as the most economical process at present. In this paper, two lab-scale UASB reactors, one of which was inoculated with the mixture of anaerobic sludge and aerobic sludge, the other with river sediments, were started up, using the inorganic synthetic water containing ammonium and nitrite as influent. After 421 days' and 356 days operation respectively, the ammonium removal efficiencies in two reactors reached 94% and 86% respectively, the total nitrogen volumetric loading rates were 2.5 and 1.6 kgN/$m^3$.d. ANAMMOX granules were obtained in both reactors; the color of most granules was brown, but some of them were red. Based on the observation and studies on the microstructure of the granules, three kinds of ANAMMOX granular sludge formation mechanisms were proposed: adhering biofilm and disintegrated granular core mechanism, adhering biofilm and inorganic core mechanism and the self-coherence mechanism. For phylogenetic characterization of anaerobic ammonium oxidizers,16S rDNA approach was performed using *Planctomycetales*-specific PCR amplification. The dominant anammox bacteria occupied more than 90% of *Planctomycetales*-specific bacteria, and 27% of all bacteria in reactors. The dominant anammox bacteria distantly related to all currently reported candidate anammox genera. Functional gene of *amoA* was analyzed to investigate the 'aerobic' ammonium-oxidizing bacteria in *β-Proteobacteria.* The 'aerobic' ammonium-oxidizing bacteria were more diverse than anammox bacteria, but most of them clustered in anoxic ammonium-oxidizing *Nitrosomonas eutropha/europaea* groups. The composition of 'aerobic' ammonium-oxidizing bacteria is only 2% of all of bacteria in reactors.

**Keywords** 16S rDNA; amoA gene; ANAMMOX granule; ANAMMOX process; phylogenetic analysis; planctomycetes

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## Introduction

The ANAMMOX (ANaerobic AMMonia Oxidation) process is a novel biological nitrogen removal process, in which the ammonia is oxidized to nitrogen gas using nitrite as the electron acceptor (Mulder *et al.*, 1995). This microbiological reaction is very attractive as it presents the opportunities for an N-removal process that improves the overall energy and material balance (Imajo *et al.*, 2004). In wastewater treatment, the combination of partial nitrification and anammox process can save operational costs of aeration and additional organic carbon, and reduce $CO_2$ emission, compared to a conventional nitrification-denitrification system.

Because anammox bacteria have a very slow growth rate and their activity is inhibited by exposure to molecular oxygen even at sub ppm levels (Van de Graaf *et al.*, 1996), thus it is believed that anammox bacteria are difficult to cultivate. To apply the anammox process to wastewater treatment, it is essential to develop a reactor configuration that is suitable for growing and accumulating the anammox bacteria. Considering the characteristics of anammox bacteria noted above, granular-sludge reactors were considered to be suitable. Among several types of reactors, UASB reactors have been successfully used

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for cultivating high concentrations of anaerobic microorganisms that have slow growth rates and low yields (Lettinga, 1991). With granular sludge it is possible to maintain a large amount of active biomass in a methanogenic reactor, thus allowing for stable operation under high loading conditions. However, the granulation mechanism is complicated and not yet adequately understood.

Currently, the dominant bacterium from the anammox sludge could not yet be isolated in pure culture. Using molecular biotechnology, three genera of anammox bacteria have been discovered: Brocadia, Kuenenia and Scalindua. The first two have been found in wastewater treatment systems (Strous *et al.*, 1999). The latter, Scalindua, has also been detected in many marine ecosystems, such as the Black Sea. The three genera share a common ancestor, but are evolutionally quite far apart (less than 85% sequence similarity on the 16S level). Still, all anammox bacteria seem to be very similar phenotypically: they all grow at the same, very slow rate, they all have an anammoxosome and ladderane lipids. In addition to these anammox bacteria yet not isolated *Planctomycetales*, classical 'aerobic' ammonium-oxidizers of the β-subclass of Proteobacteria were also found to have anaerobic ammonium-oxidizing activity (Schmidt and Bock, 1997). Some 'aerobic' ammonium-oxidizers were frequently found in anammox reactors (Tal *et al.*, 2005).

Based on these considerations, the objectives of this study are: 1) to obtain Anammox process and Anammox granular sludge in UASB reactors; 2) to propose ANAMMOX granular sludge formation mechanisms; and 3) to determine the diversity and quantity of anammox bacteria and classical 'aerobic' ammonium-oxidizers in an active anammox sludge using molecular biotechnology.

