Fast start-up, performance and microbial community in a pilot-scale anammox reactor seeded with exotic mature granules

Shou-Qing Ni [a,b,*], Bao-Yu Gao [b], Chih-Cheng Wang [c], Jih-Gaw Lin [c], Shihwu Sung [d]

[a] School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China
[b] School of Environmental Science and Engineering, Shandong University, Jinan 250100, China
[c] Institute of Environmental Engineering, National Chiao Tung University, Hsinchu 30010, Taiwan
[d] Department of Civil, Construction and Environmental Engineering, Iowa State University, Ames 50011, IA, USA

ABSTRACT

The possibility to introduce the exotic anammox sludge to seed the pilot-scale anammox granular reactor and its fast start-up for treating high nitrogen concentration wastewater were evaluated in this study. The reactor was started up successfully in two weeks; in addition, high nitrogen removal was achieved for a long period. Stoichiometry molar ratios of nitrite conversion and nitrate production to ammonium conversion were calculated to be $1.26 \pm 0.02 : 1$ and $0.26 \pm 0.01 : 1$, respectively. The Stover–Kincannon model which was first applied in granular anammox process indicated that the granular anammox reactor possessed high nitrogen removal potential of $27.8 \ kg/m^3/d$. The anammox granules in the reactor were characterized via microscope observation and fluorescence in situ hybridization technique. Moreover, the microbial community of the granules was quantified to be composed of 91.4–92.4% anammox bacteria by real-time polymerase chain reaction. This pilot study can elucidate further information for industrial granular anammox application.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Since anaerobic ammonium oxidation (anammox) reaction accounts for large amount of fixed nitrogen lose in the marine system (Jetten et al., 2009), this process is attracting more and more attention. Discovered in the middle 1990s, anammox bacteria are one of the latest additions to the biological nitrogen cycle. In the anammox process, ammonium is anaerobically oxidized to N_2 using nitrite as an oxidant. This autotrophic process uses CO_2 as the only carbon source.

The slow growth of anammox bacteria results in a very long reactor start-up and needs a high sludge retention rate period (Van der Star et al., 2007). Furthermore, anammox bacteria are obligate anaerobes, since over 2 mM oxygen can inhibit their metabolism (Strous et al., 1997). Bacteria growing in biofilms or flocs and granules were considered to be suitable for anammox process (Ni et al., 2010a,c). The first full-scale granular anammox reactor succeeded to start-up in 2007, which took 3.5 years, much longer than the originally expected 2 years (Van der Star et al., 2007). The modified upflow anaerobic sludge blanket (UASB) reactor was used in this process. The slow growth rate of anammox microorganisms

in combination with the inhibition effects and operational problems makes the start-up of the anammox process difficult. The fast start-up of anammox reactors may enhance the industrial application of anammox process.

Nowadays, the availability of anammox biomass is still the limitation of practical application. Previous studies usually seeded the reactors with activated, anaerobic or aerobic sludge, even anammox flocculent sludge (Fernandez et al., 2008; Imajo et al., 2004; Li et al., 2009; Lopez et al., 2008; Ni et al., 2009; Schmidt et al., 2004; Tran et al., 2006; Trigo et al., 2006; Yang et al., 2006,2009). Due to low level of anammox cells in the community and inhibition effect, these strategies usually had to start the process at low influent concentrations and loading rates, leading to longer start-up time. Apparently, the long start-up period is still the major obstacle for the practical application of anammox process, even though this process has been investigated for two decades. From the lesson of the first full-scale anammox reactor, which was directly scaled up from lab scale, the skipping of the pilot phase resulted in unexpected situations and a lag of start-up period (Van der Star et al., 2007). It was anticipated that some of the teething problems associated with new technologies and concepts would be identified and solved on pilot-scale. Moreover, most studies in the literature focused on the realization of anammox process in lab-scale reactors, while seldom research has been done on the pilot-scale to start up the process as soon as possible. In order to speed up anammox commercialization, the focus of this study

---

was the inoculation of mature anammox granules to fast start-up a pilot-scale reactor, which could catch potential problems and prevent costly mistakes before scaling up a laboratory-scale reactor. In 2008, anammox bacteria were found in a full-scale landfill-leachate treatment plant in East Asia (Wang et al., 2010). Until then, full-scale anammox plants were realized in both Europe and Asia. The introduction of mature sludge source as the seed may be a good choice to start-up the reactor, especially when the anammox sludge is not available nearby. To test this hypothesis for commercial application, the objective of this paper is to study the feasibility of inoculation of anammox reactor with exotic anammox sludge and evaluate the fast start-up of a pilot-scale anammox granular reactor for treating high nitrogen content wastewater. The performance of the granular anammox reactor was investigated with chemical analysis and anammox reaction stoichiometry. Microbial community of anammox bacteria was analyzed using molecular techniques. As pilot study was rare in the literature, this study can elucidate further information for industrial granular anammox application.

