Effect of influent substrate ratio on anammox granular sludge: performance and kinetics

Weiqliang Zhu · Jin Li · Huiyu Dong · Dan Wang · Peiyu Zhang

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Abstract Effect of influent substrate ratio on anammox process was studied in sequencing batch reactor. Operating temperature was fixed at 35 ± 1 °C. Influent pH and hydraulic retention time were 7.5 and 6 h, respectively. When influent $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$ was no more than 2.0, total nitrogen removal rate (TNRR) increased whereas $\text{NH}_4^+ \text{-N}$ removal rate stabilized at $0.32 \text{ kg}/(\text{m}^3 \text{ d})$. $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ increased with enhancing $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$. When $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$ was 4.5, $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ was 1.98, which was much higher than theoretical value (1.32). The IC_{50} of $\text{NO}_2^- \text{-N}$ was $289 \text{ mg}/\text{L}$ and anammox activity was inhibited at high $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$ ratio. With regard to influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$, the maximum $\text{NH}_4^+ \text{-N}$ removal rate was $0.36 \text{ kg}/(\text{m}^3 \text{ d})$, which occurred at the ratio of 4.0. Anammox activity was inhibited when influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ was higher than 5.0. With influent $\text{NO}_3^- \text{-N}/\text{NH}_4^+ \text{-N}$ of 2.5–6.5, $\text{NH}_4^+ \text{-N}$

removal rate and NRR were stabilized at 0.33 and $0.40 \text{ kg}/(\text{m}^3 \text{ d})$, respectively. When the ratio was higher than 6.5, nitrogen removal would be worsened. The inhibitory threshold concentration of $\text{NO}_2^- \text{-N}$ was lower than $\text{NH}_4^+ \text{-N}$ and $\text{NO}_3^- \text{-N}$. Anammox bacteria were more sensitive to $\text{NO}_2^- \text{-N}$ than $\text{NH}_4^+ \text{-N}$ and $\text{NO}_3^- \text{-N}$. TNRR would be enhanced with increasing nitrogen loading rate, but sludge floatation occurred at high nitrogen loading shock. The Han-Levenspiel could be applied to simulate nitrogen removal resulting from $\text{NO}_2^- \text{-N}$ inhibition.

Keywords Anammox · Influent substrate ratio · Nitrogen removal performance · Kinetic analysis

Introduction

Nowadays, anaerobic ammonium oxidation (anammox) has been successfully applied in full-scale ammonium-rich wastewater treatment (van der Star et al. 2007). It could oxidize ammonium to N_2 directly with nitrite as electron acceptor, which was very energy-saving and efficient (Carvajal-Arroyo et al. 2014a; Strous et al. 1998). Nevertheless, anammox process has not been widely applied due to low growth rate of anaerobic ammonium oxidizing bacteria (AnAOB) and many inhibitory factors such as low temperature (Zhu et al. 2017a, b), extreme pH (Li et al.

W. Zhu · J. Li (✉) · P. Zhang
School of Environmental Science and Engineering,
Qingdao University, Qingdao 266071, China
e-mail: ljin0532@126.com

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

D. Wang
National Marine Environmental Forecasting Center, State
Oceanic Administration, Beijing 100081, China

2017) and organic carbon source (Zhu et al. 2017a, b). Besides, substrate inhibition is a significant obstacle for nitrogen removal and operation stability during anammox process (Jin et al. 2013).

Both $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ are substrates for anammox reaction, and $\text{NO}_3^-\text{-N}$ is the product of it. However, these three species are all inhibitors when they are at high concentrations (Aktan et al. 2012; Dapena-Mora et al. 2007; Jin et al. 2013; Lotti et al. 2012). Although efforts were made to study their thresholds and mechanisms, it was failed to achieve uniform result. Strous et al. (1999) noted that 100 mg/L $\text{NO}_2^-\text{-N}$ would completely inhibited anammox activity, and as high as 1000 mg/L $\text{NH}_4^+\text{-N}$ or $\text{NO}_3^-\text{-N}$ did not affect anammox process. However, other studies reported that inhibitory concentration of $\text{NO}_2^-\text{-N}$ was around 280 mg/L (Isaka et al. 2007; Kimura et al. 2010). Dapena-Mora et al. (2007) indicated that 50% activity loss resulting from $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were 770 and 630 mg/L, respectively. Still, effect of influent substrate ratio on anammox process was studied little. As reported by Strous et al. (1998), influent substrate ratio ($\text{NH}_4^+\text{-N}/\text{NO}_2^-\text{-N}$) required by anammox process was 1:1.32. In order to achieve high nitrogen removal efficiency, influent substrate should be fed into anammox system with an appropriate ratio. If it deviated from the ratio, nitrogen removal performance was affected. However, it is ordinary that influent substrate fluctuates in most wastewaters. As a result, the potential negative effect result from influent substrate ratio should be studied.

