Performance and microbial community of simultaneous anammox and denitrification (SAD) process in a sequencing batch reactor

Jin Li^{a,*}, Zhimin Qiang^b, Deshuang Yu^a, Dan Wang^c, Peiyu Zhang^a, Yue Li^a

^a School of Environmental Science and Engineering, Qingdao University, Qingdao 266071, China

^b Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-environmental Sciences, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Beijing 100085, China

^c National Marine Environmental Forecasting Center, State Oceanic Administration, Beijing 100081, China

HIGHLIGHTS

- Simultaneous nitrogen and carbon removal was achieved in the SAD process.
- Contributions of anammox and denitrification to nitrogen removal were determined.
- Anammox and denitrifying bacteria dominated at low and high CODs, respectively.
- *Brocadia sinica* could grow with organic matter and tolerate high NO_3^- concentrations.

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ABSTRACT

A sequencing batch reactor (SBR) was used to test the simultaneous anammox and denitrification process. Optimal nitrogen removal was achieved with chemical oxygen demand (COD) of 150 mg/L, during which almost all of ammonia, nitrite and nitrate could be removed. Organic matter was a key factor to regulate the synergy of anammox and denitrification. Both experimental $\Delta\text{NO}_2^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ and $\Delta\text{NO}_3^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ values deviated from their theoretical values with increasing COD. Denitrifying bacteria exhibited good diversity and abundance, but the diversity of anammox bacteria was less abundant. *Brocadia sinica* was able to grow in the presence of organic matter and tolerate high nitrite concentration. Anammox bacteria were predominant at low COD contents, while denitrifying bacteria dominated the microbial community at high COD contents. Anammox and denitrifying bacteria could coexist in one reactor to achieve the simultaneous carbon and nitrogen removal through the synergy of anammox and denitrification.

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1. Introduction

Nitrogen removal from wastewater is critical to prevent the toxic effect on aquatic life, oxygen depletion and eutrophication in receiving water bodies. Conventional nitrogen removal from wastewater is accomplished through the combination of aerobic nitrification and anoxic denitrification. However, these treatment systems are energy intensive, space demanding and generate high amounts of sludge (Bagchi et al., 2012). Recently, anaerobic ammonium oxidation (anammox) process has received much attention for its cost-effectiveness and high efficiency in removing nitrogen from wastewater (Du et al., 2014). As an autotrophic oxidation process, this process converts ammonium (NH_4^+) to dinitrogen gas (N_2)

using nitrite (NO_2^-) as the electron acceptor, thus without requirement for aeration and addition of exogenous carbon sources compared to the conventional nitrification-denitrification process. Nowadays, the anammox process has been used to treat various wastewaters such as municipal wastewater (Leal et al., 2016), landfill leachate (Li et al., 2014; Wang et al., 2016), turtle breeding wastewater (Chen et al., 2013), swine wastewater (Waki et al., 2010), and high-salinity wastewater (Liu et al., 2014).

Nevertheless, an appreciable portion of nitrate (10%) can be released from anammox systems (Pathak et al., 2007; Lan et al., 2011; Du et al., 2015), which may violate wastewater discharge standards at times. Furthermore, organic matter (commonly expressed by chemical oxygen demand (COD)) in wastewater cannot be removed by the anammox process, but often induce an inhibitory effect. Considering that many wastewaters require simultaneous removal of nitrogen and organic carbon, a combina-

* Corresponding author.

E-mail address: ljin0532@126.com (J. Li).

tion of anammox and denitrification may provide a feasible solution.

With regard to the simultaneous anammox and denitrification (SAD) process, the concentration of COD seemed to play a critical role in process selection between anammox and denitrification (Chamchoi et al., 2008). Tang et al. (2010) reported that anammox bacteria were significantly suppressed by denitrifying ones at an influent COD/NO₂-N ratio of 2.92. Ni et al. (2012) also observed that high COD concentrations significantly affected ammonia and nitrite removal. However, Ahn et al. (2004) reported some opposite results. Considering that little is known about the SAD process, especially the impact of COD concentration, further research is urgently required. Besides, little study on the microbial community of SAD has been performed. Both the characteristics and interrelations of different microbial populations are still not clear.

