Analysis of key microbial community during the start-up of anaerobic ammonium oxidation process with paddy soil as inoculated sludge

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ABSTRACT
A sequencing batch reactor (SBR)-anaerobic ammonium oxidation (anammox) system was started up with the paddy soil as inoculated sludge. The key microbial community structure in the system along with the enrichment time was investigated by using molecular biology methods (e.g., high-throughput 16S rRNA gene sequencing and quantitative PCR). Meanwhile, the influent and effluent water quality was continuously monitored during the whole start-up stage. The results showed that the microbial diversity decreased as the operation time initially and increased afterwards, and the microbial niches in the system were redistributed. The anammox bacterial community structure in the SBR-anammox system shifted during the enrichment, the most dominant anammox bacteria were Candidatus Jettenia. The maximum biomass of anammox bacteria achieved 1.68 × 10^9 copies/g dry sludge during the enrichment period, and the highest removal rate of TN achieved around 75%.

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Introduction
Nitrogen removal has taken more and more attention in the field of wastewater treatment. The conventional biological nitrogen removal process has many disadvantages such as high energy and material consumptions. However, the discovery of anaerobic oxidation of ammonium (anammox) bacteria overcame these issues (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003). The anammox phenomenon was first observed in the denitrifying fluidized-bed reactor (Mulder et al., 1995), and anammox bacteria can oxidize ammonium to nitrogen gas (N_2) under anoxic conditions using nitrite as the electron acceptor. The autotrophic and anaerobic properties of microorganisms save a great deal of energy and organic carbon source consumption. The anammox bacteria are affiliated with phylum Planctomycetes (Jetten et al., 2010), and five genera of anammox bacteria have been reported, “Candidatus Brocadia”, “Candidatus Kuenenia”, “Candidatus Scalindua”, “Candidatus anammoxoglobus” and “Candidatus Jettenia” (Schmid et al., 2005, 2007; Kartal et al., 2007, 2008).

In recent years, autotrophic denitrification processes based on anammox have been successfully applied in high-strength ammonia wastewater treatments (Jetten et al., 1997; van Dongen et al., 2001; López et al., 2008). It is very important for the start-up of the anammox process to choose suitable seed sludge for enrichment of anammox bacteria. Generally, the seed sludge...
derives from anaerobic sludge, nitrifying sludge or mixed sludge (Zhang et al., 2004; Zheng et al., 2004; Chen et al., 2011).

Anammox bacteria has been found in many ecosystems such as wastewater plant (Bae et al., 2010), river (Zhang et al., 2007; Wang et al., 2012), lake (Schubert et al., 2006), constructed wetland (Zhu et al., 2011b), marine (Dalsgaard et al., 2003), paddy soil, and other natural habitats with extremely cold condition and poor nutrition (Jiang et al., 2015). It shows not only that these bacteria are widely distributed in the natural environment, but also that the anammox process has great contribution to the global nitrogen cycle flux. Previous researches indicated that anammox reaction contributed 50%–70% to N₂ production in marine oxygen and 4%–37% to N₂ production in paddy soil (Dalsgaard et al., 2003; Kuypers et al., 2003, 2005; Arrigo, 2005; Lam et al., 2007; Zhu et al., 2011a).

Rice paddy fields make a great contribution to the nitrogen cycle in terrestrial ecosystems. The long-term fertilization of paddy soil has a high concentration of nitrogen and the soil surface is flooded seasonally, which provides a suitable growth environment for anammox bacteria. The previous report has shown that the abundance of anammox bacteria in the 40–50 cm depth of paddy soil reaches to 1.2 × 10⁷ copies/g dry soil (Zhu et al., 2011a). Present researches have focused on the distribution of anammox bacteria in paddy fields. However, few studies have involved the start-up of anammox process with sludge in paddy soil and discussed the evolution of bacteria during this process.

Hence, the present study aimed at investigating the dynamic changes of key microbial community structure during the start-up of anammox process with paddy soil as seed sludge, and finally successfully starts up an anammox process for nitrogen removal from wastewater.

