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Kinetic characteristics and microbial community of Anammox-EGSB reactor

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ABSTRACT

The present study reports kinetic characteristics of Anammox (anaerobic ammonium oxidation) EGSB (Expanded Granular Sludge Bed) reactor after feeding with strong ammonium-containing synthetic wastewater. The microbial communities were analysed based on their 16S rRNA gene sequences. The results showed that the volumetric nitrogen loading rate (NLR) and volumetric nitrogen removal rate (NRR) reached up to 22.87 kg N/(m^3 d) and 18.65 kg N/(m^3 d), respectively, when the influent nitrogen concentrations were 1429.1 mg N/L. Monod and Haldane models both proved to be suitable in characterizing the kinetic behavior of the reactor. Based on Haldane model, the relationships among the ammonium, nitrite, nitrogen conversion rates and substrate concentrations were established with corresponding correlation coefficients of 0.992, 0.993 and 0.993, respectively. The maximum ammonium, nitrite and nitrogen conversion rates (q_{max}) by the granular sludge were 381.2, 304.7 and 731.7 mg N/(gVSS d), half saturation constants (K_s) were 36.75, 0.657 and 29.26 mg N/L and inhibition constants (K_i) were 887.1, 13,942.1 and 1779.6 mg N/L, respectively. Anammox-EGSB reactor was found tolerant to substrate and capable of treating strong ammonium-containing wastewater. The dominant microbial population of the granular sludge in the reactor was *Candidatus Kuenenia stuttgartiensis*.

1. Introduction

Anaerobic ammonia oxidation (Anammox) is a biotechnological process in which ammonium is directly converted to dinitrogen gas with nitrite as the electron acceptor under anoxic conditions [1]. The Anammox process which was initially discovered in a denitrifying pilot plant reactor [2]. It is a novel and promising alternative to conventional nitrogen removal processes. The application of Anammox process to nitrogen removal would lead to a reduction of operational costs up to 90% [3]. The first full-scale Anammox reactor was constructed for the Dokhaven–Sluisjesdijk wastewater treatment plant in Rotterdam in 2002, treating sludge dewatering effluent from a Sharon (single reactor high activity ammonia removal over nitrite) process. The maximum NRR for the first Anammox reactor was 9.5 kg N/(m^3 d) [4].

Ammonium is widely present in wastewaters (e.g., industrial wastewater, agricultural wastewater and domestic sewage). Most of the wastewaters contain ammonium concentrations below 1000 mg/L. However, the ammonium concentrations reach up to 10,000–17,000 mg/L in wastewaters like ion-exchange wastewater from the production units of monosodium L-glutamate. Ammonium and nitrite serve as substrates for Anammox bacteria; however, these act as inhibitors of Anammox bacteria when their concentrations exceed a certain level [5–7]. Although the Anammox process was initially designed to treat ammonium-rich wastewaters [8], the toxic substrate concentrations were mostly controlled below 1000 mg/L for the process [5,6,9,10]. The treatment of ammonium-rich wastewaters (the ammonium concentrations greater than 1000 mg/L) through Anammox will reduce the engineering investment as well as operational cost. In order to study the kinetic characteristics of Anammox reactor and to verify its adaptability to ammonium-rich wastewaters, the Anammox-EGSB reactor was run and its performance was monitored for more than 130 days, the microbial community under high substrate concentrations was also analysed. This paper reports the performances, kinetic characteristics and the microbial community of the Anammox-EGSB reactor.

2. Materials and methods

2.1. Synthetic wastewater

Ammonium and nitrite were supplemented to mineral medium as needed in the form of NH_4Cl and $NaNO_2$, respectively. The composition of the mineral medium was (g/L except for trace element solution): KH_2PO_4, 0.01; $CaCl_2{\cdot}2H_2O$, 0.18; $MgSO_4{\cdot}7H_2O$, 0.30; $KHCO_3$, 1.250; EDTA, 0.005; $FeSO_4$, 0.00625, and 1 mL/L of trace element solution. The trace element solution contained

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(g/L): EDTA, 15; H_3BO_4, 0.014; $MnCl_2{\cdot}4H_2O$, 0.99; $CuSO_4{\cdot}5H_2O$, 0.25; $ZnSO_4{\cdot}7H_2O$, 0.43; $NiCl_2{\cdot}6H_2O$, 0.19; $CoCl_2{\cdot}6H_2O$,0.24; $NaMoO_4{\cdot}2H_2O$, 0.22; $NaSeO_4{\cdot}10H_2O$, 0.21 and $NaWO_4{\cdot}2H_2O$, 0.05 (adapted from [11]).

2.2. Anammox inoculum

Anammox granular sludge was collected from a lab-scale Anammox reactor and was used as the seed sludge for Anammox-EGSB reactor. The volatile suspended solids (VSS) content of seed sludge was 31.31 g/L. The lab-scale Anammox reactor was initially inoculated with anaerobic granular sludge taken from a paper mill wastewater treatment plant (100 m^3, located in Zhejiang Province, China). The average diameter of the anaerobic granular sludge was 2.2 mm, and the VSS/TSS (total suspended solids) was 85%. The reactor was successfully started up and subsequently operated stably for 2 years before conducting the experiments.