A sequencing batch reactor (SBR) presents a great simplicity and flexibility of operation and a low cost as well, which favors the enrichment of slow-growing microbial community (Lan et al., 2011). In this work, the performance of the SAD process was assessed in an SBR at various influent COD concentrations. The individual contributions of anammox and denitrification to nitrogen removal were quantitatively determined. In addition, the structure of microbial community, including the relative abundances of anammox and denitrifying bacteria, was also explored.

2. Materials and methods

2.1. Reactor configuration and operation

An SBR made of polymethyl methacrylate was used in this study. The reactor was double-jacketed with an effective volume of 7.0 L. Its temperature was controlled at 30 ± 0.5 °C by a water bath with water recirculation through the outer chamber. The reactor was covered with a black cloth to prevent the potential growth of phototrophic micro-organisms. The operating mode of the reactor consisted of 0.5 h influent feeding, 8 h anoxic stirring reaction, 0.5 h sludge settling and 0.5 h effluent discharging. The seeding sludge was taken from another lab-scale SBR which had been operated for two years with influent COD, NH₄-N and NO₂-N concentrations of 200, 50 and 100 mg/L, respectively. The concentration of mixed liquor suspended solids was 4000 mg/L after inoculation in the studied reactor. In this study, synthetic wastewater was used as the influent, which contained desired concentrations of COD, NH₄-N, NO₂-N and trace elements with pH adjusted to 7.5. COD was simulated by glucose, with concentrations ranging from 50 to 500 mg/L at different operation phases. NH₄-N and NO₂-N concentrations were fixed at around 80 and 160 mg/L, respectively. Trace elements were added according to Du et al. (2014).

2.2. Sample pretreatment and analysis

Samples were withdrawn at the beginning and end of each operation cycle and centrifuged at 6000 rpm for 30 min. The supernatants were analyzed for COD, NH₄-N, NO₂-N and NO₃-N concentrations according to the standard methods (APHA, 1999). Each sample was analyzed in triplicate and the mean value was reported. Oxidation-reduction potential (ORP) and pH were measured by using relevant probes coupled with an S20 K ORP meter and an FE20 pH meter (Mettler Toledo), respectively. Biomass concentration was determined after filtering the washed samples through 0.45 μm membrane filter and drying at 105 °C to constant weight.

The SAD process mainly included anammox, denitrification and denitrification reactions as follows:

Based on nitrogen mass balance and Eqs. (1)–(3), the percentages of nitrogen removal (*p*) by anammox and denitrification can be calculated as follows:

$$p_{\text{ana}}(\%) = \frac{1.32\text{NH}_4^+_{\text{rem}}}{\text{NO}_2^-_{\text{inf}} + \text{NO}_3^-_{\text{inf}} + 0.26\text{NH}_4^+_{\text{rem}} - \text{NO}_2^-_{\text{eff}} - \text{NO}_3^-_{\text{eff}}} \times 100 \quad (4)$$

$$p_{\text{den}}(\%) = 100 - p_{\text{ana}} \quad (5)$$

where the subscripts represent anammox (ana), denitrification (den), removal (rem), influent (inf), and effluent (eff); and the concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ are all expressed in mg/L as N.

2.3. Construction of 16S rDNA clone library and phylogenetic analysis

DNA was extracted from sludge samples using the Fast DNA SPIN Kit for Soil (Bio 101, Vista, CA, USA). Microbial 16S rRNA genes were amplified with the bacterial primer set of 27f (5'-AGAGTTT GATCTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTACGACTT-3'). Polymerase chain reaction (PCR) was performed with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. Besides, a nested PCR was performed to amplify the anammox 16S rRNA gene. The initial amplification was carried out using the Pla46f-630r primer set (Neef et al., 1998) with a thermal profile of 96 °C for 10 min followed by 35 cycles of 1 min at 96 °C, 1 min at 56 °C, and 1 min at 72 °C. Afterwards, a 500-fold diluted PCR product was used as template for the second amplification with the Amx368f-Amx820r primer set (Schmid et al., 2005) using a thermal profile of 96 °C for 10 min followed by 25 cycles of 30 s at 96 °C, 1 min at 58 °C, and 1 min at 72 °C.