## Materials and methods

### Experimental process

In this paper, inoculated with different normal sludge, two similar UASB reactors were operated. The experimental apparatus and the process flow chart were shown in Figure 1. The total volume of the UASB reactor was 3.05 L, in which the reaction zone volume was 1.60 L; and the settling zone volume was 1.45 L. The temperature of the reaction zone was controlled at about 35°C by hot water recycle. The first reactor named R1 was inoculated with the mixture of anaerobic granular sludge (taken from an UASB reactor treating brewery wastewater) and aerobic activated sludge (taken from an aeration tank treating municipal wastewater), and the second named R2 was inoculated with river

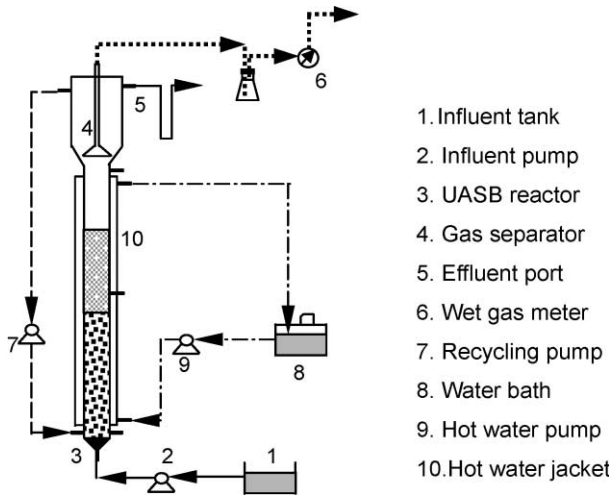


**Figure 1** Experimental apparatus and process scheme chart

sediments (taken from the river in the campus of Tsinghua University, Beijing). The sludge concentrations of two UASB reactors were 14.3 and 9.3 gVSS/L respectively.

The synthetic wastewater was used during the whole experiment. $NH_4HCO_3$ and $NaNO_2$ were added into the tap water. Concentrations of phosphorus and yeast extraction power in synthetic wastewater were both 50 mg/L. Some special trace elements (EDTA:10 mg/L; $ZnSO_4$.7H2O: 4.4 mg/L; $CoCl_2.6H_2O$: 3.2 mg/L; $MnCl_2.4H_2O$: 10.2 mg/L; $CuSO_4.5H_2O$: 3.2 mg/L; $CaCl_2.2H_2O$: 11 mg/L; $FeSO_4.7H_2O$: 10 mg/L and $(NH_4)_6Mo_7O_{24}.4H_2O$: 2.2 mg/L) were also added.



#### Analysis

1. $NH_4^+$-N: Standard colorimetry (China EPA, 2002);
2. $NO_2^-$-N and $NO_3^-$-N: Ion-chromatograph (DX-100, Dionex);
3. Gas production: LML-2 style wet gas meter (Changchun Instruments company);
4. Gas component: 1490GC-TCD style gas chromatograph (HP-Shanghai analytic instruments company);
5. Sludge concentration (VSS and SS): Standard methods (China EPA, 2002);
6. Diameter distribution and settling velocity of the granules (Zuo *et al.*, 2002);
7. Scanning electronic microscope photo: Scanning electronic microscope (QUANTA 200, FEI).

#### DNA extraction and PCR amplification of 16S rRNA and amoA gene fragments

Bulk community DNA was extracted from 1.0 ml (equal to 1.4 mg of suspended solid) of reactor sludge according to the protocol previously reported using liquid nitrogen (Hurt *et al.*, 2001). Planctomycetales-specific 16S rRNA gene was amplified from extracted DNA by using Planctomycetales-specific forward primer PLA-46F (Neef *et al.*, 1998) and universal reverse primer 1390R. The primer set composed of amoA-1F and amoA-2R (Rotthauwe *et al.*, 1997) was used for the amplification of ammonium monooxygenase A (amoA) gene. The PCR thermal profile was as follows: initial denaturation at 94 °C for 2 min and some cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1 min, and extension at 72 °C for 1.5 min, and the final elongation step was extended to 10 min (Chouari *et al.*, 2003). To decrease the bias during PCR process, different cycles (18, 21, 25, 28, 31 cycles) were used for amplification. Because the product from 18 cycles of PCR also showed smear bands in gel-electrophoresis (data not shown), the product from 18 cycles of PCR was used as template for cloning. The PCR products were purified using a AccuPrep purification kit (Bioneer, Korea).

