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ORIGINAL PAPER

Start-up and inhibition analysis of the Anammox process seeded with anaerobic granular sludge

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Abstract The longer start-up period of the Anammox process is due to the very low cellular yield and growth rates of Anammox bacteria. Nitrite inhibition is considered to be the key factor in the instability of the Anammox process during the operation. However, little attention was paid to the inhibitory effect of pH and free ammonia. This paper presents start-up and inhibition analysis of an Anammox biofilm reactor seeded with anaerobic granular sludge. Results showed that the start-up period could be divided into the sludge lysis phase, lag phase, propagation phase, stationary phase and inhibition phase. Optimization control could be implemented correspondingly to accelerate the start-up of Anammox bioreactors. Effluent pH increased to 8.7–9.1 when the nitrogen removal rate was higher than 1,200 mg l^{-1} day^{-1}. The free ammonia concentration was accompanied with a higher level of 64–73 mg l^{-1}. Inhibitory effects of high pH and free ammonia on Anammox bacteria contributed to the destabilization of the Anammox bioreactor during the first 125 days with influent $KHCO_3$ of 0.5 g l^{-1}. Increasing the suffering capacity in the inlet by dosing 1.25 g $KHCO_3$ l^{-1} effectively reduced the pH variation, and the nitrogen removal performance of the reactor was further developed.

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Introduction

Anaerobic ammonium oxidation (Anammox) is a new promising biotechnology that was initially discovered in a denitrifying reactor in Delft, The Netherlands [1]. During this process, *Planctomycete*-like bacteria consumed ammonium and nitrite to produce nitrogen gas under anoxic conditions [2]. The Anammox process combined with a preceding nitrification system called as SHARON (single reactor high activity ammonium removal over nitrite) was successfully applied in Rotterdam, The Netherlands, to treat the centrifuged digestate [3, 4]. The nitrogen removal rate (NRR) achieved was as high as 9.5 kg m^{-3} day^{-1} with a significant reduction in operational costs of aeration and exogenous electron donor, leading to a decrease to €0.75 kg^{-1} N as compared to the conventional nitrification–denitrification process (€2–5 kg^{-1} N) [4]. To date, the maximum NRR reported by Tsushima et al. [5] is 26.0 kg m^{-3} day^{-1}. The NRRs of the Anammox process described above have largely surpassed the conventional nitrification–denitrification process. Therefore, many researchers all over the world are paying increased attention to this novel and cost-effective nitrogen removal technology. The biochemical reactions of anaerobic ammonium oxidation can be represented as follows [6].

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

However, a limitation in the application of this process is its longer start-up period due to low cellular yield and

growth rates of Anammox bacteria (the doubling time reported by Strous et al. [6] is approximately 11 days) [3, 7, 8]. Furthermore, the working of Anammox bioreactors is not stable, mainly due to the substrate inhibition when high nitrogen loading rates (NLR) are applied [9, 10]. Nitrite inhibition is considered to be the key factor [6, 10]. However, little attention has been paid to the pH effect and the accompanying inhibition caused by free ammonia in the Anammox process.

It has been demonstrated that anaerobic granular sludge contains *Planctomycete* genes and has been successfully applied for the start-up of Anammox bioreactors [11–16]. In this study, we used this conventional seed sludge to start up the Anammox bioreactor, and then inhibition analysis was carried out in order to optimize the operational condition for achieving high nitrogen removal performance afterwards.

Materials and methods

The Anammox bioreactor

The upflow biofilm reactor (UBF) had an inner diameter of 50 mm, height of 750 mm and working volume of 1.1 l (Fig. 1). It was covered with black cloth to avoid light inhibition and was operated at 35 ± 1°C. The influent was flushed with argon in order to maintain anoxic conditions. Influent pH was controlled to maintain the influent pH in the range of 6.8–7.0. The UBF was packed with string-shaped three-dimensional plastic media (Yixing, China) as support materials for biofilms and was operated continuously [10]. The specific area of the strings was about 400 m^2 m^{-3}.