The PCR products were verified by electrophoresis. The amplified DNA was pooled and purified using a kit (Fermentas, Canada). The purified amplicons were then cloned by using a pMD18-T vector (Takara, Japan). The PCR-amplified products of positive recombinants were digested with the restriction enzyme HaeIII (Takara, Japan) at 37 °C for 4 h. Then they were electrophoresed in 1.5% agarose gel at 200 V for 20 min, and stained with ethidium bromide. Clones with similar banding patterns were grouped together, and one representative clone from each group was chosen for sequencing. The obtained sequences were compiled and compared to available rRNA gene sequences in GenBank using the NCBI BLAST program. Phylogenetic trees were constructed by the neighbor-joining method with the Jukes-Cantor correction through the MEGA 4 program (Molecular Evolutionary Genetics Analysis Software, Version 4.0).

2.4. Fluorescence in situ hybridization (FISH) analysis

Samples were fixed with freshly prepared 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 5 h at 4 °C. After centrifugation, the samples were washed twice with the PBS and stored at -20 °C in a storage buffer consisting of a 1:1 (v/v) mixture of PBS and ethanol. Hybridization was performed according to the standard protocol described by Amann et al. (1990). The probes used in this study were fluorescein isothiocyanate (FITC) labeled EUB338 (Amann et al., 1990), ALF968

(Nuria et al., 2008), BET42a (Nuria et al., 2008), GAM42a (Nuria et al., 2008) and AMX368 (Schmid et al., 2005). EUB338 and AMX368 could determine most bacteria and anammox populations, respectively. ALF 968, BET42a and GAM42a could determine most denitrifying bacteria. For each sample, total microscopic counts were performed with the fixed samples which had been stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA) at a final concentration of 0.1 mg/L for 10 min in the dark at 4 °C. Samples stained with DAPI were defined as total cell counts. Dual staining with DAPI and fluorescently labeled rRNA probes allowed estimation of the proportions of different bacterial groups to the total counts. Images were acquired through a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan).

2.5. Database submission of sequences

The nucleotide sequence data reported in this study have been deposited in the GenBank database (accession numbers KC907082 to KC9070118).

3. Results and discussion

3.1. Performance of the SAD process

Fig. 1a shows the concentration variations of three nitrogen species in the influent and effluent at different COD concentrations. At $\text{COD} \leq 150$ mg/L, the effluent ammonium only showed a slight increase, indicating that organic matter had little effect on anammox. At $\text{COD} = 200$ mg/L, the effluent ammonium increased to 16 mg/L; then it further increased with increasing COD, with a maximum value (i.e., 62 mg/L) reached at 400 mg/L COD. In contrast, nitrite removal showed an opposite trend, that is, the effluent nitrite decreased with increasing COD. At $\text{COD} \geq 200$ mg/L, nitrite could be removed completely. For nitrate, its effluent concentration exceeded the influent concentration at $\text{COD} \leq 100$ mg/L, and then nitrate was completely removed as the COD concentration further increased.

Du et al. (2014) reported that the ammonium removal efficiency increased when a small amount of organic matter was added. Chamchoi et al. (2008) reported that the ammonium removal efficiency dropped gradually with increasing COD; and regardless of its concentration, the organic matter could inhibit anammox but benefit denitrification. COD was a control parameter for process selection between anammox and denitrification. Xu et al. (2010) found that anammox was not suitable for wastewater with a $\text{COD}/\text{NH}_4^+\text{-N}$ ratio > 1 , because the autotrophic anammox bacteria were no longer able to outperform the heterotrophic denitrifying bacteria. In this work, anammox was greatly suppressed only at $\text{COD} > 200$ mg/L ($\text{COD}/\text{NH}_4^+\text{-N} = 2.5$). Anammox was predominant in the reactor at $\text{COD}/\text{NH}_4^+\text{-N} < 2.5$.