In addition, kinetic analysis could predict the value which could not be acquired through experiment. It could also provide an effective tool for process control. The Haldane, Edwards, Aiba and Luong models were widely applied on substrate inhibition (Jin et al. 2013; De Prá et al. 2016). Besides, they played an important part in application and development of anammox process. However, they were not suitable to all studies (Dapena-Mora et al. 2007). As a result, much more kinetic models should be constructed and analyzed, and more information concerning effect resulting from influent substrate ratio should be revealed.

A sequencing batch reactor (SBR) presents a great simplicity and flexibility of operation, which favors the enrichment of slow-growing bacteria (Lan et al. 2011). In this work, nitrogen removal performance of anammox process was assessed in an SBR with

various influent substrate ratios. Besides, kinetic analysis was performed to study the effect resulting from influent substrate ratio. Unlike single ammonia or nitrite nitrogen, influent substrate ratios on anammox granular sludge were focused on in this study. What's more, some strategies to alleviate substrate inhibition were proposed. Still, sludge floatation found at high nitrogen loading rate was explored. The results acquired from this work were also compared with previous reports.

Materials and methods

Experimental set-up

Three parallel SBRs (R1, R2 and R3) made of polymethyl methacrylate were used in this work. They were double-jacketed with an effective volume of 1.5 L. Three reactors were all inoculated with 1.65 g/L anammox sludge and covered completely with aluminum caps to create a dark circumstance. Temperature was maintained at $35 \pm 1^\circ\text{C}$ and hydraulic retention time was at 6 h. Two cycles were run each day in this experiment. Complete mixing was achieved using mechanical stirrer with rotating speed of 100 rpm. N_2 (99.99%) was purged into influent to maintain anaerobic condition. Each reactor was bubbled by N_2 for 10 min before sludge was inoculated into it.

Biomass and synthetic wastewater

Inoculated sludge was obtained from a laboratory-scale reactor with working volume of 7 L which operated steadily over 1 year with $\text{NO}_2^-\text{-N}/\text{NH}_4^+\text{-N} = 1.32$ ($\text{NO}_2^-\text{-N}$ 105 mg/L; $\text{NH}_4^+\text{-N}$ 80 mg/L) at $35 \pm 1^\circ\text{C}$. Both $\text{NO}_2^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ could be completely removed. The diameter of granular biomass was 0.5–5.0 mm, with a mean value of 1.7 mm. The digital and microscopic images of the sludge were presented in Fig. 1. $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ were added into reactors as required in the form of NH_4Cl , NaNO_2 and KNO_3 , respectively. The composition of synthetic wastewater was KH_2PO_4 0.028 g/L, CaCl_2 0.14 g/L, KHCO_3 2.0 g/L and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g/L. Trace element solutions I and II (1.2 mL per liter medium) were also added into

Fig. 1 Image of inoculated granular sludge

reactor as previously reported (van de Graaf et al. 1996). Influent pH was around 7.5 ± 0.1 .

Operational strategy

The effect resulting from influent NO_2^- -N/ NH_4^+ -N was carried out in R1. During this period, influent NH_4^+ -N was stabilized at 80 mg/L, whereas influent NO_2^- -N was added according to NO_2^- -N/ NH_4^+ -N which ranged from 1.32 to 4.5. Effect resulting from influent NH_4^+ -N/ NO_2^- -N was carried out in R2. At this period, NO_2^- -N concentration in influent was stabilized at 105 mg/L, whereas NH_4^+ -N added in influent was based on NH_4^+ -N/ NO_2^- -N which ranged from 1/1.32 to 7.0. Still, the effect resulting from influent NO_3^- -N/ NH_4^+ -N was performed in R3. During this period, both NH_4^+ -N and NO_2^- -N concentration in influent were 80 and 105 mg/L, respectively, whereas the NO_3^- -N added in influent was based on NO_3^- -N/ NH_4^+ -N which ranged from 0 to 9.5. When influent NO_2^- -N/ NH_4^+ -N test was finished in R1, influent NO_2^- -N decreased to 105 mg/L (NO_2^- -N/ NH_4^+ -N = 1.32). Then NH_4^+ -N and NO_2^- -N increased simultaneously and effect resulting nitrogen loading shock was explored.