The reactor was stated at fixed HRT, i.e., 9.1 h. When Anammox activity occurred with continuous removal of ammonium and nitrite in the bioreactor, the nitrogen loading rate (NLR) was increased stepwise by raising approximately equimolar concentrations of NH_4^+–N and NO_2^-–N after every 2–4 days.

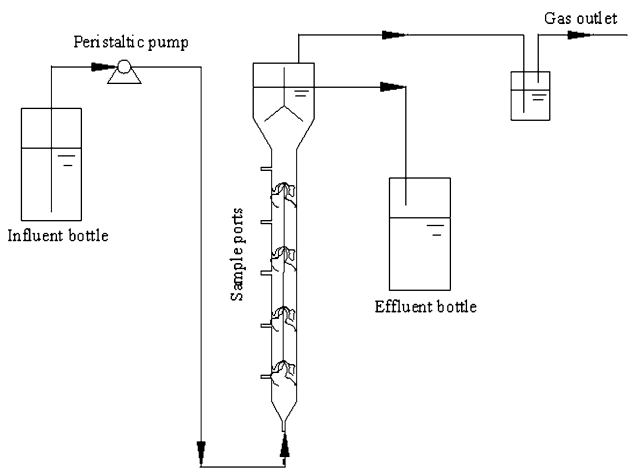

Fig. 1 Schematic drawing of an Anammox upflow biofilm reactor

Inoculation

The reactor was inoculated with anaerobic granular sludge taken from a full-scale UASB reactor for treatment of paper mill wastewater. The characteristics of the seed sludge are illustrated in Table 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): NaH_2PO_4-0.05, $CaCl_2$ $2H_2O$-0.3, $MgSO_4$ $7H_2O$-0.3, $KHCO_3$-0.5 (it was changed to 1.25 during days 126–152), $FeSO_4$-0.00625, EDTA-0.00625 and 1.25 ml l^{-1} of trace element solution. The trace element solution contained (g l^{-1}) (adapted from van de Graaf et al. [2]): EDTA-15, H_3BO_4-0.014, $MnCl_2$ $4H_2O$-0.99, $CuSO_4$ $5H_2O$-0.25, $ZnSO_4$ $7H_2O$-0.43, $NiCl_2$ $6H_2O$-0.19, $NaSeO_4$ $10H_2O$-0.21, $NaMoO_4$ $2H_2O$-0.22 and $NaWO_4$ $2H_2O$-0.050.

Specific Anammox activity assays

Completely closed vials with a total volume of 120 ml with 100 ml of liquid volume were used to perform the Anammox batch assays in the dark. Biomass concentration (VSS) at the beginning of the experiment was about 1 g l^{-1}. The pH value was fixed at 7.5 and temperature at 35 ± 1°C. Gas and liquid phases were purged with argon to remove O_2. The serum bottles were sealed tightly with butyl rubber caps. Initial concentrations of substrates were 70 mg l^{-1} of NH_4^+–N and 70 mg l^{-1} of NO_2^-–N. The concentrations of NH_4^+–N, NO_2^-–N and NO_3^-–N were periodically monitored during the incubation. Maximum specific Anammox activity (MSAA) was estimated from the maximum slope of the curve described by the decrease

Table 1 Some physical and chemical characteristics of the seed sludge

Seed sludge	SS (g l^{-1})	VSS (g l^{-1})	VSS/SS	Diameter (mm)
Anaerobic granular sludge	51.2	43.5	0.85	1–2