1. Materials and methods

1.1. Seed sludge

A long-term fertilization paddy field soil as the sampling site in Songjiang District of Shanghai in China was selected. The depth of the collected samples was 0 to 80 cm, and the samples were placed in a sterile plastic bag and brought back to the lab under low temperature conditions. One part of paddy soil samples was inoculated in the sequencing batch reactor (SBR) to start up the anammox process, and another part was stored at −80°C for the subsequent analysis.

1.2. The SBR-anammox system

The paddy soil was inoculated in an 8-L SBR reactor (effective volume, 6 L) for anammox system in this study (Fig. 1). The reactor was equipped with on-line detection devices for detecting some important parameters, such as temperature, pH, dissolved oxygen and oxidation reduction potential. The formulated wastewater was introduced from the bottom of the reactor via a peristaltic pump (BT100-2J, Longer, China). The stirring rate was about 65 r/min. The temperature was controlled at 35°C using a temperature controlling belt. The anammox process in the SBR was operated with 1-day cycle. Each cycle was composed of four phases: nitrogen aeration (20 min), continuous feeding period (20 min), anaerobic reaction period (20 hr), settling (3 hr) and withdrawal (20 min). The nitrogen aeration in the beginning of each cycle was to remove oxygen from the SBR-anammox reactor system. The pH in the influent was kept between 7.5 and 8.3. The entire reaction device is wrapped with aluminum foil to achieve the dark effect. The initial sludge concentration was 6000 mg/L.

1.3. Synthetic wastewater

The influent concentrations of NH₄Cl and NaNO₂ were gradually increased during the start-up of the anammox system, which the concentration of NH₄Cl increased from 15 to 60 mg/L, and the concentration of NaNO₂ gradually increased from 15 to 78 mg/L. The composition of the mineral medium was (g/L): NaHCO₃: 1.85; KH₂PO₄: 0.00625; FeSO₄·7H₂O: 0.018; EDTA·2H₂O: 0.0125; MgSO₄·7H₂O: 0.2; CaCl₂·2H₂O: 0.3; and 1 mL/L of trace element solution. The trace element solution contained (g/L): MnCl₂·4H₂O, 0.99; EDTA, 15; H₃BO₃, 0.014; ZnSO₄·7H₂O, 0.43; CoCl₂·6H₂O, 0.24; NaMoO₄·2H₂O, 0.22; CuSO₄·5H₂O, 0.25; NiCl₂·6H₂O, 0.19; NaSeO₄·10H₂O, 0.21; and NaWO₄·2H₂O, 0.05 (Van de Graaf et al., 1996).

Fig. 1 – Schematic diagram of the reactor configuration. DO: dissolved oxygen; ORP: oxidation-reduction potential.
1.4. Water quality analysis

The influent and effluent samples were collected and analyzed according to the standard methods immediately (APHA, 2005).

1.5. DNA isolation, polymerase chain reaction (PCR), cloning, sequencing and phylogenetic analysis

DNA was extracted from 0.25 g of soil with the Fast DNA SPIN Kit for Soil (Q, BIOgene Inc., Carlsbad, CA). Amplification of anammox was carried out using nested PCR. The first step was the use of the universal primer PLA46f/630r (Neef et al., 1998; Purkhold et al., 2000). The products amplified in step 1 were amplified in step 2 using the anammox specific primer Amnx368f/Amnx820r (Amano et al., 2007) to obtain the final product. The purified PCR products were ligated and cloned using the pGEMT-easy vector (Promega, Madison, USA). The 96 white clones were randomly selected to make positive identification of bacteria. The positive clones were typed by restriction endonuclease Hha I and Mbo I, and the strains were sequenced (Jinweizhi Biotechnology Co., Ltd., China). The DNA sequence was subjected to a vector removal procedure using DNA star software. The independent operational taxonomic unit (OTU) and the biodiversity index of each sample were analyzed by the sequences sharing 3% difference using MOTHUR software. The representative sequences from each OTU were analyzed by online BLAST alignment and then multiple sequence alignments with the published anammox bacterial 16S rRNA sequences using the NCBI database. Phylogenetic trees were constructed using the neighbor-joining (NJ) with MEGA 5 package.