2. Methods

2.1. Reactor

The pilot-scale UASB reactor used in this study was a 50 L working volume with a water jacket to maintain a temperature of 37 °C. Equipped with the gas–solid separator at the top, the reactor was fitted with several ports for gas, liquid and solid. The gas produced in the reactor was collected at the top and measured with a wet-type laboratory gas meter (Schlumberger, Sugar Land, TX, USA). The effluent was recycled from the top of the reactor at a recycling ratio of 200% based on inflow rate. Wastewater recirculation could not only create the upflow water current, which would favor the granulation process, but dilute the influent to avoid the toxic effects of the high nitrite concentrations and benefit the anammox process (Ni et al., 2010c). Before to inoculate the seed sludge, about 5 cm thickness of gravel with three sizes (approximately 2, 5 and 10 mm diameters) were placed in the bottom of the reactor. The pH in the reactor was adjusted to 7.5–8.0 with CO_2 gas. To eliminate the possibility of oxygen infiltration, all tubing and connectors were black butyl rubber or polyvinylchloride (PVC). After inoculation with 10 L anammox granules, the reactor was run in a continuously-fed sequence at a hydraulic retention time (HRT) of less than one day.

2.2. Seed sludge

The seed granules were taken from a full-scale plant treating landfill-leachate. The main processes in this plant were aeration treatment, reverse osmosis and air stripping. The aeration tank had a working volume of 384 m^3 and a less than 1.5 d HRT. The dissolved oxygen concentration, temperature and pH in the aeration tank were approximately 0.5 mg/L, 30–33 °C and 7.4. In 2008, the simultaneous partial nitrification, denitrification and anammox process were discovered in the aeration tank (Wang et al., 2010). Due to the simultaneous process, over 80% of ammonium was removed from the aeration tank. The mixed liquor suspended solid and mixed liquor volatile suspended solid of the seed sludge were 4.67 and 3.78 g/L, respectively.

2.3. Synthetic wastewater

Synthetic wastewater was constituted of ammonium, nitrite and trace elements. Ammonium and nitrite in the form of $(NH_4)_2SO_4$ and $NaNO_2$ were added to the influent according to

the need of the experiment. More information about trace elements was described by Ni et al. (2010b). In order to supply efficient substrate, the nitrite (NO_2^-–N) to ammonium (NH_4^+–N) molar ratio in the feed was maintained above one. The synthetic wastewater was deoxygenated by flushing with argon gas before feeding to the reactor.

2.4. Analysis

The concentrations of nitrite and nitrate were determined by ion-chromatography (DX 500, Dionex, Sunnyvale, CA, USA). Ammonium was measured by selective electrode according to Standard Methods (APHA, 1998). SS and VSS were determined by the weighing method after being dried at 103–105 °C and burnt to ash at 550 °C (APHA, 1998). The pH was determined via a calibrated pHS-25 acidimeter and a pH electrode (Thermo Fisher Scientific, Waltham, MA, USA). The gas produced in the reactor was collected at the top, and measured with a wet-type laboratory gas meter (Schlumberger, Sugar Land, TX, USA). The gas was sampled as needed using 1 mL Gastight syringe (Hamilton, Reno, NV, USA). Gas composition was measured with a GC-350 (GOW-MAC Instrument, Bethlehem, PA, USA) equipped with a column and a thermal conductivity detector, operated at 70 and 200 °C, respectively. To measure the granule size, images were taken using an Olympus SZH10 compound stereo microscope (Olympus America, Melville, NY, USA) and then analyzed using an image analysis software version 2.0 (Olympus Soft Imaging Solutions, Lakewood, CO, USA).

2.5. Microscope observation

For the transmission electron microscopy (TEM), samples were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, for 48 h at 4 °C. Samples were washed in buffer and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. The samples were then en-bloc stained with 2% aqueous uranyl acetate, washed with deionized water, and dehydrated in a graded ethanol series. After cleared with ultra-pure acetone, the samples were infiltrated and embedded using a modified EPON epoxy resin (Embed 812, Electron Microscopy Sciences, Hatfield, PA, USA). Resin blocks were polymerized for 48 h at 70 °C. Thick and ultrathin sections were made using a Leica UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN, USA). Ultrathin sections were collected onto copper grids and images were captured using a JEM 2100 200 kV scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA, USA).

For the scanning electron microscopy (SEM), samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer at 4 °C for 24 h. Samples were rinsed in deionized water and post-fixed in 2% aqueous osmium tetroxide followed by dehydration in a graded ethanol series up to 100% ultra-pure ethanol. The samples were fractured in liquid nitrogen and dried using a Denton DCP-2 critical point dryer (Denton Vacuum, LLC, Moorestown, NJ, USA). When dried, the samples were placed onto adhesive coated aluminum stubs, sputter coated (Denton Desk II sputter coater, Denton Vacuum, LLC, Moorestown, NJ, USA) with palladium/gold alloy, and imaged using a JEOL 5800LV SEM (Japan Electron Optics Laboratory, Peabody, MA, USA) at 10 kV with a SIS ADDA II for digital image capture (Olympus Soft Imaging Systems Inc., Lakewood, CO, USA).

2.6. Fluorescence in situ hybridization

Fluorescence in situ hybridizations (FISH) and 4,6-diamidino-2-phenylindole (DAPI) staining were performed according to the pro-

cedure described by Amann et al. (1990). The 16S rRNA-targeted oligonucleotide probe used in this study was AMX820 for anammox bacteria. The probe was synthesized and directly labeled with fluorescein isothiocyanate (FITC) at the 5' end. Image acquisition was done using an an epifluorescence microscope (Olympus BX51, Olympus Optical, Tokyo, Japan) together with the standard software package delivered with the instrument (version 4.0). The images were taken by an Olympus U-CMAD 3 camera (Olympus Optical, Tokyo, Japan) with exposure times of 0.05 s for DAPI and 0.5 s for AMX820.

