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Influence of substrates on nitrogen removal performance and microbiology of anaerobic ammonium oxidation by operating two UASB reactors fed with different substrate levels

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ABSTRACT

Both ammonium and nitrite act as substrates as well as potential inhibitors of anoxic ammonium-oxidizing (Anammox) bacteria. To satisfy demand of substrates for Anammox bacteria and to prevent substrate inhibition simultaneously; two strategies, namely high or low substrate concentration, were carefully compared in the operation of two Anammox upflow anaerobic sludge blanket (UASB) reactors fed with different substrate concentrations. The reactor working at relatively low influent substrate concentration (NO_2^--N, 240 mg-N L^{-1}) was shown to avoid the inhibition caused by nitrite and free ammonia. Using the strategy of low substrate concentration, a record super high volumetric nitrogen removal rate of 45.24 kg-N m^{-3} day^{-1} was noted after the operation of 230 days. To our knowledge, such a high value has not been reported previously. The evidence from transmission electron microscopy (TEM) showed that the morphology and ultrastructure of the Anammox cells in both the reactor enrichments was different.

1. Introduction

Anoxic ammonium oxidation (Anammox) process is a promising biotechnology initially discovered in an autotrophic denitrification reactor in The Netherlands [1], and now has been applied for the treatment of ammonium-rich wastewaters [2]. The bacterial species responsible for Anammox process can oxidize ammonium under anoxic conditions consuming nitrite as electron acceptor to produce nitrogen gas [3]. The first full-scale Anammox reactor was constructed at the Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam (The Netherlands) in 2002 [2]. It was fed with the effluent from a SHARON (single reactor high activity ammonia removal over nitrite) process [2,4]. The volumetric removal rate (VRR) for nitrogen reached 9.5 kg-N m^{-3} day^{-1} [2], which was far higher than that for the conventional nitrification–denitrification process (lower than 0.5 kg-N m^{-3} day^{-1} [5]). Moreover, the cost of Anammox process (€0.75 kg^{-1} N) was far lower than that for the conventional biological process (€2–5 kg^{-1} N) [4,6]. Because of its high-efficiency and cost-effectiveness, Anammox process is attracting more increased attention [7–10], and the number of full-scale Anammox reactor is increasing year by year [2].

Ammonium and nitrite serve as substrates of Anammox bacteria in a stoichiometric ratio of 1:1.32 according to Eq. (1) [11]:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

For achieving a higher efficiency, both the substrates should be fed into Anammox bioreactor in sufficient amount and a suitable ratio. However, both substrates act as potential inhibitors to the growth and metabolism of Anammox bacteria when their concentrations exceed the threshold inhibitory values [5,12–18]. So, the substrate inhibition must be avoided during the operation of Anammox bioreactors. In the present study, two substrate-dependent strategies were designed and compared to optimize the performance of Anammox reactors.

2. Materials and methods

2.1. Synthetic wastewater

Ammonium and nitrite were supplemented to mineral medium as required in the form of $(NH_4)_2SO_4$ and $NaNO_2$, respectively. The composition of the mineral medium was (g L^{-1} except for trace element solution) [19]: KH_2PO_4 0.01, $CaCl_2{\cdot}2H_2O$ 0.00565, $MgSO_4{\cdot}7H_2O$ 0.3, $KHCO_3$ 1.25, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL L^{-1} of trace elements solution. The trace element solution contained (g L^{-1}) [20]: 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, $NiCl_2{\cdot}6H_2O$

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Table 1
Performance of R1 with and without recirculation (average values).

Days	HRT (h)	*R*	Influent (mg-N L^{-1})		Effluent (mg-N L^{-1})			Removal (%)			VRR (kg-N m^{-3} day^{-1})
			NH_4^+-N	NO_2^--N	NH_4^+-N	NO_2^--N	NO_3^--N	NH_4^+-N	NO_2^--N	TN	
1–6	14.2	0	198	220	26	2	26	87	99	87	0.66
7–14	14.2	0	220	230	42	9	30	81	96	82	0.67
15–22	14.2	0	260	280	52	26	38	80	91	79	0.78
23–31	14.2	1.32	320	340	39	2	47	88	99	87	1.05
32–39	14.2	1.32	380	400	60	3	61	84	99	84	1.21
40–45	14.2	1.32	420	450	67	8	69	84	98	83	1.34

0.19, $NaSeO_4{\cdot}10H_2O$ 0.21, $NaMoO_4{\cdot}2H_2O$ 0.22 and $NaWO_4{\cdot}2H_2O$ 0.050.

2.2. Anammox bioreactors

Two upflow anaerobic sludge blanket (UASB) reactors (R1 and R2) were constructed each with inner diameter of 50 mm and a working volume of 1.1 L. Both reactors were covered completely by black cloth to avoid the growth of phototrophic organisms and the related oxygen production [10]. They were fed with synthetic wastewater flushed with 95% Ar–5% CO_2 continuously to maintain anoxic conditions. The temperature was set at 35 ± 1 °C [9] and the influent pH was controlled at 6.8 [18].