1.6. Scanning electron microscopy analysis

Sludge samples were taken at day 360 for microscopic analysis by scanning electron microscopy (SEM). The samples were purified using a phosphate buffer and fixed with 4% glutaraldehyde at 4°C. Then, they were dehydrated through ethanol with different concentration gradient (20%, 50%, 70%, 80%, 90%, and 100%) and dried in a Vacuum Freeze dryer (Scientz-10N, Shengkeyiqi, China). Finally, the morphology of the sludge samples was examined by SEM (Quanta 200FEG, FEI, USA).

1.7. High-throughput sequencing and data analysis

The PCR products were purified by Promega Agarose Gel DNA (Promega, Madison, USA) and measured with NanoDrop 2000 (Thermo Fisher Scientific, USA), mixed with equimolar ratio and sequenced by the Illumina MiSeq platform (Jinweizhi Biotechnology Co., Ltd., China). The measured raw sequencing data were stored and read Archive in the NCBI database. The sequence of reads was joined based on the principle of double-end pairing by Flash software. Then, the quality of the sequence was controlled by the Qiime software, and low quality sequences and short sequences were filtered out. The OTUs, rarefaction curves and the biodiversity index (Shannon, Chao 1, ACE) of each sample were generated and analyzed at 3% sequences difference using MOTHUR software. The Sequences assignment in taxonomic classifications was performed by RDP classifier. The sequences were assigned and analyzed down to the phylum, order and genus level in this study. Venn diagram and Hierarchical clustering analysis were used to describe the similarities between communities in the four samples.

1.8. Quantitative PCR assay

The abundance of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and anammox bacteria were determined by qPCR, and NOB was detected only for the abundance of Nitrospira and Nitrobacteria, which the most common species of NOB. The basic process of qPCR was as follows: (1) Preparation of standard curve: The extracted DNA samples were sent to biotechnology company (Jinweizhi Biotechnology Co., Ltd., China), and the specificity amplification, cloning, plasmid extraction, purification and quantitation of the three bacteria were completed by biology company. After the standard plasmids were obtained, they were serially diluted (7 concentration gradients) with TE buffer and quantitatively determined by the SYBR Green method. The total volume of the reaction system was 20 μL and the components were as follows: SYBR Green mix 10 μL, forward and reverse primer (concentration 5 μmol/L) 0.4 μL, DNA template 1 μL and ddH2O up to 20 μL. Primer information and reaction conditions are shown in Table 1 (Fan et al., 2016). In addition, the determination of each sample should be the standard curve of the production. The baseline fluorescence signal standard deviation of 10 times as a threshold, and then get the standard curve and C_{T} value. The amplification slope was between −3.5 and −4.2, the amplification efficiency was more than 95%, and the dissolution curve was a single peak. (2) The qPCR of the samples was determined by SYBR Green method. The primers information, the reaction system and the conditions were the same as those in the standard curve.

2. Results and discussion

2.1. Reactor performance in nitrogen removal

The anammox process was operated for 388 days in an SBR reactor. The influent concentration of NH₄Cl increased gradually from 15 to 60 mg/L and the concentration of NaN₂O₃ increased from 15 to 78 mg/L gradually during the start-up of anammox system. During the whole experiment, the SBR-anammox system performance was continuously analyzed and it could be divided into three stages (Fig. 2): Adsorption and desorption period (stage 1), transition period (stage 2), and effective period (stage 3).