2.3. Anammox EGSB reactor

The Anammox-EGSB reactor system was illustrated in Fig. 1. The total volume of reactor was 1.2 L with a working volume of 1.0 L, reactor was covered with black cloth to avoid inhibition caused by light [12]. The sampling ports were distributed vertically on the reactor with the mutual distance of 250 mm. The influent was purged with 95% Ar–5% CO_2 in order to create anaerobic conditions. The synthetic wastewater was pumped at the bottom of the reactor by using peristaltic pump. The effluent was collected in the effluent tank after passing through the gas–liquid–solid separator. The granules settled at the bottom of the reactor and the gas escaped from the top of the reactor.

2.4. Operational conditions

To investigate the performance of EGSB reactor, the NLR was gradually increased through increasing ammonium and nitrite concentrations in the influent or by shortening the hydraulic retention time (HRT). The EGSB reactor was operated at $35 \pm 1\,^{\circ}C$. Influent pH was always kept in the range of 6.8–7.0 by dosing hydrochloric acid [10]. A recycling pump was used to mix the influent (substrate) and sludge (biocatalyst) well. The ratio of recycling flow to influent flow was set at 6. Six completely-mixed reactors of 100 mL capacity were used to study the kinetic characteristics of Anammox granular sludge in Anammox-EGSB reactor for 131 days. The kinetic experiments for Anammox-EGSB reactor were conducted at controlled conditions including controlled temperature, influent pH, reflux ratio, etc. The reactors used for kinetic experiments possessed same shape, but variable volumes compared with the Anammox-EGSB reactor. The kinetic experiments were conducted at the fixed HRT using variable substrate concentrations.

2.4.1. Volumetric substrate conversion rate

Due to a greater recycling ratio of 6:1 and the large amount of gas production in Anammox-EGSB reactor, the operational pattern of the reactor tended to be completely mixed. Thus, the concentration of granular sludge could be characterized by mean value and the substrate concentrations inside the reactor were considered same as the substrate concentrations in the effluent. Under steady state, the mass balance for the reactor can be expressed as (Eq. (1)):

$$QS_0 - QS_e - V\left(\frac{dS}{dt}\right)_u = 0 \quad \text{that is} \quad \left(\frac{dS}{dt}\right)_u = \frac{Q}{V}(S_0 - S_e) \tag{1}$$

where: Q – influent flow (L/d); V – reactor working volume (L); S_0, S_e – substrate concentrations in influent and effluent (mg/L); $(dS/dt)_u$ – volumetric substrate conversion rate [mg/(L d)].

The volumetric substrate conversion rate could be calculated using Eq. (1).

2.4.2. Monod model

Monod model (Eq. (2)) is the most popular model to describe the kinetics of pollutant biodegradation [13,14]. The maximal substrate conversion rate (q_{max}) and half saturation constant (K_s) can be obtained according to Monod model. The model is represented as follows:

$$q = \frac{(ds/dt)_u}{X} = \frac{Q(S_0 - S_e)}{VX} = \frac{q_{max}S}{K_s + S} \tag{2}$$

where: q, q_{max} – specific substrate conversion rate and the maximal substrate conversion rate, respectively (1/d); X – the concentration of biomass in reactor (mg/L); K_s – half saturation constant (mg/L); and S – substrate concentration (mg/L).

2.4.3. Haldane model

A number of substrates serve as nutrients at low concentrations, but they behave as inhibitors at high concentrations [15]. High substrate concentrations inhibit microbial growth and disturb their metabolism. Haldane model (Eq. (3)) is often used to describe the kinetics of pollutant biodegradation involving inhibition [16]:

$$q = \frac{(dS/dt)_u}{X} = \frac{Q(S_0 - S_e)}{VX} = \frac{q_{max}}{1 + (K_s/S) + (S/K_i)} \tag{3}$$

where: q, q_{max} – specific substrate conversion rate and the maximal substrate conversion rate, respectively (1/d); X – the concentration of biomass in reactor (mg/L); K_s – half saturation constant (mg/L); K_i – substrate inhibition constant(mg/L); and S – substrate concentration (mg/L).

2.5. Analytical methods

The influent and effluent samples were collected on daily basis and were analysed immediately or temporarily stored at $4\,^{\circ}C$. The biomass concentration was determined as VSS. Water samples and VSS were analysed according to the standard methods [17]. The analysis of water samples was performed for ammonium concentrations, nitrite concentrations, nitrate concentrations and pH values. The dinitrogen gas was measured with a wet-gas flow meter and temperature was determined using a mercurial thermometer.

2.6. DNA extraction

For the DNA extraction, sludge was collected from the reactor during the initial (the inoculum-group 1) and final (on day 131-group 2). The granular sludge was washed several times using phosphate buffer solution prior to DNA extraction. DNA extraction was performed using DNA isolation kit v2.2 for environmental samples (Shenergy Biocolor Company, China). The extracted DNA was preserved at $-20\,^{\circ}C$.