Both reactors were inoculated on day 0 with 0.9 L settled Anammox granular sludge from a small lab-scale reactor. The diameter of granular sludge was 2.21 ± 0.47 mm. The sludge concentration in both reactors was about 25 g-VSS L^{-1}.

2.3. Operational strategies

R1 was operated at high substrate concentration for which the volumetric nitrogen loading rate (VLR) was increased gradually (initial nitrite concentration was 220 mg-N L^{-1}) at a fixed hydraulic retention time (HRT) of 14.2 h; and the recirculation ratios were maintained at 1.07–1.32. R2 was operated at low substrate concentration for which the volumetric nitrogen loading rate was increased by shortening HRT (initial HRT was set at 11.69 h) at a fixed influent NO_2^--N concentration (240 mg-N L^{-1}). In both reactors, excess-sludge was not deliberately removed throughout the operation period.

2.4. Analytical methods

The influent and effluent samples were collected on daily basis and were analyzed immediately. The determination of pH, 5-min and 30-min sludge volume indices (SVI_5 and SVI_{30}), dissolved oxygen (DO), total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were accomplished according to the standard methods [21]. Ammonium and nitrite were analyzed using colorimetric method, and nitrate was analyzed using ultraviolet spectrophotometric method. The size of granular sludge was measured by an image analyzing system (QCOLite) with a Leica DM2LB microscope equipped with a digital camera (Canon S30).

2.5. Specific Anammox activity assays

Completely closed vials with a total volume of 120 mL and 100 mL of liquid volume were used to perform the Anammox batch assays. At the beginning of the experiment, the biomass concentration was about 1 g-VSS L^{-1}. The pH was fixed at 7.5 and the temperature was maintained at 35 ± 1 °C. Gas and liquid phases were purged with argon to remove O_2. Initial NH_4^+-N and NO_2^--N concentrations were 70 mg L^{-1} each. The maximum specific Anammox activity (MSAA) was estimated from the maximum slope of the curve indicated by the decrease of substrates in the vials with the passage of time [22].

2.6. Transmission electron microscopy (TEM)

Sludge samples from the reactor were fixed in 2.5% glutaradehyde solution and left in a refrigerator at 4 °C overnight. Later, they were fixed with 1% osmium acid for 1–2 h after cleansing with phosphate buffer solution (0.1 M, pH 7.0). Subsequently, the samples were dehydrated through a graded series of 50%, 70%, 80%, 90% and 100% ethanol. After fixation and dehydration, samples were treated with pure acetone for 20 min. Then they were treated with a mixture of coating agent and acetone (v/v: 1/1; v/v: 3/1). Subsequently, the samples were infiltrated by pure coating agent and left overnight at 70 °C. Ultra thin sections of 70–90 mm size were obtained by Reichert microtome. They were stained with lead citrate solution and uranyl acetate in 50% ethanol saturated solution for 15 min, respectively. At last, the samples were observed by the transmission electron microscope (JEOL JEM-1230, Japan).

3. Results

3.1. Performance of R1 at high substrate concentration

3.1.1. Performance without effluent recirculation

During the initial operation of 22 days, no effluent recirculation was applied. The reactor performance declined as the influent NO_2^--N concentration was increased from 220 mg-N L^{-1} to 280 mg-N L^{-1} (Table 1). The maximum effluent NO_2^--N concentration went up to 58 mg-N L^{-1} on day 22 (average value, 26 mg-N L^{-1}) and, correspondingly, nitrite removal efficiency went down to 79% (average 91%, Table 1), indicating that the performance was inhibited by nitrite. Other researches also demonstrated that nitrite concentrations above 100 mg-N L^{-1} led to inhibition (Table 2).

3.1.2. Performance with effluent recirculation

To alleviate the substrate inhibition, the effluent recirculation ratio (*R*) of 1.32 (recirculation:inflow) was implemented from day 23 on to dilute the inlet substrate concentration. As shown in Table 1, the average effluent NO_2^--N concentration went down to below 10 mg-N L^{-1} with the average NH_4^+-N and NO_2^--N removal efficiencies of 84% and 98%, respectively (Table 1), although the influent NO_2^--N concentration was gradually raised to 450 mg-N L^{-1}. In fact, the NO_2^--N concentration was diluted by the recirculation to 194 mg-N L^{-1} at the bottom of the reactor, which was only 69% of that without recirculation as mentioned above (280 mg-N L^{-1}) which was lower than the reported inhibitory concentration. Thus, the performance of R1 obviously improved.

3.1.3. Performance at different substrate ratios

Various influent substrate ratios were tested at that stage in order to investigate the effects of the two substrates (ammonium

Table 2
Effects of different substrates on Anammox process.

Nitrite		Ammonium		Free ammonia		Reference
Conc. (mg-N L^{-1})	Effect	Conc. (mg-N L^{-1})	Effect	Conc. (mg-N L^{-1})	Effect	
98	+ (loss of activity)	1000	–			[11]
350	+ (IC_{50})	700	+ (IC_{50})			[13]
224	+					[9]
280	+					[14]
274	+					[15]
60	+	90	–			[16]
				13–90	+	[17]
380	+			63–74	+	[18]
280	+	1000	–	83–104	+	This study

+, inhibitory effect; –, no inhibitory effect; IC_{50}, 50% inhibition concentration.