2.1.1. The adsorption and desorption period (stage 1)

The first stage of starting the anammox process with the paddy soil as inoculated sludge is special, which exists the adsorption and desorption period. This period of the inoculated sludge was mainly from day 1 to day 80. In this period, HRT was 4 day and the concentration of NH₄-N and NO₂-N increased gradually from 15 to 30 mg/L in the ratio of 1:1 (mol/mol). In the adsorption stage, the highest removal rate of total nitrogen reached to 55%, this may be because of the adsorption effect of inorganic acid in the soil. With the operation of the reactor,
some adsorbents were gradually washed from the system and desorption of the remaining sludge occurred, resulting in a negative TN removal rate in the desorption stage.

2.1.2. Transition period (stage II)
The transition period was from day 81 to day 270. The hydraulic retention time (HRT) decreased from day 4 to day 2 gradually, and the concentration of NH$_4^+$-N and NO$_2^-$-N increased to 50 and 65 mg/L respectively, the concentration ratio of NH$_4^+$-N and NO$_2^-$-N increased from 1:1 to 1:1.32 gradually. In the first 20 days of this stage, the concentrations of ammonia and nitrite did not change significantly, but the nitrate concentration decreased. This was because of the denitrification effect from the heterotrophic bacteria in the system, and the carbon source was from the cell lysis of dead bacteria. With the reduction of organic carbon source, most of heterotrophic bacteria disappeared from the system gradually. However, the adaptability of AOB and NOB to anoxic environment (DO = 0.3 ± 0.1 mg/L) was increasing gradually with the running of the system, resulting into nitrate increase from day 120 to day 180. Through the optimization of the operation conditions and device, the nitrate concentration in the system was refrained after 180 day. The removal rate of TN was maintained at about 25% and the activity of anammox bacteria was low.

<table>
<thead>
<tr>
<th>Table 1 – Corresponding programs of the used PCR primers.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>AOB</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Nitrobacter</td>
</tr>
<tr>
<td>Nitrospira</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Anammox</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

PCR: polymerase chain reaction; AOB: ammonia oxidizing bacteria.

This was because of the denitrification effect from the heterotrophic bacteria in the system, and the carbon source was from the cell lysis of dead bacteria. With the reduction of organic carbon source, most of heterotrophic bacteria disappeared from the system gradually. However, the adaptability of AOB and NOB to anoxic environment (DO = 0.3 ± 0.1 mg/L) was increasing gradually with the running of the system, resulting into nitrate increase from day 120 to day 180. Through the optimization of the operation conditions and device, the nitrate concentration in the system was refrained after 180 day. The removal rate of TN was maintained at about 25% and the activity of anammox bacteria was low.

Fig. 2 – Nitrogen conversion and removal efficiency of total nitrogen in the SBR-anammox system. inf: influent; eff: effluent; SBR: sequencing batch reactor.
2.1.3. Effective period (stage III)
After 270 days, the activity of anammox bacteria increased (enrichment stage), the HRT was 1 day, and the concentrations of NH$_4^+$-N and NO$_2^-$-N were improved to 60 and 78 mg/L, respectively. On day 270, the effluent concentration of NH$_4^+$-N and NO$_2^-$-N decreased abruptly, and their highest removal rate was more than 95%. The removal rate of TN in this stage was also increased obviously, with the maximum of 75%. At the same time, the removal ratio of NH$_4^+$-N and NO$_2^-$-N is about 1.3, which is close to the theoretical value (1.32) of anammox reaction. At this stage, anammox phenomenon is more obvious, which is close to the theoretical value (1.32) of anammox index of the NAN$_3$ (7.18) was lower than NAN$_4$ (7.40), and finally light red.