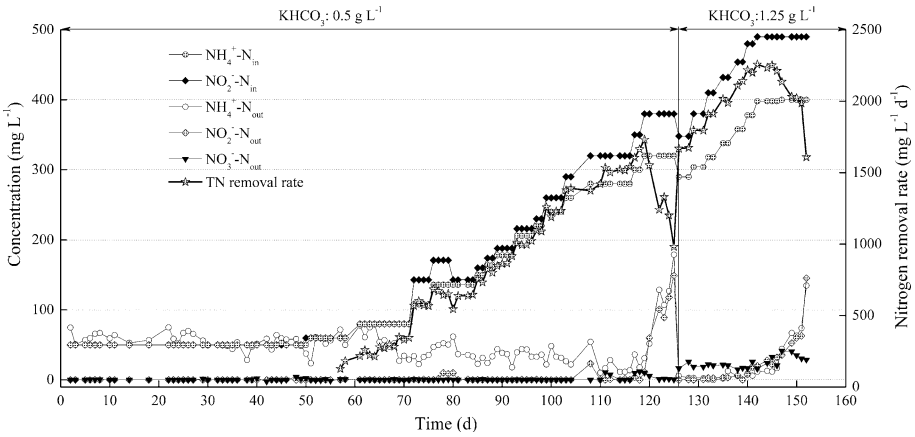

Fig. 2 Nitrogen concentration profile during the operation

of substrate concentration during the time and related to the biomass concentration in the vials [9].

Analytical methods

The influent and effluent samples were collected on a daily basis and were analyzed immediately. Water samples were analyzed according to the standard methods for the examination of water and wastewater [17]. Parameters analyzed included chemical parameters such as ammonia, nitrite, nitrate and pH. The biomass concentration was observed as suspended solids (SS) and volatile suspended solids (VSS). Ammonia was measured using the titrimetric method, nitrite was analyzed using the colorimetric method, and nitrate was analyzed using the ultraviolet spectrophotometric method. The pH meter was calibrated every 2 days according to the instruction manual.

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. Then they were fixed with 1% osmium acid for 1–2 h after being cleansed with phosphate buffer solution (0.1 M, pH 7.0). Subsequently, the samples were dehydrated through a graded series of ethanol solutions: 50, 70, 80, 90 and 100%. After fixation and dehydration, samples were treated with pure acetone for 20 min. Then they were treated with a mixed solution of coating agent and acetone (V/V: 1/1, V/V: 3/1) in sequence. Subsequently, the samples were infiltrated by a pure coating agent and left overnight at 70°C. Ultra-thin sections of 70–90 mm were obtained by Reichert microtome. They were stained with lead citrate solution and uranyl acetate in 50% ethanol saturated solution for 15 min, respectively. Finally, the samples were observed with a transmission electron microscope (JEOL JEM-1230, Japan).

Results and discussion

Start-up course and nitrogen removal

The start-up course of the UBF is depicted in Figs. 2 and 3. As evident from both figures, start-up comprised five steps based on the ammonium removal performance: sludge lysis phase (1–29 days), lag phase (30–57 days), propagation phase (58–73 days), stationary phase (74–118 days) and inhibition phase (119–125 days).

Sludge lysis phase

During this phase, the influent concentrations of NH_4^+–N and NO_2^-–N both were set at 50 mg l^{-1}; the effluent NH_4^+–N concentration was always higher than the influent concentration (Fig. 2). Accordingly, the ammonium removal efficiency was below zero. The reason why the ammonium concentration increased might be the cell lysis. As Chamchoi and Nitisoravut [18] postulated, the change of seed sludge might cause the turnover of bacteria, and the former dormant bacteria might be killed, resulting in cell lysis and breakdown of organic nitrogen to ammonium. This phenomenon was also evident by the nitrite removal. As shown in Figs. 2 and 3, nitrite was completely depleted during the sludge lysis phase, suggesting denitrification probably occurred in the reactor. As evident from feed composition, we did not include organic matter in the inlet except a very low concentration of EDTA used as the chelator for trace elements. Moreover, nitrate concentration in the effluent was below the detection limit (Fig. 2). The sludge lysis phase lasted for nearly a month.