#### Clone library

The Planctomycetales-specific 16S rRNA amplicons and large rDNA operon, and amoA gene amplicons, were cloned by using a TA cloning kit (pGEM-T Easy vector; Promega) in accordance with the manufacturer's instructions. The clones were grown in Luria-Bertani medium plates supplemented with ampicillin (100 $\mu g.ml^{-1}$). Clones were randomly selected for further analysis. Cells were lysed by boiling and 1 μl of aliquots were directly used for PCR amplification and sequencing. The sequences of rDNA operon were determined using the forward and reverse primers and internal primers located on the 16S and 23S rRNA gene respectively (Schmid *et al.*, 2001). Sequences were checked for possible chimeras using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu).

#### Phylogenetic analysis

In order to determine the phylogenetic position of the 16S rRNA and amoA gene sequences, they were compared with available database sequences *via* BLAST search and the related taxa were obtained from GenBank. The multiple alignments were performed by the Clustal X program (Thompson *et al.*, 1997). The resulting alignments were manually checked and corrected when necessary. Gaps were edited with the BioEdit program. Evolutionary distances were calculated using the method described by Jukes and Cantor. The phylogenetic trees were constructed *via* the neighbor-joining method with the MEGA 2 Program. The similarity of mostly complete rRNA gene sequence of clone AS-1 with other related anammox bacteria and other related genera were compared with paired-base comparation. The tRNA gene was found from the rDNA operon using tRNAscan-SE software (http://www.genetics.wustl.edu/eddy/tRNAscan-SE).

#### Real-time PCR analysis

To determine the frequency distribution of anammox bacteria and 'aerobic' ammonium-oxidizing bacteria and in the anammox reactor sludge, real-time PCR amplification was performed using SYBR Green qPCR Kit (Finnzymes, Finland). Real-time PCR was performed in 96-well optical plates placed in DNA Engine Opticon System (MJ Research, MA, USA). The primer set composed of amoA-1F and amoA-2R was used for the amplification of *amoA* gene. Ana-F and modified Ana-R (5′-CGATACCGAAGCAC-CATGAGT-3′) (Fujii *et al.*, 2002) was used for specific anammox bacteria 16S rRNA gene amplification. The primer set 338F/518R was used for the determination of 16S for enumeration of all bacteria (Muyzer *et al.*, 1993). Cycle thresholds determined were compared to standard curves constructed using several concentrations of clones Pla-1 and AOB-1. Relative copy numbers among target organisms were evaluated. The PCR protocol was as follows: 1 cycle at 95 °C for 15 min and 40 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s. The $R^2$ values were greater than 0.98 for all of the curves.

#### Fluorescent *in situ* hybridization

The probe S- * -Amx-0368-a-A-18 (5′-CCTTTCGGGCATTGCGAA-3′) which can cover all anammox organisms up to now was used with Cy5 labeling. The formamide concentration during hybridization was 15% (Schmid *et al.*, 2003). The hybridization process and dual staining of cells with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent oligonucleotides was performed according to Pynaert *et al.* (2003).

## Results and discussions

#### Performance of the R1 reactor

The R1 reactor had been operated for 421 days continuously; the results were shown in Figure 2.

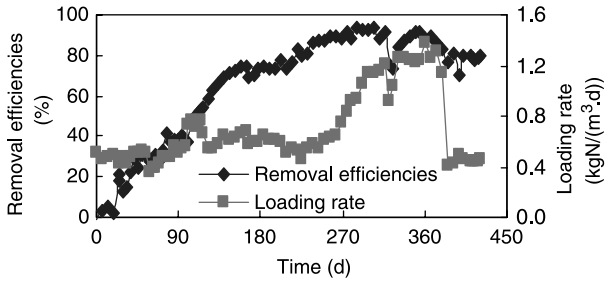


**Figure 2** $NH_4^+$-N removal efficiency and loading rate of R1 reactor

From the start-up to the 260th day of operation, the $NH_4^+$-N volumetric loading rates of the R1 reactor were kept about 0.6 kgN/($m^3$.d). From the 20th day, the $NH_4^+$-N removal efficiencies were gradually increased. As the reactor was operated to the 220th day, the $NH_4^+$-N removal efficiencies reached up to 80%. During the operational days of 221～260, the $NH_4^+$-N removal efficiencies were kept stably over 80%.