2.7. PCR amplification

PCR reactions were performed on the extracted DNA. The primer pair pla46F (5′-GGATTAGGCATGCAAGTC-3′) [18] and 630R (5′-CAKAAAGGAGGTGATCC-3′) [19] was used in PCR amplification. PCR reactions were performed using 25 μL reaction volumes containing 2.5 μL dNTPs mixtures (2.5 mM) (Takara), 2.5 μL 10× PCR buffer (containing 15 mM magnesium ions) (Takara), 1 μL of each of the primers (10 mM) (Takara), 1 μL of the extracted DNA, 0.2 μL rTaq DNA polymerase (Takara) and 17.3 μL DEPC Rnase free dH_2O.

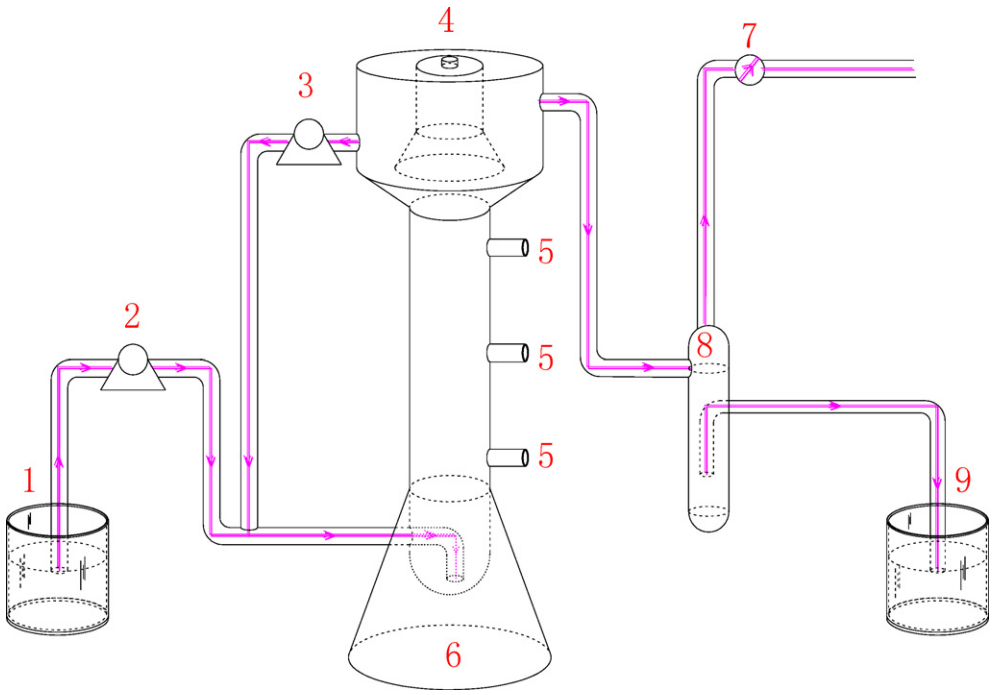

Fig. 1. Flow chart of Anammox-EGSB system.

The PCR conditions were as follows: an initial denaturation at 95 °C for 3 min; 30 cycles of denaturation (1 min at 95 °C), annealing (1 min at 56 °C) and extension (1 min at 72 °C); a final extension at 72 °C for 10 min. The amplified products were checked on 0.8% (m/v) agarose TAE gels and finally visualized under UV light.

2.8. Cloning and sequencing of 16S rRNA gene and phylogenetic analysis

PCR products were ligated into a PMD 19-T vector (Takara, Japan) and transformed into *Escherichia coli*-competent cells, following the manufacturer's instruction. The clones were grown on Luria–Bertani medium plates supplemented with ampicillin (100 mg/L). Clones were then randomly selected for further analysis.

Sequences were compared with similar sequences from the reference organisms by basic local alignment search tool (BLAST) search (http://www.ncbi.nlm.nih.gov/BLAST). Each sequence was aligned with MEGA3.1 and used for phylogenetic analysis. A phylogenetic tree was constructed by neighbor-joining method.

3. Results

3.1. Reactor performance

The Anammox-EGSB reactor was seeded with 0.75 L inoculum, the initial HRT was set at 8.0 h and the initial influent ammonium and nitrite concentrations were 112.8 and 140.4 mg N/L, respectively, with the initial NLR of 0.76 kg N/(m^3 d). Keeping other operational parameters at constant level, NLR was enhanced either by increasing the influent substrate concentrations or shortening of HRT. After continuous operation for about 80d, the influent ammonium and nitrite concentrations were elevated to 427.7, 470.9 mg N/L, respectively, and HRT was shortened to 1.5 h. The results indicated extremely high substrate conversion efficiencies (ammonium 90.6%, nitrite 99.5%) with the NLR and NRR as high as 14.38 and 13.70 kg N/(m^3 d), respectively, and the corresponding dinitrogen gas production rate reached 8.87 L/(L d) (Table 1). The NRR was quite high (2.93–26.0 kg N/(m^3 d)) as compared with other Anammox systems [18–24], thus the Anammox-EGSB reactor was believed to be started up successfully.