The routes of nitrogen removal were analyzed over the whole test period. The percentages of nitrogen removal by anammox and denitrification are presented in Fig. 1b. In general, nitrogen removal through anammox dropped with increasing COD. At $\text{COD} = 50$ mg/L, anammox accounted for 95% of nitrogen removal while denitrification only accounted for 5%. However, nitrogen removal through anammox decreased to 15% at $\text{COD} = 400$ mg/L, and then maintained constant even when more organic matter was added. The presence of organic carbon was not suitable for anammox. The addition of alternative electron source could affect the Gibbs free energy of anammox reaction. At high C/N ratios, denitrifying bacteria became greatly competitive against anammox bacteria. Although organic matter favored denitrification, anammox dominated in the reactor at $\text{COD} \leq 150$ mg/L. Besides, anammox accounted for 15% of nitrogen removal even when the COD

concentration was as high as 500 mg/L. This is different from the result of Chamchoi et al. (2008) who reported that anammox was completely inhibited at $\text{COD} > 300$ mg/L. Because anammox bacteria have a much slower growth rate than heterotrophic denitrifiers, denitrification was thermodynamically more favorable than anammox. However, anammox and denitrifying bacteria could coexist in one reactor (see Section 3.4) and the simultaneous nitrogen and carbon removal could be achieved through the synergy of anammox and denitrification.

The nitrogen removal rates at different organic loading rates are presented in Fig. 1c. The ammonium removal rate (ARR) was only slightly affected when the organic loading rate (OLR) was no more than $0.608 \text{ kg}/(\text{m}^3\cdot\text{d})$. The ARR was around $0.21 \text{ kg}/(\text{m}^3\cdot\text{d})$ and then dropped gradually. When the OLR was increased to $1.216 \text{ kg}/(\text{m}^3\cdot\text{d})$, the ARR dropped to $0.06 \text{ kg}/(\text{m}^3\cdot\text{d})$. The ARR did not decrease further with increasing OLR. The nitrite removal rate (NRR) was $0.34 \text{ kg}/(\text{m}^3\cdot\text{d})$ at $\text{OLR} = 0.152 \text{ kg}/(\text{m}^3\cdot\text{d})$, and then it increased with increasing OLR until reached a maximum (i.e., $0.48 \text{ kg}/(\text{m}^3\cdot\text{d})$) at $\text{OLR} = 0.608 \text{ kg}/(\text{m}^3\cdot\text{d})$. An early study reported that the simultaneous partial nitrification, anammox and denitrification process was affected more by nitrogen loading rate (NLR) than by OLR (Wang et al., 2011). However, the SAD process was affected greatly by OLR in this work. It was only possible when the slow-growing anammox bacteria surpassed the fast-growing denitrifiers. Anammox bacteria adapt to a low OLR while heterotrophic denitrifiers adapt to a high OLR. In this work, the ammonium removal efficiency reached 81% at $\text{OLR} = 0.608 \text{ kg}/(\text{m}^3\cdot\text{d})$, which was slightly better than the 79% removal achieved by the simultaneous partial nitrification, anammox and denitrification process at $\text{OLR} = 0.34 \text{ kg}/(\text{m}^3\cdot\text{d})$ (Chen et al., 2009). By comparison, it is seen that the SAD process was much simpler and performed better even at a higher OLR.

The stoichiometric ratios of nitrite and nitrate to ammonium (i.e., $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ and $\Delta\text{NO}_3^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$) could indicate the performance of anammox (Sun et al., 2011). Fig. 1d shows that at $\text{COD} = 50$ mg/L, the $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ was around 1.37, while the $\Delta\text{NO}_3^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ was around 0.25. The $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ ratio increased with increasing COD and reached a maximum (about 10) at $\text{COD} = 400$ mg/L, and then it fluctuated obviously when the COD further increased from 400 to 500 mg/L. In contrast, the $\Delta\text{NO}_3^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ ratio gradually dropped with increasing COD.

The theoretical values of $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ and $\Delta\text{NO}_3^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ for anammox were determined to be 1.32 and 0.26, respectively (Strous et al., 1998). At $\text{COD} = 50$ mg/L, both stoichiometric ratios were close to the theoretical values, indicating the occurrence of anammox which led to an efficient NO_2^- removal. However, the two ratios deviated from the theoretical values with increasing COD, especially at $\text{COD} > 400$ mg/L. Hence, high COD concentrations could induce a significant disturbance on the $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ ratio.