2.2. Microbial community diversity

Sludge samples were collected from the SBR-anammox system at day 1, 134, 271 and 365, and were analyzed by high-throughput sequencing. The samples were named as NAN$_1$ (day 1), NAN$_2$ (day 134), NAN$_3$ (day 271) and NAN$_4$ (day 365) in turn. Four 16S rRNA gene libraries were constructed from Illumina MiSeq sequencing data of the samples NAN$_1$, NAN$_2$, NAN$_3$ and NAN$_4$ with 110,387, 79,318, 62,241 and 103,649 high-quality reads, respectively (Table 2). In order to evaluate the diversity and abundance of the microorganisms, the effective sequences were analyzed by the MOTHUR software at the 3% distance to obtain the operational taxonomic units (OTUs) and alpha diversity index (Table 2). The Good’s coverage of all four sequencing samples was 0.98, indicating that the bacterial communities of these samples were completely characterized by the library in this study. The total number of OTUs was estimated by Chao 1 and ACE indices. The results showed that NAN$_1$ had the greatest richness than the other three samples. The bacterial sequences from the NAN$_1$ – NAN$_4$ were identified to be 3681, 1844, 1468 and 1482 OTUs, respectively, indicating that the microbial species were very rich in a stable natural habitat. The reason might be due to the perennial interaction of complex environmental factors under natural conditions, which allow bacteria in the environment to coexist and each of these occupies a stable niche (Costerton et al., 1994).

In addition, the Shannon diversity index was used to estimate microbial diversity and distribution in the samples. Table 2 showed that the Shannon diversity index in the four samples had a tendency toward decreasing firstly and then increasing slowly. The NAN$_1$ had the highest diversity (Shannon = 10.83) and the NAN$_2$ had the lowest diversity (Shannon = 7.04) among the four communities. The Shannon index of the NAN$_3$ (7.18) was lower than NAN$_4$ (7.40), and thus the diversity in the NAN$_3$ was lower than that in the NAN$_4$. At the same time, the change of Simpson’s diversity index in Table 2 can also verify this conclusion. In summary, with the operation of the reactor, the microbial diversity decreased firstly and then increased, and the microbial niches in the system were redistributed.

2.3. Comparative analysis of microbial community structure

The Venn diagram and unweighted pair group method with arithmetic mean (UPGMA) tree of the samples NAN$_1$, NAN$_2$, NAN$_3$ and NAN$_4$ showed that the similarity and differences of the four bacterial community structures in different periods, which were identified using the hierarchical cluster analysis (Fig. 3). In the Fig. 3b, NAN$_1$ was clearly isolated, NAN$_3$ and NAN$_4$ were clustered together, and this was because the system became unstable under abrupt environmental change, but it trended to be stable with the operation time. These results indicated that the microbial community structures had an apparent evolution with the time of culture, as can be seen from the Venn diagram (Fig. 3a). However, 278 OTUs always occurred in the system throughout the operation, which accounted for 18.76% of the total OTUs in the microbes of the NAN$_4$ system. The Venn diagram and OTUs were used to describe the similarity between the four communities; NAN$_3$ and NAN$_4$ had more shared OTUs (994, 59% of total) than any of others (NAN$_1$/NAN$_2$, 1304, 30.8%; NAN$_2$/NAN$_3$, 751, 29.4%; NAN$_1$/NAN$_3$, 724, 16.7%; NAN$_1$/NAN$_4$, 682, 15.3%; NAN$_2$/NAN$_4$, 648, 24.2%).

2.4. Composition analysis of microbial community from four different samples

The phylogenetic classification of effective bacterial sequences from the four samples at three different taxonomic levels (phylum, order and genus) was summarized in Fig. 4, suggesting distinct bacterial community compositions.

2.4.1. Phylum level analysis

From the phylum assignment result (Fig. 4a), it was found that 10 known bacteria phylum were detected in the paddy soil (NAN$_1$), including Proteobacteria, Chloroflexi, Nitrospirae, Chlorobi, Bacteroidetes, Verrucomicrobia and Planctomycetes, and accounting for 36.43%, 13.02%, 5.92%, 1.26%, 9.13%, 2.38% and 1.36%, respectively. Proteobacteria and Chloroflexi were the main bacterial phylum in the four different operating periods and the final relative abundance were 31.6% and 27.4%, respectively. Previous studies have shown that Chloroflexi