Keeping HRT at 1.5 h, the NLR was increased by raising the influent substrate concentrations. The influent ammonium, nitrite and nitrogen concentrations were raised from 427.7, 470.9 and 898.6 mg N/L to 661.9, 767.2 and 1429.1 mg N/L, respectively. As a result, the NLR reached up to 22.87 kg N/(m^3 d) with the subsequent NRR of 18.65 kg N/(m^3 d) and the dinitrogen gas product rate of 12.45 L/(L d). During that period, the Anammox-EGSB reactor exhibited a stable performance with the average removal ammonium and nitrite efficiencies of 85.3% and 96.6%, respectively (Table 1).

3.2. Analysis of kinetic characteristics

3.2.1. Kinetic characteristics of ammonium conversion

The information given in Table 2 demonstrates the effect of ammonium concentrations on specific ammonium conversion rates. The data fitting with Monod and Haldane models employing origin software was shown in Fig. 2. The information presented in Fig. 2 suggested that Monod and Haldane model both were very efficient to explain the process with correlation coefficients of 0.990, 0.992, respectively. The inhibition caused by substrate was not significant for the tested range of ammonium concentrations. Thus, Monod and Haldane models were both suitable to characterize the kinetic characteristics of ammonium. Based on Haldane model, the q_{max}, K_s and K_i of ammonium were 381.2 mg N/(gVSS d), 36.75 mg N/L and 887.1 mg N/L, respectively. The fitting equation was as under:

$$q = \frac{381.2}{1 + 36.75/S + S/887.1}$$

3.2.2. Kinetic characteristics of nitrite conversion

The effect of nitrite concentrations on specific nitrite conversion rates was presented in Table 2. The analytical results were

Table 1
Performance of reactor at different substrate concentrations and HRTs (average values).

Time (d)	HRT (h)	Inf. concentration (mg N/L)		Eff. concentration (mg N/L)			Removal efficiency (%)		NLR [kg N/(m³ d)]	NRR [kg N/(m³ d)]	N_2 product rate(L/(L d))
		Ammonium	Nitrite	Ammonium	Nitrite	Nitrate	Ammonium	Nitrite			
1	8	112.8	140.4	48.6	22.0	0	56.9	84.3	0.76	0.55	0.36
13	8.0	147.1	189.5	12.9	0.6	0	91.2	99.7	1.01	0.97	0.62
18	8.0	248.6	251.9	7.9	7.7	0	96.8	96.9	1.50	1.45	0.91
20	6.0	266.9	260.2	33.3	2.4	0	87.5	99.1	2.11	1.97	1.29
22	4.0	276.1	305.3	6.4	5.7	7.4	97.7	98.1	3.49	3.42	2.29
24	3.0	285.1	306.7	47.7	5.4	20.6	83.3	98.2	4.73	4.31	2.91
40	2.0	396.4	434.5	41.4	6.1	32.2	89.6	98.6	9.97	9.40	6.21
80	1.5	427.7	470.9	40.2	2.2	39.0	90.6	99.5	14.38	13.70	8.87
82	1.5	437.1	471.8	37.8	5.1	45.8	91.4	98.9	14.54	13.86	9.01
85	1.5	475.9	475	53.3	0.6	67.9	88.8	99.9	15.21	14.35	9.12
90	1.5	540.3	556.9	60.7	4.1	77.3	88.7	99.3	17.56	16.52	10.78
110	1.5	581.1	626.0	111.7	49.3	75.3	80.8	92.1	19.31	16.74	10.90
131	1.5	661.9	767.2	187.4	76.1	94.1	71.7	90.1	22.87	18.65	12.45

Table 2
Effect of substrate concentrations on specific conversion rates (average values).

Substrate	Inf. concentration (mg N/L)	Eff. concentration (mg N/L)	Volumetric loading rate [kg N/(m³ d)]	Removal efficiency (%)	Volumetric removal rate (NRR) [kg N/(m³ d)]	Specific conversion rate q = NRR/X [mg N/(gVSS d)]
Ammonium	297.4	21.9	4.76	92.6	4.41	141
	316.4	25	5.06	92.1	4.66	149
	475.8	53.4	7.61	88.8	6.76	216
	549.4	65.2	8.79	88.1	7.75	247
	590.3	106.6	9.44	81.9	7.74	247
	707.9	170.7	11.33	75.9	8.60	275
Nitrite	301.8	0.6	4.83	99.8	4.96	158
	340.3	1.9	5.44	99.4	6.83	218
	475.1	3.5	7.60	99.2	7.59	242
	618.4	16.2	9.89	97.3	9.35	299
	635.0	32.2	10.16	94.9	9.59	306
	768.1	185.6	12.29	75.8	9.32	298
Nitrogen	599.2	22.51	9.59	96.2	9.37	299
	656.7	26.9	10.51	95.9	11.49	367
	950.9	56.9	15.21	94.0	14.35	458
	1167.8	81.4	18.68	93.0	17.10	546
	1225.3	138.8	19.60	85.5	17.33	553
	1476.0	356.3	23.62	76.1	17.92	572