3.2. Nitrogen removal process in a typical operating cycle

To explore the synergistic mechanism of anammox and denitrification, nitrogen removal within different operating cycles was analyzed. Fig. 2 shows that at $\text{COD} = 50$ mg/L, both ammonium and nitrite concentrations decreased while the nitrate concentration increased with reaction time. This result indicates that anammox dominated the SAD process. At the end of the cycle, the residual nitrite could not be converted because there was no extra ammonium available. The pH increased from 7.5 to 7.8. At $\text{COD} = 150$ mg/L, both ammonium and nitrite were removed almost completely at the reaction time of 8 h. The nitrate concentration increased in the first 4 h and then dropped gradually. The effluent contained no nitrate after reaction for 8 h. The pH increased from 7.5 to 8.0. At $\text{COD} = 300$ mg/L, the removal patterns

Fig. 1. Performance of the simultaneous anammox and denitrification (SAD) process over a long-term test for 50 weeks: (a) concentrations of three nitrogen species in influent and effluent; (b) percentages of nitrogen removal by anammox and denitrification; (c) loading and removal rates of NH_4^+ and NO_2^- ; and (d) stoichiometric ratios of NO_2^- and NO_3^- removal.

Fig. 2. Nitrogen removal in a typical operation cycle of SBR at various influent COD concentrations: (a) 50 mg/L; (b) 150 mg/L; (c) 300 mg/L; and (d) 400 mg/L.

of three nitrogen species were quite similar to those at COD = 150 mg/L. The small difference is that, the ammonium concentration decreased to 31 mg/L at the reaction time of 6 h and then remained nearly constant, while nitrite and nitrate was completely removed. The pH increased from 7.5 to 8.2 after 8 h. At COD = 400 mg/L, both nitrite and nitrate were completely removed at the reaction time of 8 h. The ammonium concentration dropped in the first 5 h and then increased slowly to 59 mg/L in the effluent after 8 h. The pH increased from 7.5 to 8.5.

In general, the maximal nitrogen removal was achieved at COD = 150 mg/L, during which almost all the three nitrogen species (ammonium, nitrite and nitrate) could be removed. The anammox and denitrification exhibited a good synergy. Because of the anammox reaction, nitrate was produced in the middle of the SAD process, and then it was removed through denitrification. Nowadays, anammox has been increasingly applied to wastewater treatment due to its cost-effectiveness and high efficiency in nitrogen removal. However, a considerable amount of nitrate was produced, which may sometimes violate the wastewater discharge standards. The present study shows that if coupled with denitrification, this problem could be solved. Organic matter was a key factor to regulate the synergy of anammox and denitrification. At a low COD concentration, the effluent nitrate and nitrite concentrations were high because anammox was predominant; at a high COD concentration, the effluent ammonium concentration was high because denitrification was predominant in the process.

Under anoxic conditions, nitrate could be reduced by denitrifiers or other microbial species, thus promoting the dissimilatory nitrate reduction to ammonium (DNRA) (Dalsgaard et al., 2005). Some DNRA bacteria could also reduce nitrite to ammonium. At COD = 400 mg/L, the ammonium concentration started to increase after 5.5 h. The DNRA process occurred at a high C/N ratio, which agrees with what observed by Kumar and Lin (2010). The C/N ratio of wastewater directed the dissimilative pathway to either denitrification or DNRA. Besides, Kartal et al. (2007a) found that *Ca. Kuenenia* was able to mediate the DNRA process, so potentially were other anammox bacterial genera. In general, anammox, denitrifica-

tion and DNRA reactions may occur simultaneously at high COD concentrations, and denitrifying and DNRA bacteria were more competitive against anammox ones.

During the SAD process, the pH continuously increased with reaction time. It was found that the pH increment (ΔpH) was positively correlated with the COD concentration ($\Delta\text{pH} = 0.0019\text{COD} + 0.1983$, $R^2 = 0.97$). In addition, the effluent ammonium increased with increasing ΔpH . On the one hand, a high COD concentration inhibited anammox. On the other hand, anammox was favored under neutral pH conditions (Suneethi and Joseph, 2011). However, DNRA may occur at pH 8.5. A previous study reported that the DNRA process was positively correlated with pH (Schmidt et al., 2011). For the SAD process, ΔpH may be used as an indicator for ammonium removal and the specific reaction type.