2.7. Quantification with real-time polymerase chain reaction (PCR)

Both the seed sludge and sludge in the reactor after continuous operation were taken to quantify by real-time PCR. Total genomic DNA was extracted by the modified 2% cetyl trimethyl ammonium bromide-based protocol (Allen et al., 2006). Genomic DNA preparation was determined with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and purified DNA samples were stored in sterile deionized water at −20 °C until used. PCR reactions were performed in 25 μL volumes comprising 300 nM of each primer, DNA samples and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were conducted in optical-grade 96-well PCR plates in an ABI Prism 7900 Sequence Detection System (ABI, Foster City, CA, USA). The thermal profile was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Amplification data were analyzed with the Sequence Detection System (SDS) software v. 2.3 (Applied Biosystems, Foster City, CA, USA). Experiments were repeated two times and each sample was run in triplicate. The 16S rDNA concentrations of anammox bacteria were quantified according to Tsushima et al. (2007a).

3. Results and discussion

3.1. Reactor performance

During the whole experimental period, the pilot-scale reactor was operated with a stable performance. The nitrite and ammonium concentrations in the influent were increased gradually from 100 mg N/L to over 500 mg N/L according to the removal efficiencies. Started at a nitrogen loading rate (NLR) of 0.28 kg/m³/d, the NLR reached above 1.0 kg/m³/d gradually. To eliminate the effect of pH on reactor performance, the pH value in the reactor was tested and adjusted to 7.5–8.0 with CO_2 gas daily. The gas purge was regulated to create ultra-fine bubbles in order to avoid the breakage of the granules and to minimize biomass washout from the reactor.

Fig. 1 shows the performance of granular UASB reactor during the study period. After approximately 2 weeks start-up, excellent treatment results were obtained quickly without big fluctuation. The start-up period was shorter than reactors with other types of sludge (Fernandez et al., 2008; Li et al., 2009; Trigo et al., 2006; Yang et al., 2006), showing that the inoculation of mature anammox granules was ideal to start-up a new reactor. Nearly complete influent NH_4^+–N and NO_2^-–N were removed from the wastewater, which fully indicated that high activities of anammox bacteria were achieved in the reactor. The amazing results verified the hypothesis we delivered, which means it's possible to start a large-scale granular reactor quickly. On day 1–77, NH_4^+–N and NO_2^-–N removal efficiencies increased with the increase of influent nitrogen concentrations and NLR (Fig. 1A). After day 12, all influent NO_2^-–N was removed from the wastewater with NH_4^+–N removal efficiencies ranged from 93.2 to 99% (Fig. 1B). As all NO_2^-–N was consumed by the biomass, nitrite became the limiting nutrient and anammox
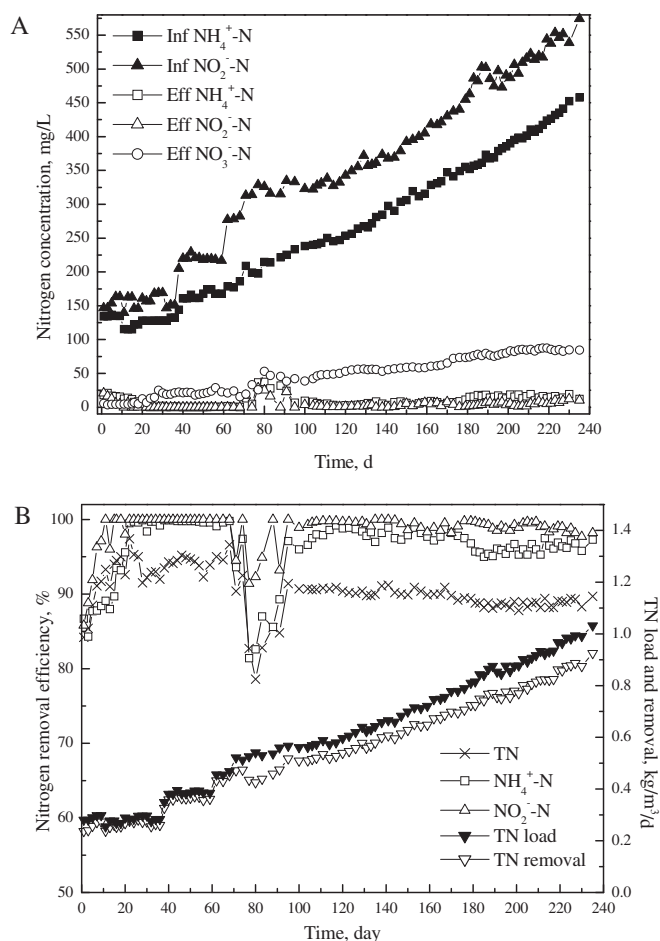

Fig. 1. Performance of the anammox granular reactor as a function of operating time. (A) Influent and effluent nitrogen compounds concentrations. (B) Nitrogen removal efficiency (total nitrogen (TN), nitrite and ammonium, left axis) and total nitrogen load (TN load and TN removal, right axis).

microorganisms grew at a growth rate lower than the maximum specific one (Lopez et al., 2008). In order to supply efficient nutrients for biomass growth, influent NH_4^+–N and NO_2^-–N were increased to approximately 200 and 330 mg/L on day 77.