shown in Fig. 3. It is evident from Fig. 3, that Monod and Haldane models both were reliable to describe nitrite conversion with correlation coefficients of 0.992, 0.993, respectively. Thus, Monod and Haldane models were also suitable to characterize the kinetic characteristics for nitrite. Based on Haldane model, the q_{max}, K_s and K_i of nitrite were 304.7 mg N/(gVSS d), 0.657 mg N/L and 13,942.1 mg N/L, respectively. The fitting equation was as follows:

$$q = \frac{304.7}{1 + 0.657/S + S/13942.1}$$

3.2.3. Kinetic characteristics of nitrogen conversion

The effect of nitrogen concentrations on specific nitrogen conversion rates was shown in Table 2. The analytical results of the

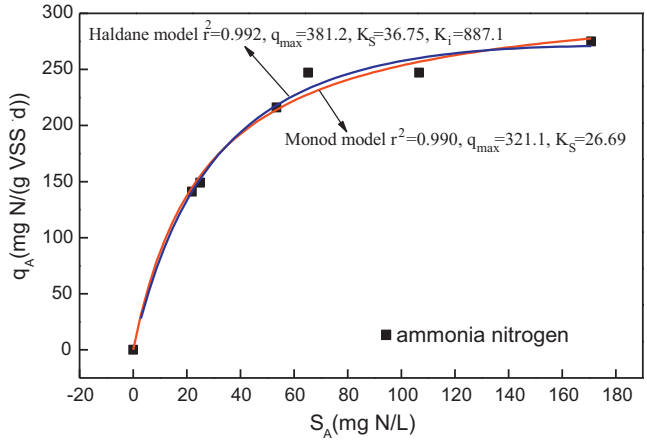

Fig. 2. Kinetic characteristic of ammonium conversion.

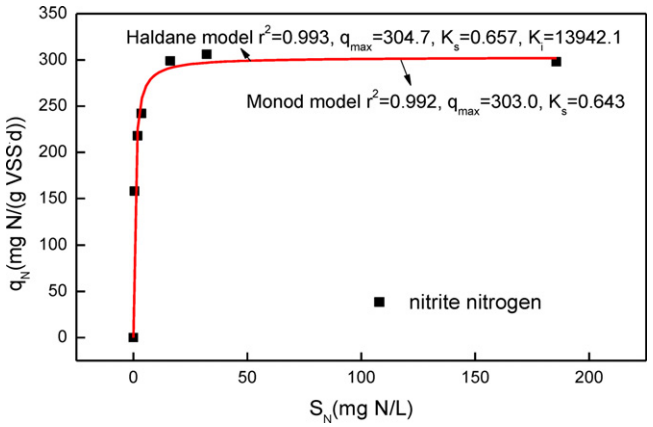

Fig. 3. Kinetic characteristic of nitrite conversion.

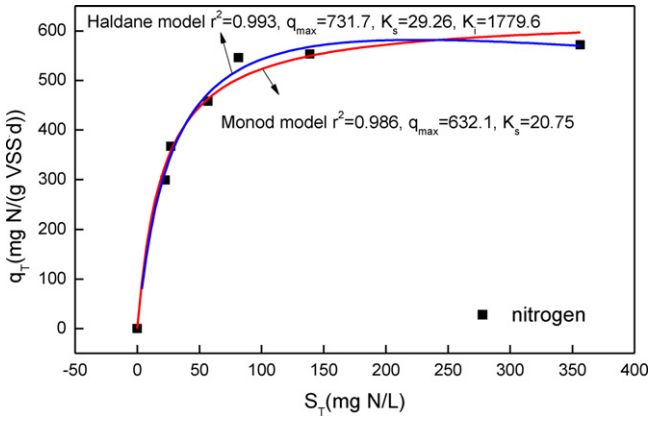

Fig. 4. Kinetic characteristic of nitrogen conversion.

data were presented in Fig. 4. Fig. 4 suggested that the Monod and Haldane models were suitable choice to describe the process with correlation coefficients of 0.986, 0.993, respectively. Based on Haldane model, q_{max}, K_s and K_i of nitrogen were 731.7 mg N/(gVSS d), 29.26 mg N/L and 1779.6 mg N/L, respectively. The fitting equation was as follows:

$$q = \frac{731.7}{1 + 29.26/S + S/1779.6}$$

3.3. Composition of microbial community

The members of microbial community found in the granular sludge were shown in Fig. 5. Overall, 8 clones were selected per sample out of 14 clones in total (7 clones per sample) and were effectively sequenced. In group 1 (the inoculum), most of the clones (6/7, 85.71%) belonged to the *Candidatus Kuenenia stuttgartiensis*, one of the clones (1/7, 14.29%) belonged to the *Candidatus Brocadia anammoxidans*. In group 2 (the granular sludge on day 131), all of the clones belonged to the *C. K. stuttgartiensis*.