Kinetic analysis

Substrate inhibition model

To demonstrate nitrite inhibition on anammox performance quantitatively, Haldane (Eq. 1), Aiba (Eq. 2) and Edwards (Eq. 3) models were applied in this work to predict substrate inhibition on anammox process (Aiba et al. 1968; Edwards 1970; Jin et al. 2013). In addition, Luong, Han-Levenspiel and Tseng-Wayman

models were also used in present research. Akaike information criterion (AIC) and F-Value were employed to evaluate model appropriateness.

$$r = \frac{r_{\max} S}{K_S + S + S^2/K_{\text{IH}}} \quad (1)$$

$$r = \frac{r_{\max} S}{K_S + S} \exp\left(-\frac{S}{K_{\text{IA}}}\right) \quad (2)$$

$$r = r_{\max} \left(\exp\left(-\frac{S}{K_{\text{IE}}}\right) - \exp\left(-\frac{S}{K_S}\right) \right) \quad (3)$$

Luong (1987) proposed a substrate inhibition model (Eq. 4) which was applied in yeast growth. However, it was first used in anammox system in this work.

$$r = \frac{r_{\max} S}{K_S + S} \left(1 - \frac{S}{S_m}\right)^n \quad (4)$$

Han and Levenspiel (1988) introduced the model (Eq. 5) which was applied in the growth of a mixed culture with n-pentane. Though it was seldom to be used in anammox process, it fitted the experimental data well (Niu et al. 2016).

$$r = r_{\max} S \frac{1 - (S/S_m)^n}{S + K_S(1 - S/S_m)^m} \quad (5)$$

The Tseng-Wayman model (Eq. 6) was proposed by Tseng and Wayman (1975) and it was used to describe inhibitory effect resulting from gluconic acid and glucose on *Gluconobacter* quantitatively.

$$r = r_{\max} \left[\frac{S}{S + K_S} \right] - K_{\text{IT}}(S - S_{\text{crit}}) \quad (6)$$

where r is substrate removal rate [$\text{kg}/(\text{m}^3 \text{ d})$]; $r_{\max}$ is maximum substrate removal rate [$\text{kg}/(\text{m}^3 \text{ d})$]; S is substrate concentration (mg/L); S_m is substrate

concentration above which net growth ceases; S_{crit} is threshold substrate concentration; K_S is half-saturation coefficient (mg/L); K_{IH} is Haldane inhibition coefficient (mg/L); K_{IA} is Aiba inhibition coefficient (mg/L); K_{IE} is Edwards inhibition coefficient (mg/L); K_{IT} is Tseng-Wayman inhibition coefficient (mg/L); n and m are coefficients.

Modified non-competition inhibition model

IC_{50} (concentration causing a 50% inhibition) of non-competition inhibitor could be calculated using modified non-competitive inhibition model (Kroiss et al. 1992) (Eq. 7).

$$I(\%) = 100 \left(1 - \frac{1}{1 + (S/a)^b} \right) \quad (7)$$

where I (%) is inhibition ratio (%); S is inhibitor concentration (mg/L); a is IC_{50} of the inhibitor (mg/L); b is fitting parameter.

Analytical methods

Influent and effluent samples were collected and analyzed immediately. NH_4^+ -N, NO_2^- -N and NO_3^- -N concentrations were analyzed according to standard methods (APHA 1998). Temperature and pH were measured by WTW 340i/SET pH/Oxi portable multi parameter measuring meter. Sludge concentration was measured after filtering the washed samples through 0.45 μm membrane filter and drying at 105 °C to constant weight. Granular sludge size was calculated by image analysis using software Nano-Measurer 1.2. Each data point represented in present research was the mean value of three replicates, and standard deviation was lower than 10% of the mean value.

Results and discussion

Performance of anammox process with different influent substrate ratios.

Influent NO_2^- -N/ NH_4^+ -N

Nitrogen removal performance of R1 was presented in Fig. 2. With influent NO_2^- -N/ NH_4^+ -N no more than 2.0, total nitrogen removal rate (TNRR) increased

whereas ammonium removal rate (ARR) stabilized at 0.32 kg/(m³ d). When the ratio increased from 2.0 to 4.5, both TNRR and ARR decreased gradually. TNRR and ARR were 0.15 and 0.06 kg/(m³ d), respectively, when the ratio was 4.5. Anammox activity was inhibited at high NO_2^- -N/ NH_4^+ -N ratio. Tao et al. (2012) indicated that NO_2^- -N/ NH_4^+ -N higher than 1.5 might negatively inhibit anammox process. Simulated IC_{50} of nitrite through modified non-competition inhibition model was 289 mg/L in present research. It was higher than Strous et al. (1999) and Egli et al. (2001) who found nitrite inhibition threshold values were 100 and 185 mg/L, respectively. However, it was lower than Cho et al. (2010) and Fernandez et al. (2012) who indicated IC_{50} value were 400 and 350 mg/L. The anammox activity comparison resulting from nitrite effect was presented in Table 1. The discrepancy between present work and early studies might be attributed to different influent ammonium loading rate, operational duration time, reactor configuration and operational mode. In addition, different Anammox species also had different inhibition threshold. Therefore, the inhibition levels might vary depending on the dominant species.