Lag phase

This phase was characterized by sharp variations in effluent ammonium concentrations (Fig. 2) and ammonium removal efficiency (Fig. 3). During this period (days

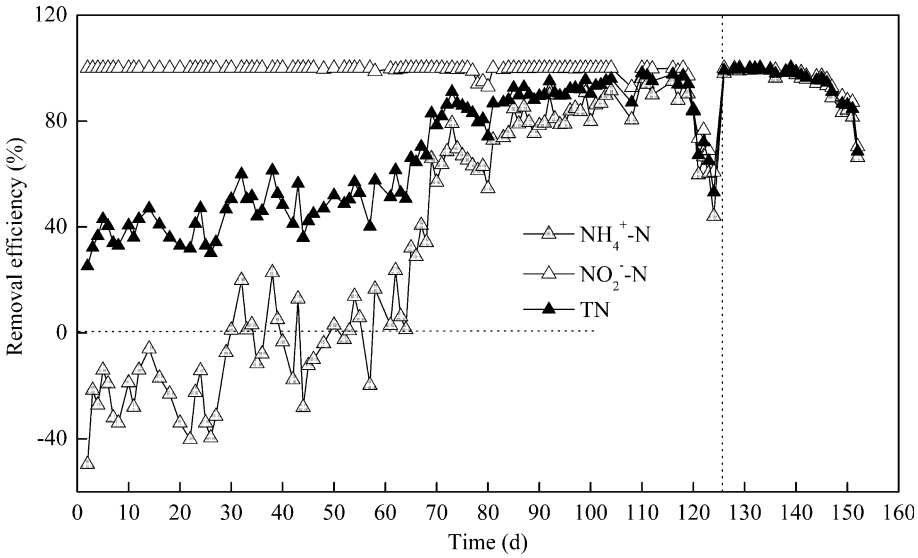

Fig. 3 Nitrogen removal efficiency profile during the operation

30–57), a reduction of ammonium was observed, but it only lasted for a few days (less than 3 days), and then an increase of ammonium was detected. The maximum and minimum NH_4^+–N concentrations in the effluent were 72 mg l^{-1} on day 57 and 24 mg l^{-1} on day 51, respectively. On the whole, the average NH_4^+–N concentration in the outlet and the average ammonium removal efficiency were 53 mg l^{-1} and −1.3%, respectively, during this phase. Ammonium decrease occurred for 10 days (days 30, 33–35, 38–39, 43 and 49–51) during this phase, suggesting that the sludge in the reactor was undergoing a transition period. The sludge in the lower part near the inlet changed from dark to a bit brownish. Nevertheless, no Anammox activity was detected in batch tests during this period. Nitrite and nitrate concentrations were still very low in the outlet (Fig. 2), suggesting that denitrification was still prevalent.

Propagation phase

Ammonium removal was continuously monitored in this phase (days 58–73). With influent NH_4^+–N and NO_2^-–N concentrations both of 60 mg l^{-1} on day 58, the effluent NH_4^+–N concentrations decreased to 50 mg l^{-1} (Fig. 2). Ammonium removal efficiency increased to 16.6% (Fig. 3). Hereafter, the ammonium decrease was always detected when both of the influent concentrations of NH_4^+–N and NO_2^-–N were initially raised to 80 mg l^{-1} each on days 61–71 and then to 108 and 143 mg l^{-1} for NH_4^+–N and NO_2^-–N, respectively (Fig. 2). The ammonium removal performance increased sharply during the propagation phase. NH_4^+–N removal efficiency also significantly increased from 2.9% on day 58 to 79.1% on day 73 (Fig. 3). Nitrite and nitrate concentrations in the outlet were still the same as mentioned in the sludge lysis and lag phases. The nitrite removal to ammonium conversion molar ratios decreased from 3.11–3.46 to 1.67 (as shown in Fig. 4). Ammonium and total nitrogen (TN) removal rates increased to 225 and 601 mg l^{-1} day^{-1}, respectively. To our optimal observation, the brownish part of the sludge enlarged, and several particles floated to the surface. The specific Anammox activity (SAA) of 0.041 g NH_4^+–N g-VSS^{-1} day^{-1} (0.092 g TN g-VSS^{-1} day^{-1}) was detected on day 73.