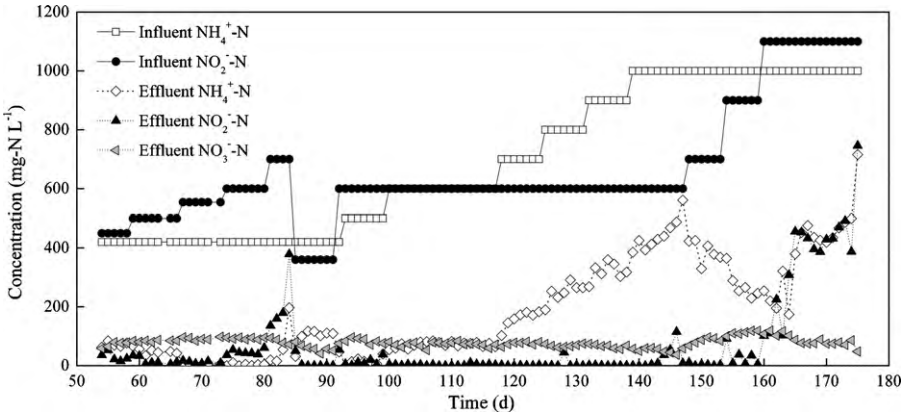

Fig. 1. Profile of nitrogen concentration of R1.

and nitrite) on reactor performance. HRT and recirculation ratio (R) were fixed at 11.57 h and 1.07 h, respectively throughout these tests. The results are shown in Fig. 1.

3.1.3.1. Effect of nitrite. During days 51–84, keeping the influent NH_4^+-N concentration fixed at 420 mg-N L^{-1}, five different influent NO_2^--N/NH_4^+-N ratios, i.e., 1.07, 1.19, 1.32, 1.43 and 1.67 were investigated by stepwise increase of ammonium concentration. The NO_2^--N concentration in the effluent was 12 ± 7 mg-N L^{-1} when the influent NO_2^--N/NH_4^+-N ratios were lower than 1.32. However, it rose significantly to 379 mg-N L^{-1} when the ratio increased to 1.67 with the ammonium and nitrite removal efficiencies of 53% and 46%, respectively. Consequent to decrease in the influent NO_2^--N concentration to 360 mg-N L^{-1} during days 85–91 (influent NO_2^--N/NH_4^+-N ratio 0.85), the effluent NO_2^--N concentration also went down immediately to lower than 3 mg-N L^{-1}, implying that the malfunction of the reactor could be recovered quickly. The ammonium and nitrite removal to nitrate production molar ratio was $1:(1.31 \pm 0.01):(0.22 \pm 0.01)$, which was close to the reported values [8,9,11,20].

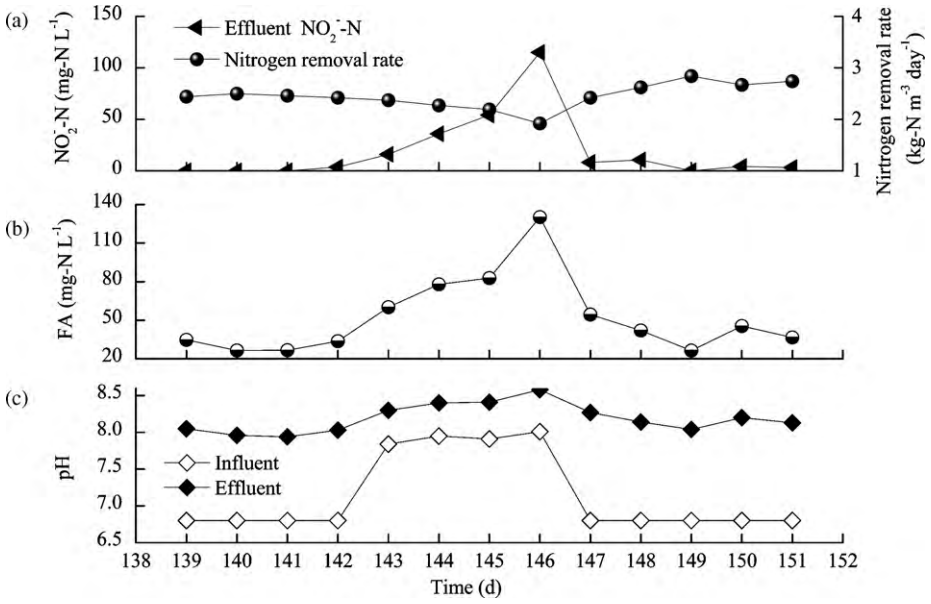

Fig. 2. Influence of pH and free ammonia on the nitrogen removal performance.