During the operational days of 261～330, the $NH_4^+$-N volumetric loading rates were gradually increased from 0.6 to 1.2 kgN/($m^3$.d), and the $NH_4^+$-N removal efficiencies kept about 85%. Then the R1 reactor was operated for 50 days at the $NH_4^+$-N volumetric loading rate of 1.2 kgN/($m^3$.d). During these 50 days, the $NH_4^+$-N removal efficiencies were about 85～94%.

As the reactor was operated to the 381st day, some sludge was taken from the R1 reactor for batch test, and the $NH_4^+$-N volumetric loading rate was decreased to 0.4 kgN/($m^3$.d) for keeping high removal efficiencies. During this period, the $NH_4^+$-N removal efficiencies were about 75～80%.

During the whole operation, the $NO_2^-$-N was removed together with $NH_4^+$-N. Before the 90th day, the ratios of $NH_4^+$-N removed to $NO_2^-$-N removed were gradually increased from 0.18 to 1.18. From the 91st day, the ratios were kept stable about 1.10. The ratio was different from that reported by Graaf (Van de Graaf *et al.*, 1997). In Graaf's article, the ratio of $NH_4^+$-N removed to $NO_2^-$-N removed was about 0.76.

When the reactor was operated to the 120th day, the color of granular sludge in the R1 reactor changed from black to brown.

#### Performance of the R2 reactor

R2 reactor had been operated for 366 days continuously; the results were shown in Figure 3.

It was different from the R1 reactor in that the $NH_4^+$-N removal efficiencies of the R2 reactor were about 20% when the start-up. From the 14th day to the 130th day, the $NH_4^+$-N volumetric loading rates were kept about 0.3 kgN/($m^3$.d), and the $NH_4^+$-N removal efficiencies were about 40～50%.

During the operational days of 131～270, the $NH_4^+$-N volumetric loading rates were fluctuated from 0.4 to 0.8 kgN/($m^3$.d), and the $NH_4^+$-N removal efficiencies were gradually increased from 50% to 79%.

As the reactor was operated to the 271st day, the $NH_4^+$-N volumetric loading rate was decreased to 0.4 kgN/($m^3$.d). Then, the $NH_4^+$-N volumetric loading rates were gradually increased. When the R2 reactor operated to the 350th day, the $NH_4^+$-N volumetric loading rate reached up to 0.8 kgN/($m^3$.d). During this period, the $NH_4^+$-N removal efficiencies were gradually increased from 70% to 82%.

From the 351st day, the $NH_4^+$-N volumetric loading rates kept about 0.8 kgN/($m^3$.d), and the $NH_4^+$-N removal efficiencies were about 82～86%.

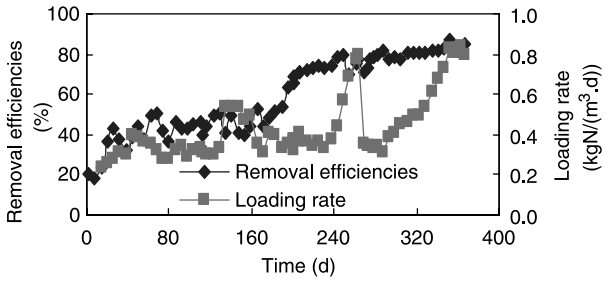


**Figure 3** $NH_4^+$-N removal efficiency and loading rate of R2 reactor

During whole operation of the R2 reactor, the $NO_2^-$-N was removed with $NH_4^+$-N in some ratio. Before the 50th day, the ratios of $NH_4^+$-N removed to $NO_2^-$-N removed were increased from 0.25 to 1.97. During the operational days of 51～155, the ratios were fluctuated from 1.12 to 1.88. From the 156th day, the ratios were gradually decreased from 1.45 to 1.10. In the end of the experiment, the ratios of $NH_4^+$-N removed to $NO_2^-$-N removed in the R2 reactor were similar to those in the R1 reactor.