Nitrogen removal efficiencies dropped when influent NO_2^-–N concentration was increased to above 300 mg/L on day 77. Similar phenomena were observed by Ni et al. (2010a). Previous research showed that the substrate (ammonium and nitrite) could inhibit anammox bacteria (Dapena-Mora et al., 2007). In this study, the fluctuation of nitrogen removal might be the response to a sudden shock of loading rates. Also, this may be caused by the toxic effects of ammonium and nitrite. Usually nitrite concentrations above 100 mg N/L would inhibit anammox activity (Ni et al., 2010c; Strous et al., 1999), while the value was much higher for ammonium, i.e. 50 mM (Dapena-Mora et al., 2007). In this study, the disturbance was observed when influent concentrations increased above 200 mg NH_4^+–N/L and 330 mg NO_2^-–N/L. Hence, ammonium was not the potential inhibitor, as the concentration of ammonium on day 77 was far lower than the inhibition values. Dapena-Mora et al. (2007) also indicated that 20 and 25 mM nitrite would lead to the 20% and 50% inhibition of anammox activity, respectively. In this study, nitrite could be the possible inhibitor, since its toxicity to anammox bacteria was well accepted. The inhibition could recover by hydrazine and hydroxylamine addition or production (Ni et al., 2010a; Strous et al., 1999). Other research in our lab showed that the recovery of anammox activity from flocculent sludge reactor took nearly one month (Ni et al., 2010a). Tang

Table 1
Comparison of different anammox UASB and granular anammox process.

Reactor type[a]	Inoculum	Operation day	Ammonium removal efficiency (%)	NLR or NRR (kg N/m³/d)	Final influent (mg/L)		Purity [c](%)	References
					NH_4^+-N	NO_2^--N		
UASB	Anaerobic/aerobic granules	421	85–94	2.5 (NLR)	–	–	27.0	Yang et al. (2006)
UASB	Anaerobic granules	336	60	–	50	70	–	Tran et al. (2006)
UASB	Anaerobic biomass	220	99	0.52 (NLR)	40	50	–	Schmidt et al. (2004)
UASB	Anaerobic granules	400	86.5–92.3	6.4 (NRR)	1027	1409	–	Imajo et al. (2004)
UASB	Aerobic granules	300	90	0.5 (NLR)	200	220	–	Ni et al. (2009)
SBR	Activated sludge	365	99.9	1.6 (NLR)	1268	1661.4	85.0	Lopez et al. (2008)
UASB	Activated sludge	516	74	–	380	357	16	Li et al. (2009)
UASB	Nitrifying sludge	220	97	0.16 (NLR)	115	130	–	Yang et al. (2009)
SBR	Anammox sludge	320	99–100	0.43 (NLR)	180	250	–	Fernandez et al. (2008)
MSBR	Anammox sludge	375	<80	0.71 (NRR)	390	390	–	Trigo et al. (2006)
CTR	Anammox sludge	340	81.3 (TN)	3.6 (NLR)	300	300	–	Qiao et al. (2009)
SBR	Anammox sludge	400	60 (TN)	1.0 (NRR)	700	0	–	Vazquez-Padin et al. (2009a)
SBR	Anammox sludge	400	78 (TN)	0.36 (NRR)	200–250	0	<35	Vazquez-Padin et al. (2009b)
SBR	Anammox granules	218	98	0.3 (NLR)	150	150	–	Arrojo et al. (2006)
UASB[b]	Nitrifying and anammox sludge	>1400	<90	9.5 (NLR)	∼400–550	575 ± 175	50–60	Van der Star et al. (2007)
UASB	Anammox granules	235	99.290 (TN)	1.03 (NLR)	458	575	91.2–92.4	This study

[a] MSBR: membrane sequencing batch reactor. CTR: column type reactor.
[b] First full-scale anammox reactor.
[c] Purity means percentage of anammox cells in the bacteria community.

et al. (2009) found that after inhibition the reactor was not recovered after 25 days even at the end of the experimental period. Since anammox process produced hydrazine/hydroxylamine continuously, without addition of any chemicals, the pilot-scale reactor in this study was recovered by stopping increasing influent loading rates and changing the feed strategy. This strategy was proved to be successful as the disturbance stopped in 14 days. Furthermore, no fluctuation happened again after the recovery, even later the influent NH_4^+-N and NO_2^--N concentrations increased to above 450 and 570 mg N/L, respectively. If controlled properly, granular anammox biomass can tolerate high substrate contents.

The fluctuation of anammox bacteria affected nitrite removal less significantly than ammonium removal, resulting in fewer drops in nitrite removal (Fig. 1B). Meanwhile, during that period, nitrate production increased together with the increase of nitrogen loading. Bacteria diversity analysis, which was conducted in the future research and not included as a part of this study, indicated that nitrifiers, denitrifiers, and anammox microorganisms coexisted in the community. During the fluctuation period, the coexisted nitrifiers could oxidize ammonium or nitrite to nitrate and lead to the increase of nitrate production, compensating for the loss of anammox activity. This could be responsible for above phenomena.