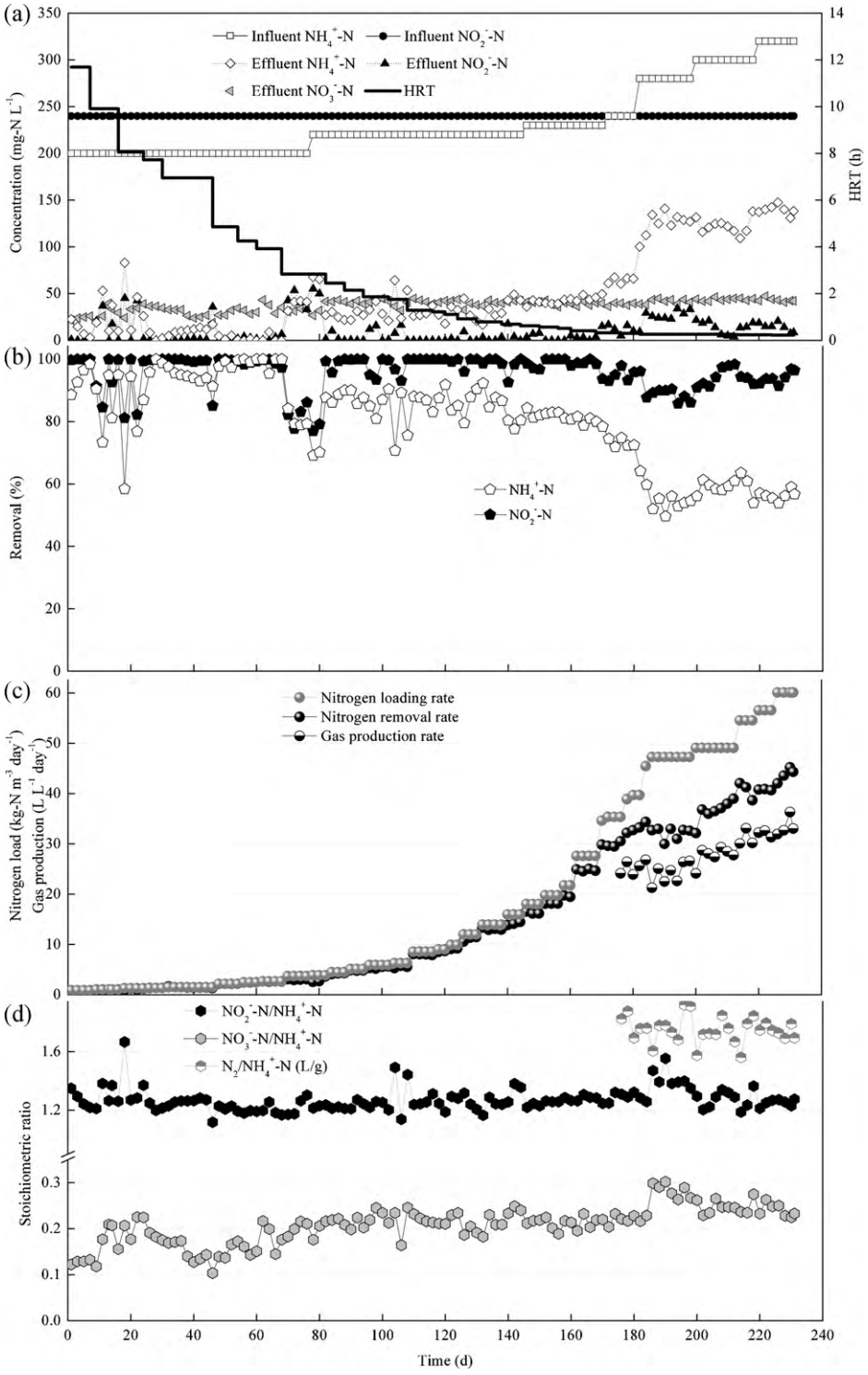

Fig. 3. Nitrogen removal performance of R2.

3.1.3.2. Effect of ammonium. For days 92–142, the influent NO_2^--N concentration was set constant at 600 mg-N L^{-1}, while the influent NH_4^+-N concentration was gradually raised to 1000 mg-N L^{-1} (Fig. 1). At this stage, the effluent NO_2^--N concentration stayed at a low level, i.e., 3 ± 4 mg-N L^{-1} (Fig. 1), and the volumetric nitrogen removal rate went up slightly to 2.46 ± 0.04 kg-N m^{-3} day^{-1}. Our results were in agreement with earlier reports stating that the Anammox process was not inhibited by the ionized form of ammonium concentrations up to 700–1000 mg L^{-1} (Table 2).

3.1.3.3. Effect of free ammonia. Recently, free ammonia (FA) was proposed to inhibit the Anammox process [17,18]. Free ammonia concentration can be calculated by Eq. (2) [23]:

$$\frac{C_{FA}}{C_{TAN}} = \frac{10^{pH}}{e^{6344/(273+T)} + 10^{pH}} \quad (2)$$

where C_{FA} is free ammonia concentration (mg-N L^{-1}); C_{TAN} is total NH_4^+-N concentration (mg-N L^{-1}); and T is temperature (°C).