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of sequences</th>
<th>Average length of sequences (bp)</th>
<th>OTUs *</th>
<th>ACE *</th>
<th>Chao 1 *</th>
<th>Shannon *</th>
<th>Simpson *</th>
<th>Good’s coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAN$_1$</td>
<td>110,387</td>
<td>422</td>
<td>3681</td>
<td>3908</td>
<td>3919</td>
<td>10.83</td>
<td>0.998</td>
<td>0.98</td>
</tr>
<tr>
<td>NAN$_2$</td>
<td>79,318</td>
<td>425</td>
<td>1844</td>
<td>2506</td>
<td>2475</td>
<td>7.04</td>
<td>0.974</td>
<td>0.98</td>
</tr>
<tr>
<td>NAN$_3$</td>
<td>62,241</td>
<td>426</td>
<td>1468</td>
<td>1949</td>
<td>1976</td>
<td>7.18</td>
<td>0.979</td>
<td>0.98</td>
</tr>
<tr>
<td>NAN$_4$</td>
<td>103,649</td>
<td>420</td>
<td>1482</td>
<td>2034</td>
<td>2006</td>
<td>7.40</td>
<td>0.982</td>
<td>0.98</td>
</tr>
</tbody>
</table>

OTUs: operational taxonomic unit. * They were defined by the 3% dissimilarity level (i.e., 97% identity threshold).
might play a very important role in the formation of aggregates (Chu et al., 2015), which provides a good condition for the cultivation of anammox bacteria that like agglomeration growth. Meanwhile, the abundance of Chlorobi and Planctomycetes increased to 10.39% and 8.48%, respectively. Nevertheless, Bacteroidetes, Verrucomicrobia and Nitrospirae showed a trend of being eliminated. This may be due to the absence of carbon sources in the system, resulting that the heterotrophic bacteria died out; on the contrary, the inorganic autotrophic bacteria could selectively grow. Denitrifying bacteria and inorganic autotrophic denitrifying bacteria have been detected in previous reports as Chloroflexi, Chlorobi and Proteobacteria (Wang et al., 2016). Most of Nitrospirae was not eliminated in the four systems because of the small amount of dissolved oxygen in the influent. However, some hypoxic-tolerating Nitrospirae have been domesticated after a long period of operation. The increase in Planctomycetes is exciting (from NAN_1 to NAN_4), because the anammox bacteria belonging to this phylum, the results may indicate that the anammox bacteria have been enriched.

2.4.2. Order level analysis

The bacterial identification at the order level was shown in Fig. 4b. The results indicated that the abundance of Anaerolineales increased from 7.54% to 21.39%, while the abundance of Caldilineales changed little during the system operation period. Meanwhile, Anaerolineales and Caldilineales belong to filamentous Chloroflexi, and several similar observations of filamentous bacteria in other anammox process have also been reported (Park et al., 2010). Filamentous bacteria are considered to play an important role in the aerobic granulation sludge process, which could provide a stable three-dimensional framework for the attachment of other bacteria (Gao et al., 2011). In addition, when the anaerobic ammonium oxide bacteria agglomeration is getting bigger and bigger, the interior of the three-dimensional frame will form an anaerobic environment conducive to the growth of bacteria. It also can be seen in the dynamic changes of the order level bacteria belonging to Planctomycetes from Fig. 4b. It was shown that the abundance of Phycisphaerales increased in the NAN_2, but the abundance rapidly decreased with the reactor running time, and the final relative abundance was 1.54%. However, the abundance of Brocadiales showed an increasing trend in the whole operation time. The other studies have confirmed that anammox bacteria belonged to the order Brocadiales (Jetten et al., 2010); therefore, the anammox bacteria in the present system may also increase in the whole operation time.