4. Discussion

4.1. Reactor performance at high substrate concentrations

The reactor performance under different influent substrate concentrations and HRTs were shown in Table 1. The influent nitrogen concentrations were gradually increased to 1000 mg N/L on the 90th day where NRR was as high as 18.65 kg N/(m^3 d) and was still on the rise.

In theory, Anammox is the reaction between equimolar ammonium and nitrite concentrations. For the ammonium-containing wastewater, half of ammonium is required to be oxidized to nitrite. In laboratory studies, the nitrogen concentrations of synthetic wastewater represent the ammonium concentrations in actual wastewater that can be converted by Anammox process. Although the nitrogen concentrations exceeded 1400 mg N/L, the reactor performance still remained stable and efficient in the present study indicating that the Anammox-EGSB reactor was able to tolerate high ammonium–nitrogen concentrations and it may be used to treat strong ammonium-containing wastewaters. Such a superior performance is of quite practical value for Anammox process. The nitrogen loading in the present investigation was around 1400 mg N/L which were even higher than two-fold concentrations cited by other researchers. So, the reactor volume could be reduced by half when treating strong nitrogen-containing wastewater (nitrogen concentrations > 1400 mg N/L). Moreover, the demands of water to dilute the wastewater and costs of electricity to run the pumps could also be saved by half. Therefore, the Anammox process capable to tolerate high substrate concentrations may cut down engineering investment and operation costs.

The highest NRR reported in the literature [20] for the lab-scale Anammox biofilm reactor was 26.0 kg N/(m^3 d), but the influent nitrogen concentrations were less than 900 mg N/L. Once the

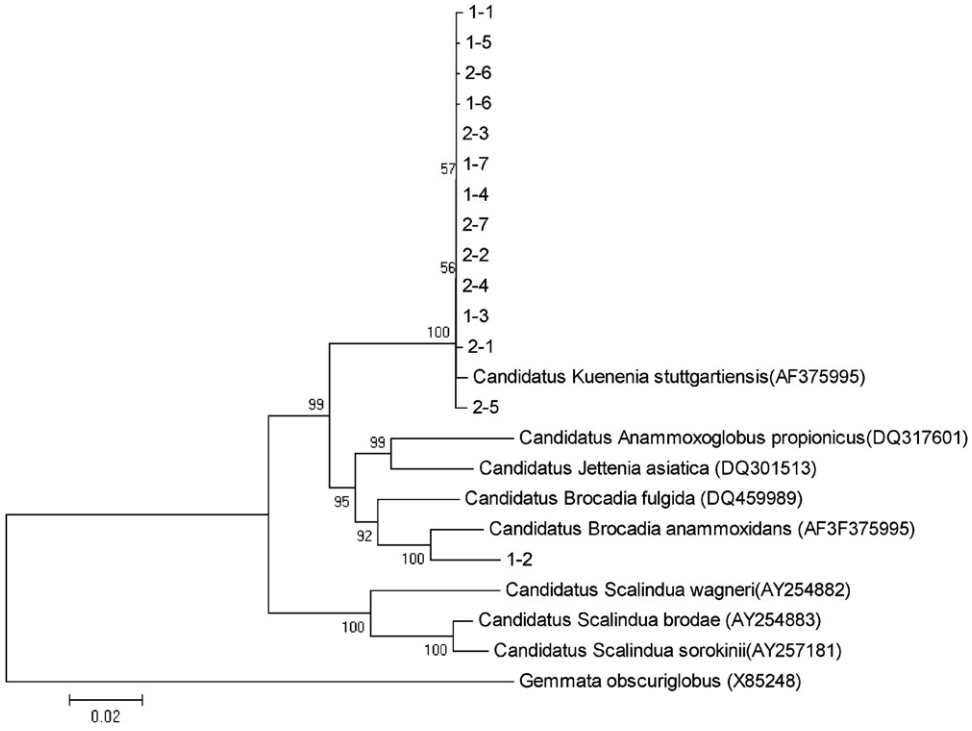

Fig. 5. Phylogenetic tree of Anammox bacteria showing the positions of the clones obtained from the granular sludge in the Anammox-EGSB rector in the initial and final [group 1 (1-1–1-7) represented the inoculum and group 2 (2-1–2-7) represented the granular sludge on day 131].

influent nitrogen concentrations exceeded 1000 mg N/L, the NRR dropped to 9.8 kg N/(m^3 d). It may be inferred that the reactor performance was still promising even ammonium–nitrogen loading beyond 1000 mg N/L in present work.

4.2. Maximum specific conversion rate

The theoretical maximum specific ammonium conversion rate was 381.2 mg N/(gVSS d) in the present work, which was greater than 38.65–297.2 mg N/(gVSS d) as reported in the literature [10,21–23]. The actual specific ammonium conversion rate was 275 mg N/(gVSS d) and the ratio of actual value to theoretical value was 0.721, which indicated that the tested Anammox granular sludge might have a higher conversion rate.