Stationary phase

Nitrogen removal efficiency of the system was relatively stable during this phase (days 74–118) when the influent concentrations of NH_4^+–N and NO_2^-–N were raised step by step from 108 and 143 mg l^{-1} to 300 and 350 mg l^{-1}, respectively (Fig. 2). Ammonium, nitrite and total nitrogen removal efficiencies were 80.3 ± 10.3%, 99.2 ± 2.0% and

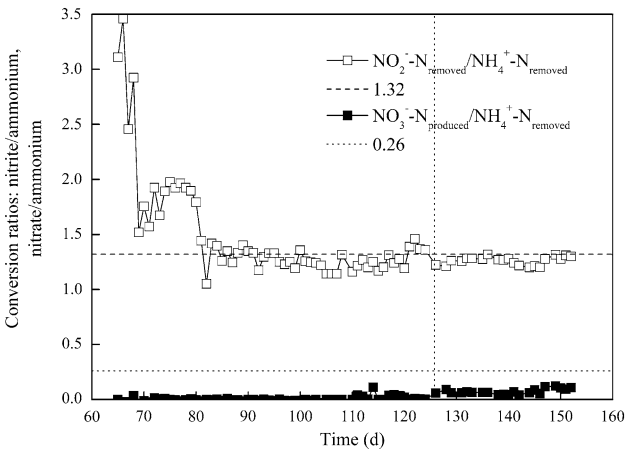

Fig. 4 Stoichiometric characteristics of the UBF after significant ammonium removal

90.3 ± 5.3%, respectively. Ammonium, nitrite and total nitrogen removal rates finally increased to 742, 921 and 1,663 mg l^{-1} day^{-1}, respectively (day 118). In comparison with the propagation phase, the ratios of NO_2^-–$N_{removed}$/NH_4^+–$N_{removed}$ further decreased to 1.20–1.34 (Fig. 4), which were very close to the value reported by Strous et al. [6]. However, surprisingly, a scanty amount of nitrate in the outlet was detected throughout the start-up course. The maximum NO_3^-–$N_{produced}$/NH_4^+–$N_{removed}$ ratio obtained in the stationary phase was only 0.04, which is far from the value of 0.22 reported by van de Graaf et al. [2] and Tsushima et al. [5] and of 0.26 reported by Strous et al. [6], but was close to that of Chamchoi and Nitisoravut [18]. The concurrent reactions of ammonium oxidation and nitrate reduction were also reported by Mulder et al. [1] and Jianlong and Jing [16]. SAA increased to 0.072 g NH_4^+–N g-VSS^{-1} day^{-1} (0.163 g TN g-VSS^{-1} day^{-1}) on day 118.

Inhibition phase

Nitrogen removal performance deteriorated during this period (days 119–125) when influent NH_4^+–N and NO_2^-–N concentrations were further increased to 320 and 380 mg l^{-1}, respectively. Effluent NH_4^+–N and NO_2^-–N concentrations significantly increased to 179 and 149 mg l^{-1} (on day 125, Fig. 3). Ammonium, nitrite and total nitrogen removal efficiencies were decreased to 44, 61 and 53%, respectively (Fig. 4). Ammonium, nitrite and total nitrogen removal rates sharply declined to 371, 608 and 979 mg l^{-1} day^{-1}, respectively, which were only 50, 66 and 59% of the corresponding removal rates obtained in the stationary phase described above. The appearance of these typical dysfunctions suggested that the Anammox biomass in the reactor might be inhibited. The performance of bioreactors reached the maximum value when the substrate concentration was elevated continuously. Severe performance deterioration of the bioreactor would be induced with a heavy increase of substrate concentrations [6, 19, 20].

Variations in the ammonium removal rate during the start-up of the Anammox UBF are shown in Fig. 5. We assumed that ammonium removal in our experiment was definitely carried out by the Anammox pathway; however, nitrite removal may partly be the function of denitrification. Indeed, the start-up of Anammox bioreactors is the course of activation, amplification and enrichment of Anammox bacteria from conventional seed sludges. Along with the increase of Anammox biomass in the bioreactor, ammonium removal performance improved progressively. However, the maximum biomass concentration was limited by the reactor space. Therefore, nitrogen removal performance did not improve permanently and would reach a stable level. This characteristic of the start-up course of Anammox bioreactors was in accordance with logistic Eq. (2) [21].

$$N_t = \frac{K}{1 + e^{a-rt}} \tag{2}$$

where N_t is the number of microorganisms; a is a constant; K indicates the maximum capacity of microorganisms in the reactor; r is the instant growth rate of the microorganisms; t is time.