As shown in Fig. 2, in the absence of a deliberate control of the influent pH, the effluent pH fluctuated between 7.8 and 8.1 (Fig. 2b

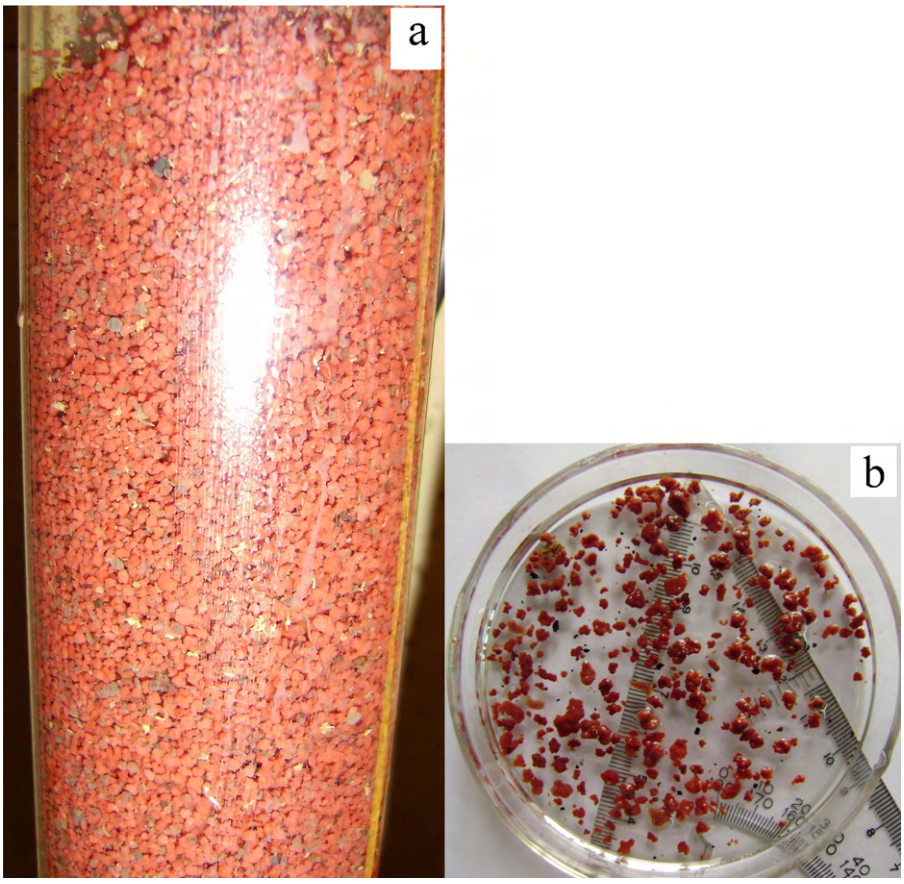

Fig. 4. Images of the Anammox granules in R2.

and c) while the effluent NO_2^--N concentration went up to 115 mg-N L^{-1} with a decrease in the volumetric nitrogen removal rate to 1.92 kg-N m^{-3} day^{-1} (Fig. 2a). This value was 22% lower than that in the case when the influent pH was controlled. After re-adjustment of the influent pH at 6.8, the effluent NO_2^--N concentration decreased to values lower than 10 mg-N L^{-1} immediately (Fig. 2a). Since the effluent pH in this stage was 8.41–8.58 (Fig. 2c) and Anammox reactors were reported to be stable for effluent pH range of 8.5–9.3 [24,25], so the direct inhibition from pH may not be considered as the dominant reason for the deterioration of reactor performance. However, the free ammonia concentration reached 83–130 mg-N L^{-1} (Fig. 2b), which was among or even higher than the reported inhibitory concentration value (Table 2). This might be the cause responsible for reactor destabilization in this stage.

3.1.3.4. Overloading. As shown in Fig. 1, upon further increase in the influent NO_2^--N concentration to 1100 mg-N L^{-1} during days 160–175 (influent NH_4^+-N concentration was set at 1000 mg-N L^{-1}), the effluent NO_2^--N concentration went up sharply to 746 mg-N L^{-1}, and the ammonium and nitrite conversions decreased to 28% and 32%, respectively. Although the influent NO_2^--N/NH_4^+-N ratio was still lower than the theoretic stoichiometric value mentioned above, the performance seemed to be saturated or even overloaded.