During the initial stage of the study, the influent NO_2^--N concentration was raised by a step of 50 mg/L. At low concentrations, this strategy did not cause fluctuation. On day 77, when nitrite concentration increased to 330 mg N/L directly, disturbance happened in the reactor. The fluctuation indicated that the instant increase of nitrite was not suitable for running pilot-scale anammox reactors, which could result in anammox activity drop. Since then, the nitrite concentration was increased gradually. This information is valuable for the operation of full-scale reactors, in which the operational problem is one of the obstacles. This study focused on the fast start-up, rather than the limitation of reactor ability or maximum nitrogen removal potential. After day 95, NLR increased to 1.03 kg/m³/d at an average speed of 3.5 mg/L/d, while influent NH_4^+-N and NO_2^--N increased at speeds of 1.6 and 1.8 mg/L/d (Fig. 1B). During this period, the NH_4^+-N and NO_2^--N removal efficiencies varied from 95% to 99.2% and from 97.7% to 100%, respectively. The average TN removal efficiency was 89.6 ± 0.96%. High nitrogen removal was achieved for a long period with a maximum nitrogen removal rate (NRR) of 0.93 kg/m³/d. During the whole experiment, the NO_2^--N to NH_4^+-N molar ratios

in the feed varied from 1.21 to 1.43, with an average value of 1.31. Obviously, the marked variation did not affect the reactor performance significantly, which was also described in the literature (Ni et al., 2010a). Consequently, the granular anammox UASB reactor was able to deal with high nitrogen contained wastewater, characterized by high anammox bacteria activity and high resistance to high strength nitrogen contained wastewater.

Table 1 shows the comparison of different anammox UASB and granular anammox process. Most studies usually started up laboratory-scale reactors at low influent nitrogen concentrations in an extended long period of time. In this study, the pilot-scale reactor could start up at high nitrogen contents and took shorter time to treat high nitrogen concentration wastewater. Most anammox UASB reactors were inoculated with granules and activated sludge at low loading rates (Imajo et al., 2004; Li et al., 2009; Lopez et al., 2008; Ni et al., 2009; Schmidt et al., 2004; Tran et al., 2006; Yang et al., 2006,2009). Usually, in those processes the removal efficiencies decreased with the increase of NLR. High nitrogen removal was realized in a SBR reactor after 365 days of operation, leading to an anammox bacteria purity of 85% (Lopez et al., 2008). While in this study, the percentage of anammox cells in bacterial community was much higher in a short operation period. Seeded with anammox biomass, this study exhibited high performance at a relatively high NLR. The reactor could endure high nitrogen concentration from initial period. The comparison proves that the introduction of sludge source from exotic reactors will benefit the reactor start-up and performance. The experience of the pilot-scale reactor demonstrated that this strategy could be adopted to run full-scale anammox reactors in order to speed up the start-up and fulfill the designed loading rate.

3.2. Stoichiometry of the anammox process

Stoichiometry is the calculation of quantitative relationships of the reactants and products in a balanced chemical reaction. Stoichiometry is simply the math behind chemistry, which can be used to calculate quantities such as the amount of products that can be produced with given reactants and percent yield (the percentage of the given reactant that is made into the product). Fig. 2 shows the amounts of nitrite removal and nitrate production against ammonium removal. In accordance with the regression line in the figure, stoichiometry molar ratios of NO_2^--N conversion and nitrate (NO_3^--N) production to NH_4^+-N conversion were calculated to be
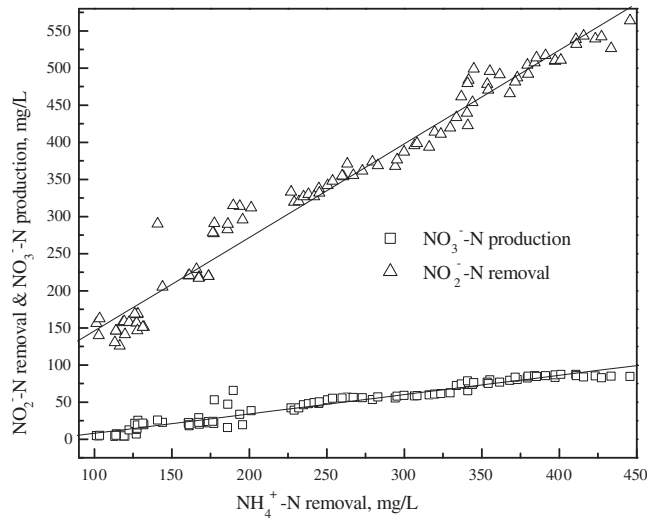
Fig. 2. Nitrite removal and nitrate production vs. ammonium removal as a function of operating time.

Fig. 3. Nitrogen removal kinetics of granular anammox process.

1.26 ± 0.02:1 and 0.26 ± 0.01:1, respectively. Yang et al. (2009) indicated that the average ratio of nitrate to ammonium conversion varied between 1.15:1 and 1.62:1 during different periods. Generally, stoichiometry molar ratios of NO_2^-–N to NH_4^+–N conversion varied in different anammox reactors at a range of 0.5 to 4, relying on substrate, operation and reactor configuration (Ahn, 2006). Furthermore, stoichiometry molar ratio of nitrogen gas produced in the reactor to NH_4^+–N conversion was 0.97 ± 0.04:1, similar to well-accepted theoretical value.