3.3. Microbial community of the SAD process

The microbial community structure in the SAD process was analyzed through constructing 16S rDNA clone libraries. Sludge was extracted from the reactor in Week 15. From the constructed 16S rDNA clone libraries, 28 positive clones were selected and analyzed. All clones with 16S rDNA sequence similarity of at least 97% were grouped into an operational taxonomic unit (OTU). Totally 16 OTUs were identified. The phylogenetic tree construction of universal bacteria was presented in Fig. 3 and the phylogenetic tree construction of anammox bacteria was presented in Fig. 3b. Table 1 indicates the distribution of sequence and OTU from the universal and anammox bacteria clone libraries and their correlation with various phylogenetic groups.

In terms of universal bacteria, β -Proteobacteria accounted for 52% and were predominant. Besides, γ -Proteobacteria accounted for 9%. Neither α -Proteobacteria nor δ -Proteobacteria clone were identified. The second most abundant phylogenetic group was Acidobacteria (18%), and others included Chlorobi (8%), Chloroflexi (6%), Gemmatimonadetes (4%), Nitrospirae (2%) and Planctomycetes (1%). In the anammox bacteria clone library, *Candidatus Brocadia sinica* accounted for 96% (predominant), and uncultured

Fig. 3. Phylogenetic trees constructed with the neighbor-joining method: (a) universal bacteria; and (b) anammox bacteria. Reference sequences are obtained from GenBank with parenthesized accession numbers.

Fig. 3 (continued)

Table 1

Distribution of sequence and OTU from universal and anammox bacteria clone libraries and their correlation with various phylogenetic groups.

Phylogenetic group		OTU (clones)
β-Proteobacteria	Universal bacteria	
	<i>Rhodocyclaceae</i> bacterium*	1(18)
	<i>Thauera</i> sp.	1(13)
	<i>Azoarcus</i> sp.*	1(4)
	<i>Burkholderia</i> sp.	1(9)
γ-Proteobacteria	<i>Enterobacter ludwigii</i>	1(1)
	<i>Raoultella ornithinolytica</i>	1(1)
	<i>Acinetobacter</i> sp.	1(3)
	<i>Enterobacter</i> sp.	1(3)
	<i>Acidobacteria</i> bacterium*	1(15)
Acidobacteria	<i>Chlorobi</i> bacterium*	1(7)
Chloroflexi	<i>Bellilinea</i> sp.*	1(5)
Gemmatimonadetes	<i>Gemmatimonadetes</i> bacterium*	1(3)
Nitrospirae	<i>Nitrospira</i> sp.	1(2)
Planctomycetes	<i>Planctomycetales</i> bacterium*	1(1)
Total number		14(85)
Planctomycetes	Anammox bacteria	
	<i>Candidatus Brocadia sinica</i>	1(43)
	<i>Planctomycetales</i> bacterium*	1(2)
Total number		2(45)

* Uncultured.

Planctomycetales bacteria accounted for 4%. Compared to the universal bacteria, the microbial diversity of anammox bacteria was less abundant. It was reported that there were large phylogenetic distances between different anammox bacteria and different anammox species were rarely found in the same ecosystem (Kartal et al., 2007b). This indicates that each anammox species occupied a distinct ecological niche.

Among the microorganisms identified, denitrifying bacteria exhibited a good diversity and abundance, which mainly belonged to β-Proteobacteria (*Rhodocyclaceae* sp., *Thauera* sp., *Azoarcus* sp.). A previous study reported that β-subclass Proteobacteria played a key role in the activated sludge for nitrogen removal (Lee et al., 2002), which could contribute to both organic matter degradation and nitrogen removal. This also agrees with the nitrification-anammox biomass which was characterized with a high diversity of denitrifiers of β-Proteobacteria (Langone et al., 2014). The β-subclass Proteobacteria (denitrifiers) played an important role in both anammox-nitrification and anammox-denitrification processes. Besides, Wang et al. (2016) reported that *Thauera* could

coexist with anammox bacteria and contribute to the simultaneous denitrification and anammox.