When the reactor was operated to the 210th day, the red granular sludge was found for the first time from the sludge sample taken from the R2 reactor. Most of the red granules were smaller than 0.4 mm.

#### ANAMMOX granular sludge formation mechanisms

The microstructure of the ANAMMOX granules in the two reactors was observed under scanning electronic microscope; some of the photos were shown in Figure 4.

Under the SEM, a kind of cocci with irregular morphology could be found to be the dominating microorganisms in the surface of the granules, which were very similar to the ANAMMOX bacteria described in references (Van de Graaf *et al.*, 1996; Strous *et al.*,

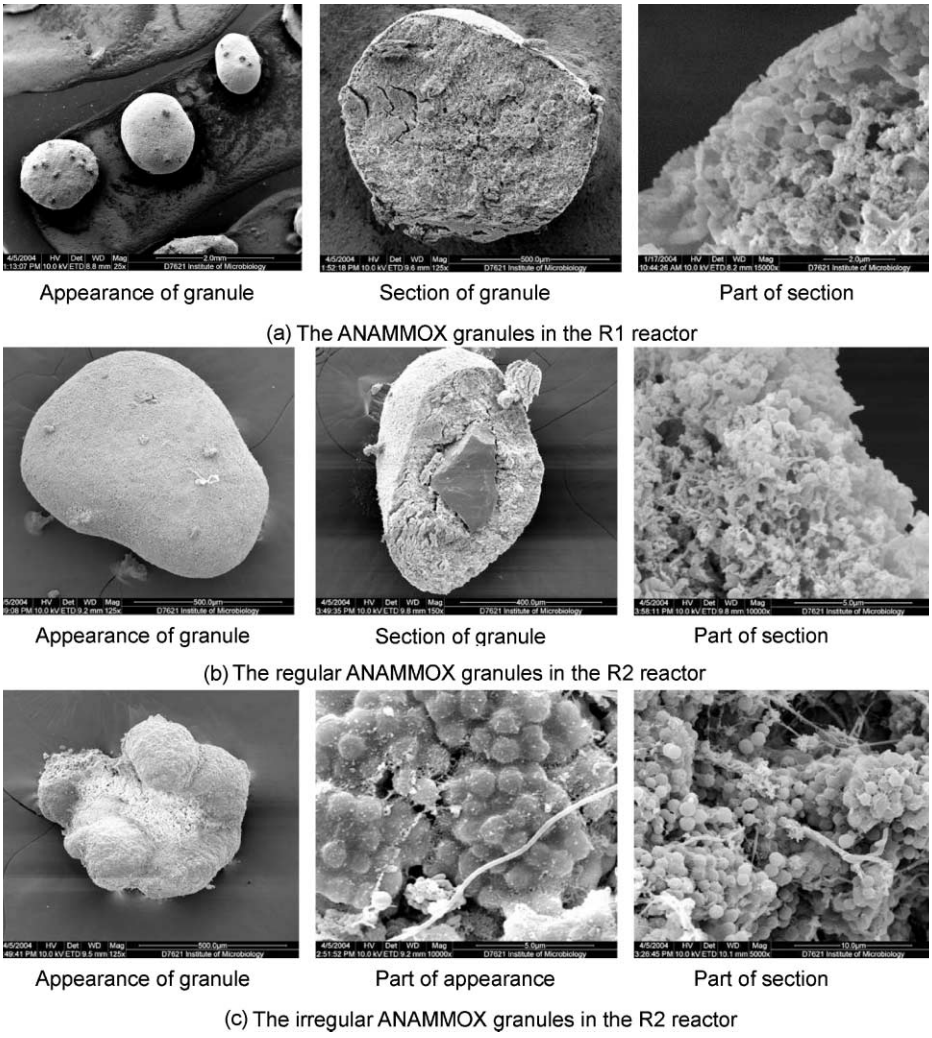


**Figure 4** SEM photos of the ANAMMOX granular sludge

1999; Jetten *et al.*, 2001). Based on the observation and studies on the microstructure of the granules, three kinds of ANAMMOX granular sludge formation mechanisms were proposed: adhering biofilm and disintegrated granular core mechanism, adhering biofilm and inorganic core mechanism and the self-coherence mechanism.

*Adhering biofilm and disintegrated granular core mechanism.* The process of adhering biofilm and disintegrated granular core mechanism was shown in Figure 5.