3.3. Kinetics model of granular anammox process

The process kinetics for granular anammox reactor was investigated by the Stover-Kincannon (S–K) model, which is one of the most widely used mathematical models. Even this model was originally developed for attached growth process (Stover and Kincannon, 1982); it was successfully applied to UASB reactor (Isik and Sponza, 2005). This model has seldom been applied in granular anammox process. The S–K model was described as the following equation (Isik and Sponza, 2005):

$$\frac{ds}{dt} = \frac{Q}{V}(S_0 - S) \tag{1}$$

where Q is the flow rate (L/d), V is the working volume of the UASB reactor (L), S_o and S are influent and effluent substrate concentrations (g/L), dS/dt is the substrate removal rate (kg/m³/d) and is defined as follows:

$$\frac{ds}{dt} = \frac{U_{max}(\frac{QS_0}{V})}{k_B + (\frac{QS_0}{V})} \tag{2}$$

where U_{max} is the maximum substrate utilization rate (kg/m³/d), k_B is the saturation constant (kg/m³/d). So Eq. (1) can be illustrated as follows:

$$\left(\frac{ds}{dt}\right)^{-1} = \frac{V}{Q(S_0 - S)} = \frac{k_B}{U_{max}} \frac{V}{QS_0} + \frac{1}{U_{max}} \tag{3}$$

Fig. 3 shows the S–K model simulation by plotting total substrate removal rates against loading rates. The k_B and U_{max} constants were calculated to be 27.5 and 27.8 kg/m³/d. This indicated that the maximum total substrate removal rate was 27.8 kg/m³/d in this granular UASB anammox reactor, suggesting that the granular anammox reactor in this study possessed an
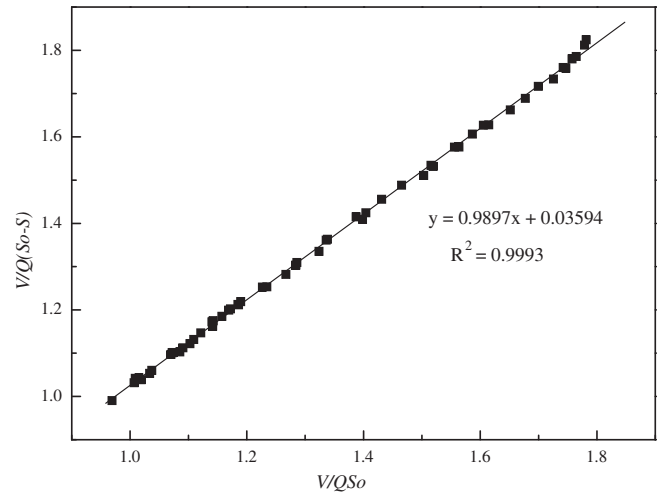
excellent nitrogen removal potential. Tsushima et al. (2007b) developed a fix-bed column biofilm reactor using non-woven fabric sheets as biomass carrier, achieving to an extremely high NRR of 26.0 kg/m³/d at a HRT of 1 h. Since the nitrogen loading rate was 58.5 kg/m³/d, the fix-bed column reactor could only remove ∼44.4% nitrogen from the wastewater, much lower than that in this study (∼90%). In current research, it was easy to increase the NRR by lowering the HRT without consideration of nitrogen removal and running cost. Furthermore, the correlation coefficient (R^2) is 0.9993, which means that the nitrogen removal in the granular anammox reactor fits the S–K model perfectly. Apparently, the S–K model may be applied to simulate the performance of granular reactor and design the anammox granular reactor.

3.4. Anammox granules and morphological characterization

During the last period of the experiment, the mean granule size in the reactor was 3.44 ± 0.37 mm (Fig. S1A). Meanwhile, the granules were sampled from the reactor to do the microscope observation. As shown in Fig. S1B, the granules in the reactor were reddish brown, semitransparent and easy to congregate with each other. The structure of the granules was different to anaerobic and aerobic granules; each part was densely integrated with others, which favored the granule joining tightly and existing stably (Fig. S1C). This structure may be caused by the shear forces of the effluent recirculation currents. High magnification observation showed that a network of anammox species with cavities was visible in the granular structure (Fig. S1D). Granular consortia consisted of a number of cavities, which could be the possible gas vents for nitrogen production. Similar phenomena were also observed in the anaerobic methanogenic granules (Baloch et al., 2008).

Spherical shaped bacteria, which were supposed to be anammox bacteria (Jetten et al., 1999), were evident to coexist with rod shaped bacteria (Fig. S1E). The dominant of anammox microorganisms was proved by quantitative PCR later. Transmission electron micrograph shows that the anammox bacterial cells have an unusual irregular morphology (Fig. S1F). The anammox species in this study displayed typical features of anammox bacteria: a single membrane bound anammoxosome and riboplasm with ribosome-like particles separated from paryphoplasm by an intracytoplasmic membrane, as presented by Jetten et al. (1999) and Kartal et al. (2008). The cells contain three distinct membrane bound compartments: the paryphoplasm, cytoplasm and anammoxosome. The nucleoid closely juxtaposed with the anammoxosome. In this paper, the cells displayed an identical pattern of

organization to other anammox species, such as *Candidatus Brocadia fulgida* (Kartal et al., 2008).

3.5. Anammox biomass characterization with molecular techniques

To locate the bacterial strains in the reactor, FISH analysis using the DAPI and AMX820 probes was performed to study the group morphology of anammox granules during the treatment period. Image of epifluorescence microscopy presented a high density of cells growing in clusters and emitting blue fluorescence (Fig. S2A). Anammox bacteria stained with AMX820 probe formed high-density clusters (Fig. S2B). The comparison of images stained with general DNA stain DAPI and anammox targeted stain AMX820 shows that anammox cells were the major bacteria in the reactors. FISH analysis can only identify the available anammox bacteria, while the PCR analysis can amplify the signals over the anammox bacteria. Quantitative PCR is more sensitive than FISH method, especially in low enriched cultures (Lopez et al., 2008).