Thus, fitting the logistic equation to predict the ammonium removal rate (which was proportional to the growth rate of the Anammox biomass [6]) variations of the Anammox UBF during the start-up course, curve fitting produced better results with a R^2 value of 0.984, which was achieved in the former four phases (Fig. 5). However, during the inhibition phase, ammonium removal decreased sharply, as shown in Fig. 5 (solid line), which was far away from the logistic equation curve (Fig. 5 dot line).

Based on the characteristics during the start-up course, we can implement the proper controlling strategies to optimize the operation conditions for Anammox bioreactors. In the sludge lysis phase, denitrification should be controlled to maintain pH in the optimum range. In the lag phase, substrate concentration, especially nitrite concentration, should be maintained at a lower level to prevent substrate inhibition. In the propagation phase, the nitrogen loading rate should be elevated step by step, and in the stationary phase, the nitrogen loading rate should not be raised further in order to avoid substrate inhibition.

pH increase and inhibition analysis

Influent pH of the reactor was strictly controlled at 6.8 throughout the start-up. During the days when the

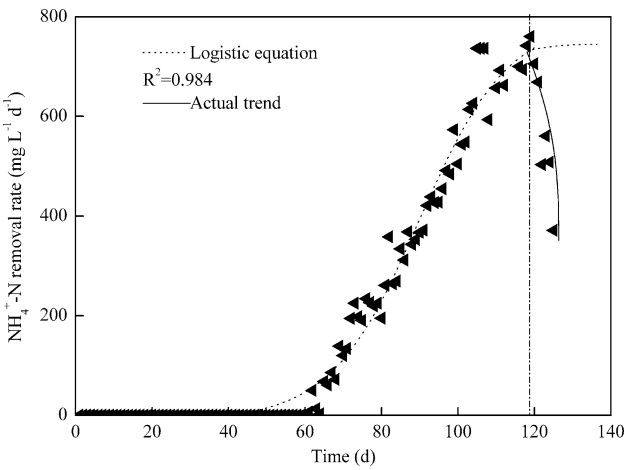

Fig. 5 Prediction of the start-up course of the UBF with logistic equation

Anammox reaction did not occur, effluent pH was in the range of 7.8–8.3 (Fig. 6a), which might be the function of denitrification. Thereafter, effluent pH increased to a higher level as depicted in Fig. 6a when the Anammox reaction appeared in the UBF. It could be seen that effluent pH increased with the advancement of operation of the bioreactor, which corresponded to a higher nitrogen loading rate and nitrogen removal rate.

The phenomenon of a significant increase in effluent pH for Anammox bioreactors was also reported by Liu et al. [22]. Szatkowska et al. [23] proposed that the Anammox process can increase the pH value to some extent due to cellular synthesis. However, Strous et al. [6] reported that the pH variation in one cycle of a SBR was not large. Chamchoi and Nitisoravut [18] showed that the pH values in the effluent were always in the range of 7.7–8.4, which were somewhat lower than that in the influent.

There are several reasons to explain the effluent pH increase in Anammox bioreactors. Firstly, considering the stoichiometry of the Anammox reaction (Eq. 1), 0.13 mol H^+ is consumed when 1 mol of ammonium is converted. Van de Graaf et al. [2] predicted that 0.09 mol OH^- was produced when 1 mol of ammonium was consumed. The consumption of acidity results in a pH increase in the Anammox process [22]. The linear relationship between effluent pH and nitrogen removal rates (NRR) (Fig. 6b) clearly suggested that this function may be the main reason for a pH increase when Anammox occurred in the UBF.