The theoretical maximum specific nitrite conversion rate was 304.7 mg N/(gVSS d), which was higher than 202.96 mg N/(gVSS d) as reported by Zheng and Hu [21]. The actual specific nitrite conversion rate was 306 mg N/(gVSS d) with the ratio of actual value to theoretical value of 1.004. Such information suggested that the nitrite removal ability of the tested Anammox granular sludge was fully utilized.

The theoretical maximum specific nitrogen conversion rate was 731.7 mg N/(gVSS d), which was in the range of 163–1800 mg N/(gVSS d) as reported in the literature [6,10,24,25]. The actual nitrogen specific conversion rate was 572 mg N/(gVSS d) with actual value to theoretical value ratio of 0.783, which indicated that the tested Anammox granular sludge still had potential Anammox activity.

4.3. Substrate affinity

When reactor was fed with ammonium, nitrite and nitrogen concentrations up to 707.9, 768.1, 1476 mg N/L, respectively; the K_s values of Anammox granular sludge for ammonium, nitrite and nitrogen were 36.75, 0.657 and 29.26 mg N/L, respectively, while the corresponding values of Anammox granular sludge reported in the literature were 48.41–87.1 [21,26], 6.552–15.39 [21,26] and 84.37 mg N/L [27], respectively. Strous et al. [5] reported that the Anammox affinity constants for ammonium and nitrite were equal to or less than 0.1 mg N/L while the diameters of aggregated Anammox bacteria they used were mostly (80%) less than 50 μm. However, the diameters of Anammox granular sludge of the present study were in millimeter range, so the mass transfer resistance in our study was much stronger. As a result, the substrate affinity constants were greater than those reported by Strous et al. [5]. However, compared to the reported values for Anammox granular sludge, the tested Anammox granular sludge had significantly higher substrate affinity in the present study.

In China, the national discharge standard for ammonium concentration is 15.0 mg/L. The K_s value for ammonium in the present study was 36.75 mg N/L. It is hard to utilize the maximum potential of the Anammox granular sludge if the effluent ammonium concentration should meet the discharging standard. Therefore, the Anammox-EGSB reactor is relatively more suitable for the pretreatment of ammonium-rich wastewaters.

4.4. Substrate tolerance

The K_i values for ammonium, nitrite and nitrogen using Anammox granular sludge were 887.1, 13,942.1 and 1779.6 mg N/L, respectively. The K_i values of ammonium and nitrite reported in the literature were 1123 and 159.5–720.6 mg N/L, respectively [21,26]. The K_i of ammonium was less than the reported values while the value for nitrite was greater than the reported values.

It is generally believed that the toxicity caused by nitrite is stronger than that of ammonium. However, the results obtained during the present work were contrary. The present influent ammonium to nitrite ratio is 1:1.096 while the consumption ratio of ammonium to nitrite is 1:1.253. It implied that the ammonium was excessive and the nitrite was relatively insufficient. Furthermore, it is well established that the inhibition occurs when the ammonium and nitrite are in free state, and the proportion of their free state is related to medium pH. As Anammox is a basification reaction, the higher the NRR, the higher is the effluent pH. The influent pH values were in range of 6.8–7.0, while the effluent pH values ranged 8.01–8.80 in the present study. In aqueous solution, the ratio of free ammonium (FA) and nitrite (FNA) to total ammonium and nitrite could be calculated by:

$$\frac{C_{FA}}{C_{t,NH_3}} = \frac{10^{pH}}{K_b/K_w + 10^{pH}} \cdot \frac{C_{FNA}}{C_{T,NO_2^+}} = \frac{1}{1 + K_a \times 10^{pH}}$$

where: C_{FA} – FA concentration; C_{t,NH_3} – total ammonium concentration; K_b – ammonium dissociation constant, $K_b = 10^{-4.7}$ (20 °C); K_w – water dissociation constant, $K_w = 1 \times 10^{-14}$ (20 °C); C_{FNA} – FNA concentration; C_{T,NO_2^+} – total nitrite concentration; K_a – nitrite dissociation constant and $K_a = 10^{-4.5}$ (20 °C).

Fig. 6a and b shows the relationship between $C_{FA}/C_{t,NH_3}$, $C_{FNA}/C_{T,NO_2^-}$ and pH at 20 °C. It can be inferred from Fig. 6 that the proportion of $C_{FA}/C_{t,NH_3}$ increases sharply and the proportion