Quantitative real-time PCR analysis was used to quantify the microbial community of anammox bacteria in the reactor after 200 days of operation. The PCR assay based on the 16S rRNA gene-specific set of primers AMX809F/AMX1066R allowed quantification of anammox bacterial population in the bioreactor. The set of the primers had a high specificity to 16S rRNA gene sequences to anammox biomass. The applicability of real-time PCR to anammox bacteria was well discussed by Tsushima et al. (2007a).

The detection limit of the real-time PCR was 25 fg. The standard curve was constructed from a series of 10-fold dilutions of DNA extract ranging from 25 fg to 25 ng of DNA (data not shown). The consistency of the real-time PCR assay with these primers was confirmed by the strong linear inverse relationship between the Ct values and the DNA contents of anammox bacteria ($P = 0.01$, $r^2 = 0.998$). The amplification efficiency was more than 96% (the slope was −3.27).

At least three anammox species were confirmed in the anammox granules: *Candidatus Kuenenia, Brocadia* and *Jettenia* with corresponding similarities of 99%, 93% and 92%. The level of anammox cells in the community increased from 60–70% in the first day to more than 85% after 4 months of continuous operation. The data showed that the anammox bacteria comprised 91.4–92.4% cells of the microorganisms' community after 200 days of operation. This indicates that most biomass in the reactor were anammox bacteria, resulting in high NH_4^+–N and NO_2^-–N removal efficiencies. Even over 95% influent NH_4^+–N and NO_2^-–N were removed from the system, the production of NO_3^-–N by anammox bacteria led to less than 90% TN removal efficiency. The stoichiometry molar ratio of NO_2^-–N conversion to NH_4^+–N conversion was 1.26 ± 0.02:1, smaller than well-accepted value. The reason may be that small amount of nitrosifyers (ammonia-oxidizing bacteria, AOB) coexisted with anammox bacteria in the community. Those nitrosifying bacteria oxidized ammonium to nitrite using the trace oxygen in the influent, which could be the reason of less NO_2^-–N conversion. Morphological observation and molecular techniques confirmed the occurrence of AOB species. Further study is in process to investigate the bacterial diversity towards the identification of nitrifiers, denitrifiers, and anammox species.

4. Conclusions

The adoption of mature anammox sludge from exotic sources could shorten the start-up period and lead to high performance. The pilot-scale granular anammox reactor showed high recovery ability after disturbance. According to the well-fitted Stover–Kincannon model, it had high substrate removal potential. At least three anammox species were found in the granules. The typical

features of anammox cells were evident by electron microscopes. The dominance of anammox cells was obtained by FISH analysis and further quantified to comprise 91.4–92.4% bacteria community by quantitative real-time PCR. The experience of the pilot-scale reactor stated that it's possible to speed up the start-up of full-scale anammox reactors.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.11.006.

References

Ahn, Y.H., 2006. Sustainable nitrogen elimination biotechnologies: a review. Process Biochem. 41, 1709–1721.

Allen, G.C., Flores-Vergara, M.A., Krasnyanski, S., Kumar, S., Thompson, W.F., 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. Nat. Protoc. 1, 2320–2325.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

APHA, AWWA, WEF, 1998. Standard Methods for the Examinations of Water and Wastewater, 20th ed. American Public Health Association, Washington DC, USA.

Arrojo, B., Mosquera-Corral, A., Campos, J.L., Mendez, R., 2006. Effects of mechanical stress on anammox granules in a sequencing batch reactor (SBR). J. Biotechnol. 123, 453–463.

Baloch, M.I., Akunna, J.C., Kierans, M., Collier, P.J., 2008. Structural analysis of anaerobic granules in a phase separated reactor by electron microscopy. Bioresour. Technol. 99, 922–929.

Dapena-Mora, A., Fernandez, I., Campos, J.L., Mosquera-Corral, A., Mendez, R., Jetten, M.S.M., 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. Enzyme Microb. Technol. 40, 859–865.

Fernandez, I., Vazquez-Padin, J.R., Mosquera-Corral, A., Campos, J.L., Mendez, R., 2008. Biofilm and granular systems to improve Anammox biomass retention. Biochem. Eng. J. 42, 308–313.

Imajo, U., Tokutomi, T., Furukawa, K., 2004. Granulation of Anammox microorganisms in up-flow reactors. Water Sci. Technol. 49 (5–6), 155–163.

Isik, M., Sponza, T., 2005. Substrate removal kinetics in an upflow anaerobic sludge blanket reactor decolorising simulated textile wastewater. Process Biochem. 40, 1189–1198.

Jetten, M.S.M., Strous, M., van de Pas-Schoonen, K.T., Schalk, J., van Dongen, U.G.J.M., van de Graff, A.A., Logemann, S., Muyzer, G., van Loosdrecht, M.C.M., Kuenen, J.G., 1999. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22, 421–437.

Jetten, M.S.M., Niftrik, L., Strous, M., Kartal, B., Keltjens, J.T., Op den Camp, H.J.M., 2009. Biochemistry and molecular biology of anammox bacteria. Crit. Rev. Biochem. Mol. Biol. 44, 65–84.