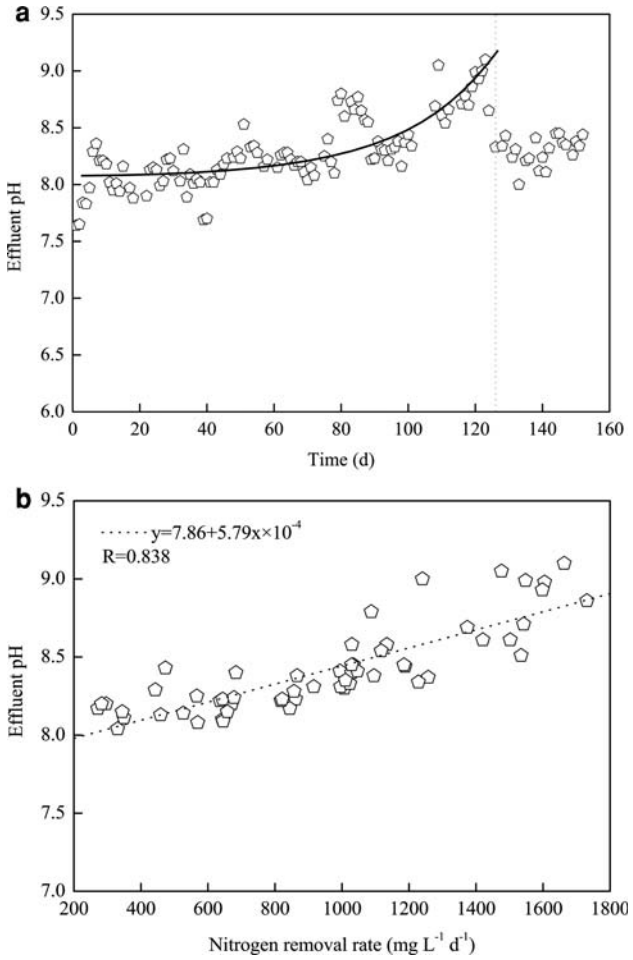

Fig. 6 Effluent pH profile during the operation (**a**) and relationship between effluent pH and nitrogen removal rate (**b**)

Pietsch et al. [24] reported that hydrostatic pressure decreases from the bottom to the top of an upflow reactor. The carbon dioxide partial pressure in the gas phase typically decreases from the bottom zones to the upper sections of the reactor because of the lowered hydrostatic pressure. This reduction leads to a degassing of the dissolved CO_2 from the liquid into the gas phase, which is then followed by a rising pH. Stripping of the dinitrogen gas that is produced could also be a cause.

The buffering capacity of the solution was another important factor contributing to the pH variations. Higher alkalinity leads to smaller pH variations. In our study, the influent $KHCO_3$ was 0.5 g l^{-1} during the first 125 days, and influent pH was always controlled at 6.8 by dosing HCl solution, which leads to a lower influent alkalinity. A weaker buffering capacity of the solution was then achieved. This may be the reason why the results obtained in the present study were different from Strous et al. [6] and Chamchoi and Nitisoravut [18]. In their research, no deliberate influent pH control was carried out, and sufficient buffering capacity was achieved by flushing with CO_2 gas mixture into the reactor continuously.

The pH is an important control parameter during the operation of Anammox bioreactors. The effect of pH on Anammox process has been tested by Strous et al. [14] by using batch experiments. Results showed that the specific Anammox activity at pH of 9 was only 1/5 of that at of pH 8. In this study, effluent pH values ranged from 8.7 to 9.1, which resulted from applying NLR of more than 1,200 mg l^{-1} day^{-1} (Fig. 6b); such values surpassed the optimum pH range for Anammox bacteria (6.7–8.3) [25]. Under high pH, operational conditions were not suitable for the growth and metabolism of Anammox bacteria. Performance failure of Anammox reactors may occur when reactors are operated under such high pH for a longer time.

Correspondingly, high pH was accompanied by a high free ammonia concentration (Eq. 3) [26].

$$\mathrm{FA}\ (\mathrm{mg\ L^{-1}}) = \frac{17}{14} \times \frac{\sum \mathrm{NH_4^+ - N(mgL^{-1})} \times 10^3}{(k_b/k_w) + 10^{\mathrm{pH}}} \qquad (3)$$

where $k_b/k_w = e^{6344/(273+T(^\circ\mathrm{C}))}$.