Granule formation using seed granules can be considered to proceed as follows: 1) ongoing growth of the bacteria; 2) attachment and growth of biofilm on the seeded granule; 3) ongoing growth of the biofilm; and 4) development of new granules from the detached biofilm (Imajo *et al.*, 2004). In this process, it is clear that the first step of providing a suitably large attachment surface is quite important to enhanced granulation.

*Adhering biofilm and inorganic core mechanism.* The process of adhering biofilm and inorganic core mechanism was shown in Figure 6.

The process of granule formation using inorganic core was similar to that of using seeded granule. The result showed that inorganic core can serve as an effective and inexpensive attachment material for development of Anammox granules.

*Self-coherence mechanism.* The process of self-coherence mechanism was shown in Figure 7.

Granule formation by self-coherence can be considered to proceed as follows: 1) ongoing growth of the bacteria; 2) smaller particles formation by self-coherence of bacteria; 3) self-coherence of smaller particles; 4) development of new granules from cohered small particles.

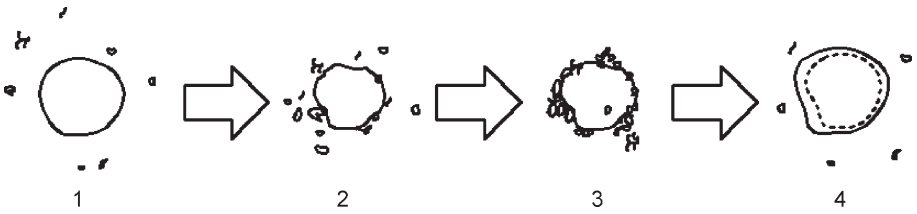


**Figure 5** Process of the adhering biofilm and disintegrated granular core mechanism

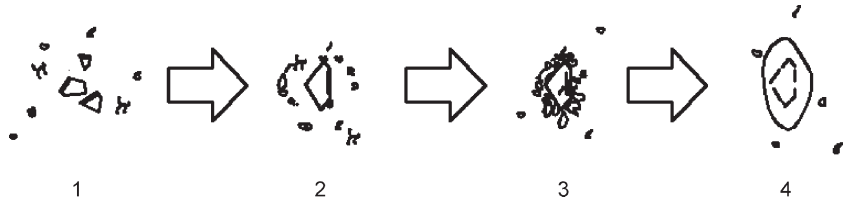


**Figure 6** Process of the adhering biofilm and inorganic core mechanism

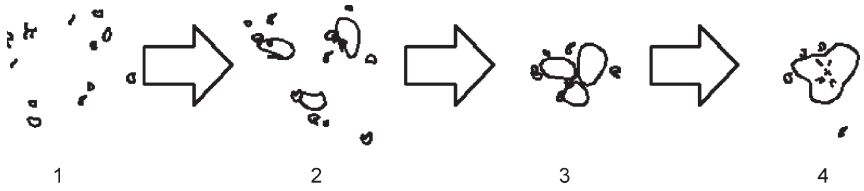


**Figure 7** Process of the self-coherence mechanism

#### Phylogenetic analysis of 16S rDNA from ANAMMOX sludge

The DGGE results show that the microbial population of R1 and R2 was very similar (date not shown). So, we selected R2 to construct clone library.

The 16S rRNA gene sequences of planctomycetes were amplified with the primers Pla46F and 1390R, and cloned. Sixteen clones of the resulting clone library were randomly sequenced and 15 sequences were highly similar to each other (more than 99.5% sequence similarity) and grouped together with P8. This coincides with the report that anammox bacteria of different genera rarely occur in the same wastewater treatment plants or enrichment culture (Schmid *et al.*, 2003). The main clones were distantly related to all other sequences presently represented in public 16S rRNA databases (lower than 92%) except for the clone KSU-1 (97.0%). Fujii *et al.* (2002) reported that the clone KSU-1 was dominant among detectable members of anammox biofilm cultures. Subsequent phylogenetic analysis (Figure 8) showed that the 15 sequences were branched relatively close to the known anammox genera and formed sequence cluster with clone KSU-1. And the other one sequence of clone P4 was clustered with *Candidatus Brocadia anammoxidans*.