Kartal, B., van Niftrik, L., Rattray, J., van de Vossenberg, J.L.C.M., Schmid, M.C., Damsté, J.S., Jetten, M.S.M., Strous, M., 2008. Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol. Ecol. 63, 46–55.

Li, X.R., Du, B., Fu, H.X., Wang, R.F., Shi, J.H., Wang, Y., Jetten, M.S.M., Quan, Z.X., 2009. The bacterial diversity in an anaerobic ammonium-oxidizing (anammox) reactor community. Syst. Appl. Microbiol. 32, 278–289.

Lopez, H., Puig, S., Ganigue, R., Ruscalleda, M., Balaguer, M.D., Colprim, J., 2008. Start-up and enrichment of a granular Anammox SBR to treat high nitrogen load wastewaters. J. Chem. Technol. Biotechnol. 83, 233–241.

Ni, B.J., Chen, Y.P., Liu, S.Y., Fang, F., Xie, W.M., Yu, H.Q., 2009. Modeling a granule-based anaerobic ammonium oxidizing (ANAMMOX) process. Biotechnol. Bioeng. 103, 490–499.

Ni, S.Q., Lee, P.H., Fessehaie, A., Gao, B.Y., Sung, S., 2010a. Enrichment and biofilm formation of Anammox bacteria in a non-woven membrane reactor. Bioresour. Technol. 101, 1792–1799.

Ni, S.Q., Lee, P.H., Sung, S., 2010b. The kinetics of nitrogen removal and biogas production in an anammox non-woven membrane reactor. Bioresour. Technol. 101, 5767–5773.

Ni, S.Q., Fessehaie, A., Lee, P.H., Gao, B.Y., Xu, X., Sung, S., 2010c. Interaction of anammox bacteria and inactive methanogenic granules under high nitrogen selective pressure. Bioresour. Technol. 101, 6910–6915.

Qiao, S., Kawakubo, Y., Cheng, Y., Nishiyama, T., Fujii, T., Furukawa, K., 2009. Identification of bacteria coexisting with anammox bacteria in an upflow column type reactor. Biodegradation 20, 117–124.

Schmidt, J.E., Batstone, D.J., Angelidaki, I., 2004. Improved nitrogen removal in upflow anaerobic sludge blanket (UASB) reactors by incorporation of Anammox bacteria into the granular sludge. Water Sci. Technol. 49 (11–12), 69–76.

Stover, E.L., Kincannon, D.F., 1982. Rotating biological contactor scale-up and design. In: Proceedings of the 1st International Conference on Fixed Film Biological Processes. Kings Island, Ohio, USA. pp. 1–21.

Strous, M., van Gerven, E., Kuenen, J.G., Jetten, M.S.M., 1997. Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (anammox) sludge. Appl. Environ. Microbiol. 63, 2446–2448.

Strous, M., Kuenen, J.G., Jetten, M.S.M., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248–3250.

Tang, C.J., Zheng, P., Mahmood, Q., Chen, J.W., 2009. Start-up and inhibition analysis of the Anammox process seeded with anaerobic granular sludge. J. Ind. Microbiol. Biotechnol. 36, 1093–1100.

Tran, H.T., Park, Y.J., Cho, M.K., Kim, D.J., Ahn, D.H., 2006. Anaerobic ammonium oxidation process in an upflow anaerobic sludge blanket reactor with granular sludge selected from an anaerobic digestor. Biotechnol. Bioprocess Eng. 11, 199–204.

Trigo, C., Campos, J.L., Garrido, J.M., Mendez, R., 2006. Start-up of the Anammox process in a membrane bioreactor. J. Biotechnol. 126, 475–487.

Tsushima, I., Kindaichi, T., Okabe, S., 2007a. Quantification of anaerobic ammonium oxidizing bacteria in enrichment cultures by real-time PCR. Water Res. 41, 785–794.

Tsushima, I., Ogasawara, Y., Kindaichi, T., Satoh, H., Okabe, S., 2007b. Development of high-rate anaerobic ammonium-oxidizing (anammox) biofilm reactors. Water Res. 41, 1623–1634.

Van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.W., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Res. 41, 4149–4163.

Vazquez-Padin, J., Fernadez, I., Figueroa, M., Mosquera-Corral, A., Campos, J.L., Mendez, R., 2009a. Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. Bioresour. Technol. 100, 2988–2994.

Vazquez-Padin, J.R., Pozo, M.J., Jarpa, M., Figueroa, M., Franco, A., Mosquera-Corral, A., Campos, J.L., Mendez, R., 2009b. Treatment of anaerobic sludge digester effluents by the CANON process in an air pulsing SBR. J. Hazard. Mater. 166, 336–341.

Wang, C.C., Lee, P.H., Kumar, M., Huang, Y.T., Sung, S., Lin, J.G., 2010. Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. J. Hazard. Mater. 175, 622–628.

Yang, Y., Zuo, J., Quan, Z., Lee, S., Shen, P., Gu, X., 2006. Study on performance of granular ANAMMOX process and characterization of the microbial community in sludge. Water Sci. Technol. 54 (8), 197–207.

Yang, Z., Zhou, S., Sun, Y., 2009. Start-up of simultaneous removal of ammonium and sulfate from an anaerobic ammonium oxidation (anammox) process in an anaerobic up-flow bioreactor. J. Hazard. Mater. 169, 113–118.