Free ammonia was toxic to the anabolic and catabolic processes of microorganisms [27]. Waki et al. [28] presumed that free ammonia concentrations of 13–90 mg l^{-1}

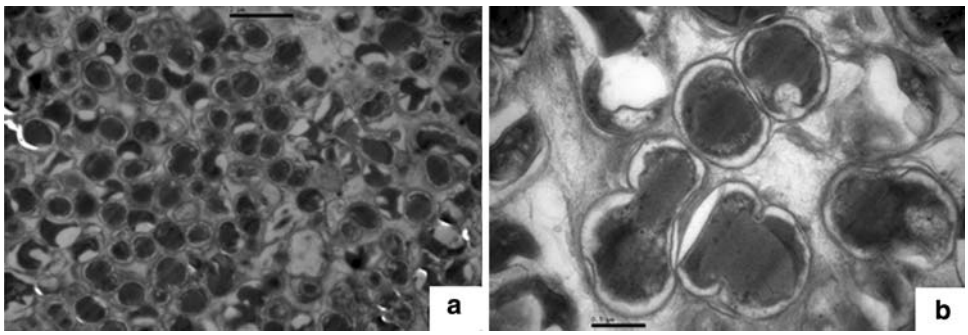

Fig. 7 TEM photographs of the granular sludge

could negatively affect the performance of the Anammox process. The nitrogen removal performance was lower than half of that in the control reactor with free ammonia concentrations lower than 21 mg l^{-1}. In the present study, the free ammonia concentration increased to 64–73 mg l^{-1} because of the high pH values during days 122–125.

Based on the analysis, in order to increase the buffering capacity of the solution and finally to reduce the inhibition caused by high pH and free ammonia, we increased the influent alkalinity by dosing $KHCO_3$ to 1.25 g l^{-1} during days 126–152 (Fig. 2), which was 2.5 times higher than that used before. From Fig. 6a, we can see that the effluent pH did not vary very much and was maintained stably at 8.0–8.4 throughout this period. Correspondingly, nitrogen removal performance was further improved to 2,260 mg l^{-1} day^{-1} when the influent ammonium and nitrite concentrations were finally raised to 400 and 490 mg l^{-1} (Fig. 2), respectively, which was 1.36 times higher than that obtained at the condition of 0.5 g $KHCO_3$ l^{-1}. Nitrate production was enhanced during this period. On day 147, it increased to 41 mg l^{-1} (Fig. 2) and the nitrate-to-ammonium removal ratio increased to 0.12 (Fig. 4). Figure 7 shows photographs of the cultivated sludge obtained from the UBF. It can be seen that that the granular sludge contained many Anammox-like cells as depicted in Toh et al. [29]. The Anammox activity reached 0.243 g TN g-VSS^{-1} day^{-1}. However, reactor performance began to decline again. On day 152, concentrations of ammonium and nitrite in the outlet were as high as 135 and 145 mg l^{-1}, respectively (Fig. 2). Ammonium removal efficiency went down to 66.3% (Fig. 3).

In conclusion, the pH increase in the Anammox process led to a high pH range of 8.7–9.1 during the first 125 days with a $KHCO_3$ concentration of 0.5 g l^{-1} when the nitrogen removal rate was higher than 1,200 mg l^{-1} day^{-1}. Moreover, the free ammonia concentration of about 64–73 mg l^{-1} was accompanied by high pH, which was among the inhibition values presumed by Waki et al. [28]. Both contributed to the performance deterioration of the Anammox UBF. Increasing the suffering capacity of the solution (1.25 g $KHCO_3$ l^{-1}) resulted in a relatively low and stable pH range (8.30 ± 0.12). Thus, free ammonia was always below 30 mg l^{-1}. The relatively low pH and free ammonia concentration ensured that the UBF achieved a better nitrogen removal performance. However, when influent nitrite increased to 490 mg l^{-1} on days 142–152 (Fig. 2), nitrite inhibition finally occurred.

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