The diversity of "aerobic" ammonium-oxidizing bacteria was also determined using *amoA* clone library. Twenty-seven clones of the resulting clone library were randomly sequenced, and grouped according to >99% *amoA* gene sequence similarity (Figure 9). About 52% of clones were grouped together with clone A25. The sequences of these clones were very similar to isolate K5F which was isolated from enriched anammox biofilm. About 26% of clones were grouped together with clone A7. The sequence of clone A7 was similar to clone LDD56-8 and this group of clones was reported to the main ammonium-oxidizing group in ammonium-oxidizing activated sludge with low dissolved oxygen (0.6% oxygen) (Park and Noguera, 2004). About 11% of clones grouped together with clone A24. These clones grouped together with *Nitrosomonas eutropea* M103 (Pynaert *et al.*, 2003). The clones A7 and A24 clustered with two of three "aerobic" ammonium-oxidizing groups S5 and S3 respectively, which was reported from the biofilm of the RBC where the *Candidatus "Kuenenia stuttgartiensis"* was identified (Schmid *et al.*, 2000). The other two groups of clones were clustered with *Nitrosomonas eutropha* Nm57, and *Nitrosolobus multiformis* ATCC25191, respectively.

#### Quantification of ammonium-oxidizing bacteria

In the current study, real-time PCR was investigated for applications in monitoring nitrifying populations in wastewater treatment because it combines high throughput with high analytical sensitivity and precision. One nanogram of extracted DNA from the