3.2. Performance of R2 at low substrate concentration

During days 1–68, the influent NO_2^--N/NH_4^+-N ratio of R2 was set at 1.20:1 and the HRT was gradually shortened from 11.69 h to 3.92 h (Fig. 3a). The average NO_2^--N removal was 99% (Fig. 3b). However, the NO_2^--N removal descended to 76% when the HRT was further shortened to 2.84 h (Fig. 3b), implying that NH_4^+-N was deficient relative to NO_2^--N at this stage. Raising the influent NH_4^+-N concentration to 220 mg-N L^{-1} during days 78–83, effluent NO_2^--N concentration progressively decreased to 0 (Fig. 3a). The results revealed that the increase of influent NH_4^+-N concentration resulted in increased NH_4^+-N removal which in turn led to the increase of NO_2^--N removal according to the stoichiometry of Anammox reaction. The nitrogen removal performance was then improved. Thereafter, the influent NH_4^+-N concentration was raised at a step of 10–20 mg-N L^{-1} where the effluent NO_2^--N concentration higher than 30 mg-N L^{-1} was detected and lasted for 3 days (Fig. 3a). It proved to be a useful strategy to improve the reactor performance throughout the operation. A super high volumetric nitrogen removal rate of 45.24 kg-N m^{-3} day^{-1} (average, 43.73 ± 1.05 kg-N m^{-3} day^{-1}) and nitrogen gas production rate of 36.32 L L^{-1} day^{-1} (average, 33.51 ± 1.93 L L^{-1} day^{-1}) were reached after an operation of 230-day with volumetric nitrogen loading rate up to 60.13 kg-N m^{-3} day^{-1} (flow-rate, 118 L day^{-1}; HRT, 0.22 h) (Fig. 3c). As evident in Fig. 3d, the stoichiometric ratio of nitrite removal, ammonium consumption and nitrate production was $1:(1.25 \pm 0.02):(0.23 \pm 0.01)$, which were close to the reported values aforementioned; the ratio of nitrogen gas production (L) to ammonium removal (g) (1.73 ± 0.05) was also close to the theoretic value (1.687 according to Eq. (1)) under the tested temperature.

The Anammox granules in R2 also showed some good characteristics having scarlet color (Fig. 4), implying that they were rich in haemachrome [20]. The diameter was 2.51 ± 0.91 mm, and the 5-min sludge volume index (SVI_5) was 25 mL g VSS^{-1} with a thickening process verified by an SVI_5 to SVI_{30} ratio of 1. These observations suggested a good sedimentation property (SVI_{30} of Anammox granules was reported by Dapena-Mora et al. [22] and Dosta et al. [26] was 40–110 mL g VSS^{-1}). Moreover, the sludge concentration inside the reactor was 29.3 g VSS L^{-1} and the specific

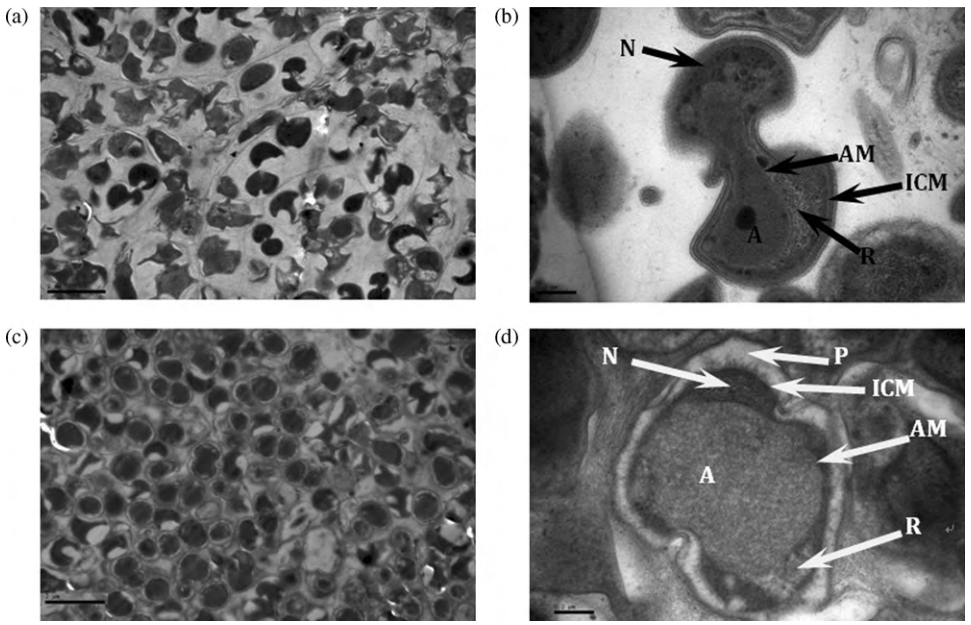

Fig. 5. Transmission electron micrographs of the Anammox sludges taken from R1 (a and b) and from R2 (c and d) at the end of the experiment. The innermost compartment, the anammoxosome (A), filled with material of moderate electron density and granular texture, but devoid of ribosome-like particles, is surrounded by a single membrane (anammoxsome membrane, AM). A riboplasm compartment (R) containing both ribosomes and a fibrillar nucleoid (N) completely surrounds the anammoxosome; the nucleoid appears attached to the anammoxosome membrane. The riboplasm is surrounded by a single intracytoplasmic membrane (ICM) and the paryphoplasm (P), in this case relatively electron-transparent, surrounds the rim of the cell. The scale bar in a and c = 2 μm; in b and d = 0.2 μm.

Anammox activity of the granules at the end of the experiment was 1.772 ± 0.168 kg-N kg VSS^{-1} day^{-1} (maximum value, 1.921 kg-VSS^{-1} day^{-1}).

Throughout the operation, the effluent pH remained stable at 7.9–8.2, and the free ammonia concentrations inside R2 were below 12.3 mg-N L^{-1}, which were far lower than the inhibition values (Table 2) providing a reasonable explanation of the good performance.