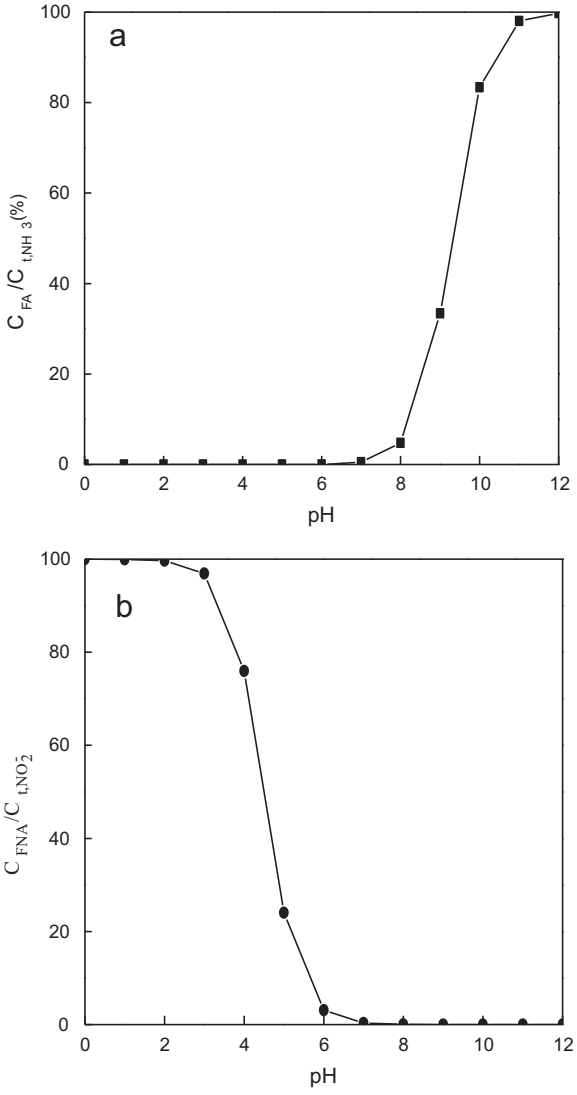

Fig. 6. Relationships between $C_{FA}/C_{t,NH_3}$, $C_{FNA}/C_{T,NO_2^-}$ and pH at 20 °C.

of $C_{FNA}/C_{T,NO_2^-}$ declines suddenly when pH is higher than 8.00. The average pH in the reactor was higher than 8.00 (actually 8.35), so under those circumstances, the K_i of uncharged ammonium and nitrite was 89.5 and 0.623 mg/L, respectively. The FNA concentration was far less than FA concentration in the reactor which proved that the toxicity of FNA was stronger than FA.

It has been reported in the literature that Anammox activity would be significantly inhibited when the nitrogen concentrations exceeded 1000 mg N/L [5,6,10,20,28]. In the present work, however, the Anammox-EGSB reactor could achieve high NLR when nitrogen concentrations reached up to 1476 mg N/L. That may be due to the following reasons: firstly, the reflux ratio of the Anammox-EGSB reactor was as high as 6:1 which could dilute the substrate concentrations in the reactor which could reduce the substrate concentrations in the reactor with high efficiency; secondly, the substrate was fed and removed at the same time that was why the maximum specific ammonium, nitrite and nitrogen conversion rates were high compared to earlier reports. Finally, after long-term acclimation (more than 4 months), the Anammox granular sludge in the reactor might have adapted to the high substrate concentrations. Therefore, the Anammox granular sludge in the present work was more resistant to high substrate concentrations.

4.5. Composition of microbial community

The dominant Anammox bacterial communities of inoculum were *C. K. stuttgartiensis* and *C. B. anammoxidans*. After the reactor operation at high substrate concentrations for a long time, the dominant Anammox bacteria in the granular sludge on day 131 were *C. K. stuttgartiensis* while the *C. B. anammoxidans* were not detected. *C. K. stuttgartiensis* and *C. B. anammoxidans* were the dominant Anammox bacteria in various enrichments. Therefore, a clear niche difference existed between "*Brocadia*/*Kuenenia*" clade and other genera [12]. However, the niche differentiation between "*Brocadia*" and "*Kuenenia*" is still unresolved. Gaul et al. [29] suggested that "*Brocadia*" cells were more susceptible to nitrite inhibition, and therefore not enriched in reactor systems at high nitrite levels. The results in this study are consistent with this hypothesis.

5. Conclusion

(1) The Anammox-EGSB reactor is suitable to treat strong ammonium-containing wastewaters. When the influent nitrogen concentrations were up to 1429.1 mg N/L, the NLR and NRR were as high as 22.87 kg N/(m^3 d) and 18.65 kg N/(m^3 d), respectively.
(2) Anammox granular sludge displayed excellent Anammox activity. The theoretical ammonium, nitrite and nitrogen specific conversion rates of Anammox granular sludge were 381.2, 304.7 and 731.7 mg N/(gVSS d), respectively, the measured values were 275, 306 and 572 mg N/(gVSS d), respectively.
(3) Anammox granular sludge possessed great substrate affinity and excellent substrate tolerance. When the ammonium, nitrite and nitrogen concentrations were 707.9, 768.1 and 1476.0 mg N/L, the corresponding K_s values of Anammox granular sludge were 36.75, 0.657 and 29.26 mg N/L, respectively. While the K_i values of Anammox granular sludge to ammonium, nitrite and nitrogen were 887.1, 13,942.1 and 1779.6 mg N/L, respectively.
(4) The 16S rRNA sequencing analysis confirmed that the enriched granular sludge contained Anammox cells with *C. K. stuttgartiensis* as the dominant population.

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