Additionally, the dominant orders in phylum Proteobacteria were Nitrospirales (5.92%), Burkholderiales (3.22%), Nitrosomonadales (2.83%), Pseudomonadales (2.29%) and Xanthomonadales (2.47%) in the NAN_1, while only Burkholderiales (7.56%) was the dominant bacteria in the sample NAN_4. It was found that the abundance of Nitrospirales and Nitrosomonadales increased to 15% and 7.75% from NAN_1 to NAN_2, respectively. This may be because a small amount of dissolved oxygen occurred in the influent and the constant stirring in the system also caused reoxygenation. Subsequently, the nitrifying bacteria growth was successfully inhibited by injection with pure N₂ and reduced the agitation speed from 100 to 60 r/min. This resulted in the decrease in the relative abundance of the Nitrospirales and Nitrosomonadales in the system, accounted for 1.08% and 0.75% in the sample NAN_4, respectively.

2.4.3. Genus level analysis

The key microbes were analyzed at the genus level in the different periods (Fig. 4c). It could be found that there are many unknown bacteria in the system based on high-throughput sequencing results, which showed that many of the sequences in the sample were not detectable at the genus level (19.9%, 36.6%, 35.8% and 30.3% for day 1, day 134, day 271 and day 365, respectively). As shown in the Fig. 4c, the dominant genus in the paddy field soil (NAN_1) was identified as Flavobacterium (1.26%), Perlucidibaca (1.26%) and Thiobacillus (1.15%), which belonged to order Bacteroidetes, Proteobacteria and Betaproteobacteria, respectively, but most of the bacterial genuses were eliminated with the operation time. During the period from day 1 to day 134, there was no observation of growth and enrichment of the genus planctomycetes. However, the genus planctomycetes...
including the uncultured planctomycete and Candidatus Brocadia was enriched based on the results of sample NAN_3 (Fig. 4c). In the sample NAN_4, Candidatus Brocadia gradually decreased 0.2%, in contrast the Candidatus Jettenia increased to 1.75%, indicating that anammox bacteria evolved with the operation of the reactor.

2.5. Phylogenetic analysis of anammox population

The sequences of similarity greater than 97% were divided into one OTU by MOthur software. The results showed that the biological diversity indices of anammox bacteria based on 16S rRNA under different time. The N1–N4 represent clone
library names for the samples from days 1, 134, 271 and 365, respectively. The scarcity curve showed that the number of clones in the four gene libraries was close to saturation (Fig. 5), which indicated that the clone library was constructed in the present study covering the anammox bacterial diversity in the sample. It can be seen from Table 3 that the diversity of the anammox bacteria showed a tendency to increase initially and then decrease gradually during the start-up. The highest diversity was observed on day 271, this may be because the anammox bacteria gradually adapted to the system environment. However, the Shannon index of anammox bacteria was the lowest during the experimental period, which caused the anammox bacterial community to become simple in the final stage.

The phylogenetic tree of the anammox bacteria, shown in Fig. 6, indicated that anammox bacteria evolved gradually throughout the start-up of the anammox process. The 37.5% sequences were identified as Candidatus Brocadia (Similarity ≥ 98%), and the 42.5% sequences were identified as Candidatus Kuenenia (Similarity ≥ 97%). This indicated that the predominant species of the anammox bacteria in the reactor eventually evolved into Candidatus Jettenia and the diversity of anammox bacteria became simple. This was consistent with other studies, where the results showed that the diversity of anammox bacteria was low in one particular habitat, and the predominant species was a single species (Penton et al., 2006; Schmid et al., 2007; Kartal et al., 2008; Hu et al., 2010). In addition, previous study has shown that the anammox bacteria were enriched from a paddy soil in column reactor which could maintain the original soil structure, and finally the dominant species were Candidatus Anammoxoglobus and Candidatus Jettenia (Hu et al., 2013). However, in our study, Candidatus Anammoxoglobus was not found in the enrichment process because the agitation of the SBR system destroyed the soil structure of particulate aggregates which were suitable for Candidatus Anammoxoglobus growth (Kartal et al., 2007).