3.3. TEM observation

A typical characteristic of the previously described Anammox organisms is the presence of a membrane-bound intracytoplasmic compartment known as the anammoxsome [3,27–33]. TEM was performed on thin sections prepared from the enriched biomass taken from the bottom of the two UASB reactors. The dominant cells in both enrichments displayed typical ultrastructural features of Anammox bacteria: a single membrane-bound anammoxsome containing tubule like structure, and riboplasm with ribosome-like particles separated from paryphoplasm at the cell rim by an intracytoplasmic membrane [30] (Fig. 5). However, the evidence from TEM images depicted two major differences in the two reactor enrichments. First of all, the number of the Anammox cells in R1 enrichment was much lower than that in R2 enrichment (Fig. 5a and c). Secondly, the morphology of the cells differed either. The Anammox cells in R2 enrichment clearly show the paryphoplasm (P), which is relatively electron-transparent [28] (Fig. 5d), while in R1 enrichment cells, it seems difficult to distinguish this part (Fig. 5b). The denser Anammox cells in R2 enrichment could be ascribed to the super high nitrogen removal rate, while the difference in cellular structure might be probably the cause of substrate feeding strategy, considering that the same mineral medium and the same seed sludge were applied to both reactors. As Gaul et al. [34] suggested, "Brocadia" are more susceptible to nitrite inhibition, and therefore are not enriched in reactor systems at high nitrite levels. As from Fig. 6, the nitrite concentration in the bottom of R1 was as high as 300 mg L^{-1} when the reactor was stably oper-

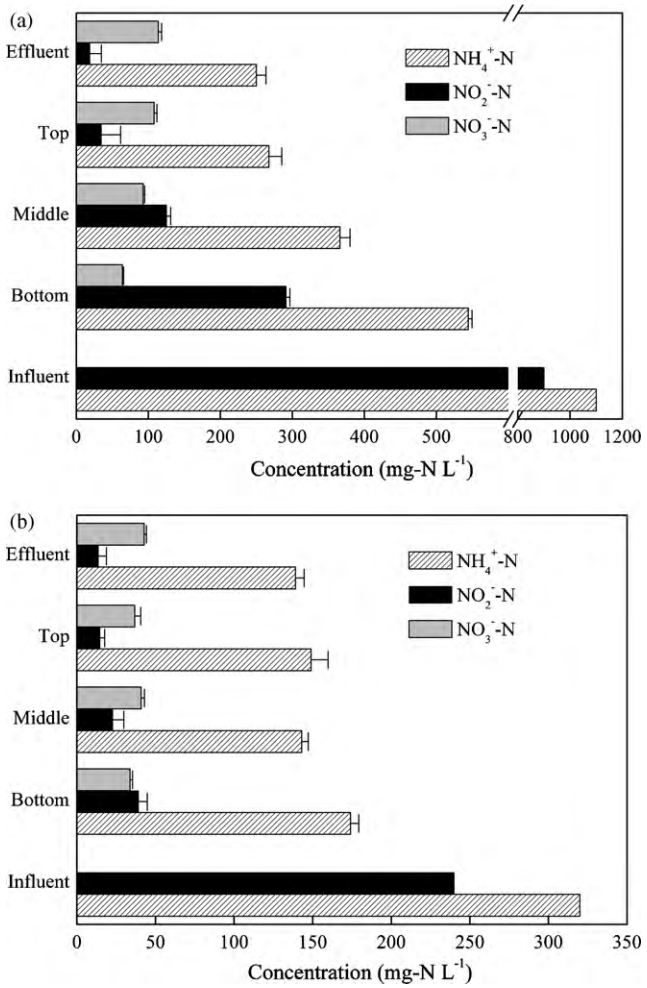

Fig. 6. The axial distribution of nitrogen compounds in R1 (A) and R2 (B).

Table 3
Overview of the high volumetric nitrogen removal rates for anaerobic ammonium oxidation.

Type of reactor	HRT (h)	VRR (kg N m^{-3} day^{-1})	Influent nitrite concentration (mg-N L^{-1})	Organism	Reference
UASB	0.22	45.2	240	Brocadia	This study
	11.07	3.78	900–1100 (effluent recycling ratio, 1.07)	Kuenenia	
Fixed-bed biofilm column reactor	0.24	26.0	260–334[a]	N.A.	[9]
Anaerobic biological filtrated reactor	0.67	11.5	<250	N.A.	[14]
Granular sludge bed reactor (full-scale)	2.6[a]	9.5	575 ± 175 (effluent recycling ratio, N.A.)	Bracadia	[2]
Gas-lift reactor	6.7	8.9	1420	N.A.	[8]
UASB (pilot-scale)	N.A.	6.4	1409(effluent recycling ratio, 14[a])	N.A.	[35]

N.A., not available.
[a] Calculated value.

ated under influent nitrite concentration of 900 mg L^{-1} (Fig. 6a), while the nitrite concentrations at different heights of R2 were just lower than 40 mg L^{-1} (Fig. 6b). The subsequent phylogenetic analysis also confirmed this hypothesis. The Anammox phylotype in R2 reactor had 95% sequence similarity to *Candidatus* "Brocadia anammoxidans", while the Anammox phylotype in R1 had 100% sequence similarity to *Candidatus* "Kuenenia stuttgartiensis" (Hu et al., Manuscript submitted for publication).