Granulation was achieved in the SAD process. Most of the denitrifiers identified in the present work were reported to facilitate the formation of aggregates thereby preventing biomass from getting washed out. *Thauera* sp. could produce lots of extracellular polymeric substances which contributed to the granulation process (Heylen et al., 2006). *Chloroflexi* bacteria were facultative anaerobes and were important for granulation (Chen et al., 2013). Besides, *Chloroflexi* bacteria were present in a highly enriched anammox biomass (Cho et al., 2010). Anammox bacteria grew slowly and were difficult to enrich in the reactor, which makes the anammox process still a challenge in full-scale wastewater treatment (Ali et al., 2014). However, anammox-based granulation could be achieved through synergy with denitrifying bacteria in the SAD process, which helped anammox bacteria to enrich in the reactor.

Although anammox species belonged to bacteria domain, they were not identified by universal bacterial primer set (27f-1492r). Anammox bacteria were deeply branched in the *Planctomycetes* phylum and had low 16S rRNA similarities to other *Planctomycetes* genera (Schmid et al., 2005). The detailed reason for this lack of similarity still remains unknown. However, anammox species could be identified through nested PCR. 43 of 45 clones were closely related to *Brocadia sinica*. It was reported that *Brocadia sinica* was able to outcompete other anammox bacteria only under high ammonium and nitrite conditions (Oshiki et al., 2011). In this work, the influent ammonium and nitrite concentrations were around 80 and 160 mg/L, respectively which are much higher than those in natural ecosystems. As a result, *Brocadia sinica* could be enriched in the reactor. Besides, Hu et al. (2010) found that “*Ca. Brocadia*” was the abundant anammox species in wastewater treatment where organic compounds, ammonium, nitrite and nitrate were all present. *Brocadia sinica* was able to grow in the presence of organic matter and tolerate high nitrite concentrations.

3.4. Quantification of microbial community structure

Fluorescence in situ hybridization (FISH) was employed to quantify the microbial community composition in the SAD process. Sludge samples were extracted from the reactor in Weeks 5, 15, 30 and 40 (with COD concentrations of 50, 150, 300 and 400 mg/L, respectively). The abundances of bacteria, anammox bacteria and

Fig. 4. Composition of microbial community in SBR during different operation periods.

denitrifying bacteria were presented in Fig. 4. At COD = 50 mg/L, anammox bacteria accounted for 95% and dominated the microbial community. Although the abundance of anammox bacteria decreased with increasing COD concentration, it still accounted for 16% at COD = 400 mg/L. Denitrifying bacteria accounted for only 4% at COD = 50 mg/L, but they outcompeted anammox bacteria at COD = 300 mg/L. When the COD concentration was increased to 400 mg/L, denitrifying bacteria reached a maximum abundance (79%) and dominated the SAD process.

Anammox bacteria were predominant at low COD concentrations, while denitrifying bacteria were predominant at high COD concentrations. Denitrifying bacteria could outcompete anammox ones in an environment rich in organic matter, because anammox bacteria have a low activity and growth rate and denitrification is thermodynamically more favorable than anammox. However, similar to Daverey et al. (2015), this study found that anammox and denitrifying bacteria could coexist in one reactor and the simultaneous carbon and nitrogen removal could be achieved through the synergy of anammox and denitrification. The FISH results were also in accordance with the performance of the SAD process (see Section 3.1).

4. Conclusions

The SAD process was studied in an SBR. The optimal nitrogen removal was achieved at COD = 150 mg/L, during which almost all of ammonium, nitrite and nitrate could be removed. Both $\Delta\text{NO}_2^- - \text{N} / \Delta\text{NH}_4^+ - \text{N}$ and $\Delta\text{NO}_3^- - \text{N} / \Delta\text{NH}_4^+ - \text{N}$ ratios deviated from the theoretical values with increasing COD concentration. Denitrifying bacteria exhibited a better diversity and abundance than anammox bacteria. *Brocadia sinica* was able to grow in the presence of organic matter and tolerate high nitrite concentrations. Anammox and denitrifying bacteria could coexist in one reactor and the simultaneous carbon and nitrogen removal could be achieved.

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