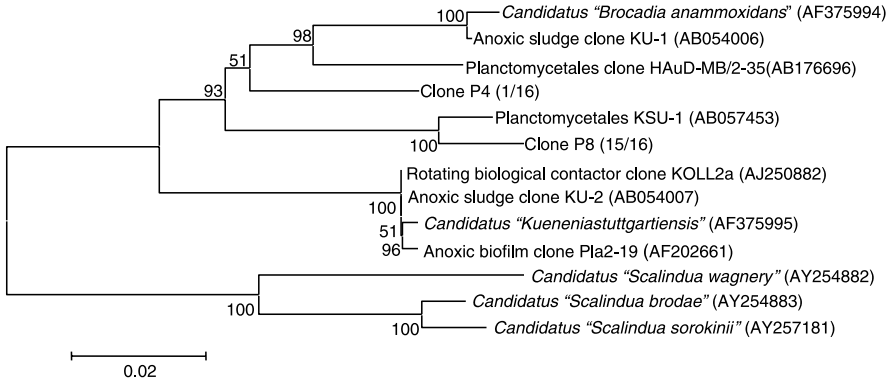


**Figure 8** Phylogenetic tree based on the results of 16S rDNA sequence comparisons

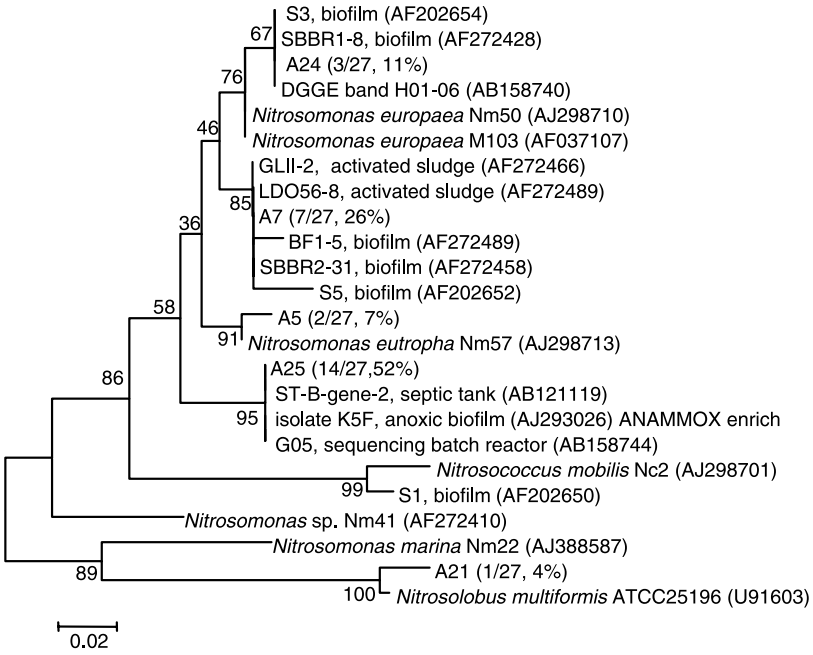


**Figure 9** Phylogenetic tree based on the results of *amoA* gene comparisons

reactor sludge includes $7.02 \times 10^{10}$ copies of Bacterial genes and $1.89 \times 10^{10}$ copies of P8 type anammox bacteria and $3.12 \times 10^{9}$ copies of *amoA* gene. The *Nitrosomonas europaea/eutropha* were assumed to contain two copies of *amo* operon (Norton *et al.*, 2002) and one copy of 16S rRNA per cell (Klappenbach *et al.*, 2001). Therefore, the copy number in ammonium-oxidizing bacteria would be about $1.56 \times 10^{9}$. Compare the 16S rRNA gene copy numbers; new type anammox bacteria were 27% and 'aerobic' ammonium-oxidizing bacteria were 2%. β-subclass ammonium-oxidizers analyzed to date possess specific rates for anaerobic ammonium oxidation which are 20 fold lower than the rate measured for the anaerobic ammonium-oxidizer affiliated with the Planctomycetales (Jetten *et al.*, 1998). So, the main ammonium-oxidizing activity would be contributed by the anammox bacteria.

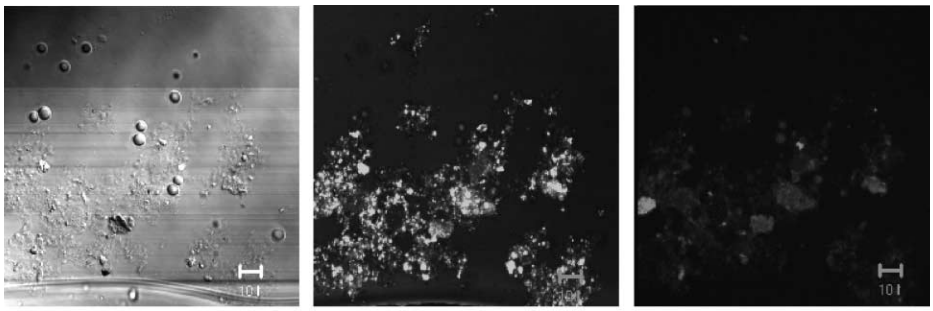


**Figure 10** FISH images of the anammox sludge (a) Phase contrast microscopy. (b) Epifluorescence microscopy of cells stained with DAPI (green). (c) Epifluorescence after hybridization with Cy5-labeled anammox probe (red)

### Identification of anammox bacteria

To determine the existence of anammox bacteria in the sludge, the probe S-*-Amx0368-a-A-18 which could cover most anammox bacteria up to now (Schmid *et al.*, 2003) was used. DAPI was also used to stain all the bacteria. Imaging using epifluorescence microscopy revealed anammox bacteria growing within the sludge (Figure 10).

## Conclusions

(1) Two lab-scale UASB reactors, one of which was inoculated with the mixture of anaerobic sludge and aerobic sludge, the other with river sediments, were started-up, while using the inorganic synthetic water containing ammonium and nitrite as influent. After 421 days' and 356 days' operation respectively, the ammonium removal efficiencies in two reactors reached 94% and 86% respectively, the total nitrogen volumetric loading rates were 2.5 and 1.6 kgN/$m^3$.d.

(2) Based on the observation and studies on the microstructure of the granules, three kinds of ANAMMOX granular sludge formation mechanisms were proposed: adhering biofilm and disintegrated granular core mechanism, adhering biofilm and inorganic core mechanism and the self-coherence mechanism.

(3) 16S rDNA approach was performed using *Planctomycetales*-specific PCR amplification for phylogenetic characterization of anaerobic ammonium oxidizers. The dominant anammox bacteria occupied more than 90% of Planctomycetales-specific bacteria, and 27% of all bacteria in reactors. The dominant anammox bacteria distantly related to all currently reported candidate anammox genera. Functional gene of *amoA* was analyzed to investigate the 'aerobic' ammonium-oxidizing bacteria in *β-Proteobacteria.* The 'aerobic' ammonium-oxidizing bacteria were more diverse than anammox bacteria, but most of them clustered in anoxic ammonium-oxidizing *Nitrosomonas eutropha/europaea* groups. The composition of 'aerobic' ammonium-oxidizing bacteria is only 2% of all of the bacteria in reactors.

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