4. Discussion

4.1. Nitrogen removal performance

As far as the performance comparison of Anammox reactors with two operation modes was concerned, it could be concluded that the Anammox process was severely inhibited by nitrite concentration. Therefore, the influent nitrite concentration may serve as a control parameter during the operation of Anammox process. Based on the nitrite conversion capacity of the reactor, the volumetric loading rate can be effectively controlled to avoid nitrite inhibition.

The effluent recirculation seemed to be a useful strategy to dilute the influent substrate concentration that subsequently relieved the nitrite inhibition. Nitrogen removal performance varied significantly when the influent NO_2^--N concentration was up to 280 mg-N L^{-1}. The performance improved further after implementing the effluent recirculation (R, 1.07–1.32). The highest volumetric nitrogen removal rate of 3.78 kg-N m^{-3} day^{-1} was achieved on day 164. Nevertheless, the recirculation was ineffective when the influent NO_2^--N concentration was further raised to 1100 mg-N L^{-1} (recirculation ratio, 1.07). Upon increasing the recirculation ratio further would help to treat higher substrate concentrations. Additionally, inhibition caused by free ammonia at high substrate concentration could also contribute to the performance deterioration in the absence of pH control.

When the reactor was operated at lower substrate concentration, no severe inhibition was observed throughout the operation. The final volumetric nitrogen removal rate was 45.24 kg-N m^{-3} day^{-1} by progressive shortening of HRT to 0.22 h. To our knowledge, such a high VRR for anaerobic ammonium oxidation has never been reported earlier (Table 3). The granular sludge containing high concentration of Anammox bacteria retained in the UASB reactor was considered to contribute to the significantly high nitrogen removal performance.

After the comparison of both operation strategies, it was clear that the application of low substrate concentration at high flow-rate was more effective to achieve high-rate nitrogen removal than the application of high substrate concentration at low flow-rate. The maximum VRR of the former was 11 times higher than that of the latter. Similar results were reported in the literatures when Anammox bioreactors were operated at low substrate concentration and shorter HRT [8,9,14]. Obviously, operation at lower substrate concentration could avoid substrate inhibition leading to the higher nitrogen removal rates. Tsushima et al. [9] offered another explanation that some self-inhibitory by-products like dissolved organic carbon (DOC) derived from Anammox reaction could be washed out at shorter HRTs. On the other hand, the external mass transfer was enhanced at a high flow-rate and high nitrogen loading rates [36–38]. Furthermore, the effluent pH remained in the optimum range of 7.9–8.2 and the free ammonium concentrations were lower than the threshold inhibitory values, which helped to stabilize the reactor operation. The strategy with low substrate concentration at high flow-rate not only satisfied substrate requirement, but also avoided substrate inhibition.

The Anammox granules developed in R2 under high inflow rate possessed good settling property resulting in the biomass concentration up to 29.3 g VSS L^{-1} even under the very high inflow rate (109 L L^{-1} day^{-1}). The scarlet Anammox granules in R2 indicated the high amount of Anammox cells (Figs. 4 and 5c). Thus, the bacterial activity was up to 1.921 kg-N kg VSS^{-1} day^{-1}. The high biomass concentration and high bacterial activity also contributed to the super high nitrogen removal performance of R2. While the biomass concentration in R1 maintained at 24.3–26.0 g VSS L^{-1} during the whole operation; but the bacterial activity (0.212 kg-N kg VSS^{-1} day^{-1}) and the settling property (SVI_{30}: 58 mL g VSS^{-1}) of the granules were relatively low.

4.2. Microbiological characterization

Clear niche differences exist among the Anammox genera [10]. For example, *Candidatus* "Scalindua" was detected under marine conditions [29] and *Candidatus* "Anammoxoglobus" was enriched by adding propionate [30]. In the present study, the morphology and ultrastructure of the Anammox cells seemed different under the two operational models although the same seed sludge was inoculated and the same medium was applied for both reactors, suggesting that the dominant populations in the two reactor enrichments would probably be different. The results of subsequent sequencing analysis clearly proved this hypothesis. Thus, it could be deduced that the operation at high nitrite concentration led to selective enrichment of *Candidatus* "Kuenenia stuttgartiensis". Our results seemed to support the hypothesis that "Brocadia" cells were not enriched in reactors fed with high nitrite level [34]. However, the reason why paryphoplasm of the cells from reactor enrichments under different substrate concentrations possessed obvious differences still remains unclear. Further researches are needed to investigate this phenomenon.

5. Conclusions

The effects of substrates on nitrogen removal performance of Anammox process were investigated by operating two UASB reactors fed with different substrate levels. A super high nitrogen removal rate of 45.24 kg-N m^3 day^{-1} was recorded when the reactor was operated under relatively low nitrite concentration. The

morphology of the Anammox cells in the two enrichments differed either. With the subsequent confirmation of sequencing analysis, it seemed that high nitrite concentration was found to favorite enrichment of *Candidatus* "Kuenenia stuttgartiensis".

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