The results of high-throughput sequencing showed that only Candidatus Brocadia was detected in N1 and N2, while Candidatus Brocadia and Candidatus Kuenenia were detected in the clone library. This was because the content of Candidatus Kuenenia is too low at the high-throughput sequencing detection limit. The results from the high-throughput sequencing and clone libraries showed that the abundance of Candidatus Brocadia increased firstly and then decreased, and Candidatus Jettenia was dominant in the final stabilized system. This indicated that the type of anammox bacteria has changed during the start-up of anammox process. In the high-throughput sequencing and clone libraries, uncultured planctomycete was also found during the whole process, and the results of clone library showed that some uncultured planctomycete had close relationship with anammox bacteria.

### Table 3 - Alpha diversity results of anammox bacteria under different time.

<table>
<thead>
<tr>
<th>Sample</th>
<th>The name of the clone library</th>
<th>The numbers of clones</th>
<th>The numbers of OTU</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>N1</td>
<td>40</td>
<td>3</td>
<td>0.95</td>
</tr>
<tr>
<td>Day 134</td>
<td>N2</td>
<td>40</td>
<td>5</td>
<td>1.06</td>
</tr>
<tr>
<td>Day 271</td>
<td>N3</td>
<td>40</td>
<td>6</td>
<td>1.64</td>
</tr>
<tr>
<td>Day 365</td>
<td>N4</td>
<td>40</td>
<td>3</td>
<td>0.82</td>
</tr>
</tbody>
</table>

* They were defined by the 3% dissimilarity level (i.e., 97% identity threshold).

2.6. Monitoring anammox and nitrifying bacteria with qPCR and SEM

The abundance of the anammox and nitrifying bacteria in the reactor was detected by real-time quantitative polymerase
chain reaction (qPCR). The result was shown in Fig. 7. The abundances of anammox bacteria, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the initial sludge samples were $1.98 \times 10^7$ copies/g dry soil, $2.11 \times 10^6$ copies/g dry soil and copies/g dry soil, respectively. It can be seen from Fig. 7 that the abundance of the three types of bacteria were still low ($\leq 1 \times 10^8$ copies/g dry soil) and played much smaller role on the TN removal in the first 40 days, thus, TN removal was mainly owing to the adsorption of soil at this stage. In the subsequent operation time, the quantity of NOB began to decrease due to the decrease of the dissolved oxygen concentration, while the AOB adapted to the system environment because they had higher affinity for oxygen than the NOB (Laanbroek and Gerards, 1993). The abundance of anammox decreased little and the activity of anammox was low. However, the abundance of AOB and NOB decreased rapidly with the optimization of reactor after 180 days of operation, and decreased to $1.09 \times 10^6$ copies/g dry sludge and $3.77 \times 10^4$ copies/g dry sludge, respectively. The anammox phenomenon became more obvious and the denitrification efficiency increased. At this time, the abundance of anammox bacteria gradually increased to $1.68 \times 10^{-9}$ copies/g dry sludge. Previous studies have shown that anammox bacteria could exhibit high activity when the abundance of gene copy numbers reaches this order (Cao et al., 2016). SEM was also used to observe the sludge samples in the stable stage. Some crater-like spherical bacteria were clearly observed and the particle size of bacteria was about 900 $\mu$m (Fig. 8), and which was similar to previous description (Egli et al., 2001).

Fig. 6 – Phylogenetic trees of the anammox bacteria under different time. The scale bar represents 2 nucleotide substitutions per 100 nucleotides; In OTU A (N/40), N represents the number of sequences contained in OTU A.

Fig. 7 – The number variation of the function bacterial in the reactor during start-up process. NOB: nitrite oxidizing bacteria.
3. Conclusions

The anammox bacteria were successfully enriched in the paddy soil, and the system was started up successfully. In this study, the abundance of anammox bacteria reached $1.68 \times 10^9$ copies/g dry sludge finally, and the highest TN removal efficiency was 75%. During the enrichment process, the total bacterial community structure within the system was changed, and the reduction of Nitrospira and the increase of Chloroflexi were beneficial to the growth and enrichment of anammox bacteria. In addition, the anammox bacteria in the system have undergone significant evolution. The most dominant anammox bacteria were *Candidatus Jettenia*.

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REFERENCES