In terms of stoichiometric ratio, ΔNO_2^- -N/ ΔNH_4^+ -N increased with enhancing NO_2^- -N/ NH_4^+ -N ratio. When NO_2^- -N/ NH_4^+ -N was 4.5, ΔNO_2^- -N/ ΔNH_4^+ -N was 1.98, which was much higher than theoretical value (1.32). It indicated that over-consumption of nitrite occurred when nitrite surplus existed during anammox process. This did not agree with previous report (Aktan et al. 2012). Different reactor type and operation mode might devote to the discrepancy. ΔNO_3^- -N/ ΔNH_4^+ -N also increased with enhancing NO_2^- -N/ NH_4^+ -N ratio. When NO_2^- -N/ NH_4^+ -N was 4.5, ΔNO_3^- -N/ ΔNH_4^+ -N was 0.68, which was much higher than theoretical value (0.26). That meant each mole ammonium removal was accompanied by more nitrate production at high NO_2^- -N/ NH_4^+ -N. The change tendency of ΔNO_2^- -N/ ΔNH_4^+ -N and ΔNO_3^- -N/ ΔNH_4^+ -N was closed to Lotti et al. (2012) who indicated that ΔNO_2^- -N/ ΔNH_4^+ -N and ΔNO_3^- -N/ ΔNH_4^+ -N increased to 2.97 and 0.75, respectively, with 3000 mg/L NO_2^- -N. In addition, the result also agreed with Strous et al. (1999) that ΔNO_2^- -N/ ΔNH_4^+ -N increased from 1.3 at 140 mg/L NO_2^- -N to 4.0 at 700 mg/L NO_2^- -N. van Hulle et al. (2010) hold the point that electron donor of AnAOB did not limited in NH_4^+ -N, and other internal electron

Fig. 2 Nitrogen removal performance with different influent $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$: **a** concentrations of three nitrogen species in influent and effluent; **b** nitrogen removal efficiency; **c** loading and removal rates of $\text{NH}_4^+ \text{-N}$ and $\text{NO}_2^- \text{-N}$; **d** stoichiometric ratio. (Color figure online)

donor might generate in cell to reduce nitrite at high nitrite concentration. The ammonification of organic nitrogen derived from cell lysis (Bettazzi et al. 2010) as well as survival of heterotrophic bacteria in anammox system (Wang et al. 2016) could also

devoted to increase of $\Delta \text{NO}_2^- \text{-N}/\Delta \text{NH}_4^+ \text{-N}$ and $\Delta \text{NO}_3^- \text{-N}/\Delta \text{NH}_4^+ \text{-N}$.

When influent $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$ decreased back to 1.32, anammox activity recovered with 8 cycles' operation (data not shown). This indicated that nitrite

Table 1 Comparison of anammox activity resulting from NO_2^- -N effect

Reactor	Operation mode	T (°C)/pH	NO_2^- -N/(mg/L)	Inhibition/%	References
Vials	Batch tests	30/7.8	350	– 50	Dapena-Mora et al. (2007)
Up-flow column	Continuous	(33 ± 1)/–	465	No inhibition	Yang et al. (2011)
Up-flow column	Continuous	(23 ± 2)/–	436	No inhibition	Yang et al. (2011)
UASB	Continuous	(35 ± 1)/6.8	280	Inhibition	Tang et al. (2010)
UBF	Continuous	(35 ± 1)/(6.8–7.0)	380	Inhibition	Tang et al. (2009)
Vessels	Batch	35/–	> 60	Inhibition	Bettazzi et al. (2010)
Monod test tubes	Batch	36/–	274	Inhibition	Kimura et al. (2010)
Cylindrical reactor	Continuous	30/–	750	– 90	Kimura et al. (2010)
ABF	Continuous	37/–	280	Inhibition	Isaka et al. (2007)
Serum bottles	Batch	37/7	185	– 100	Egli et al. (2001)
Vials	Batch	30/7.5	400	– 50	Lotti et al. (2012)
Serum flasks	Batch	(30 ± 2)/(7.4–7.5)	210	– 100	Carvajal-Arroyo et al. (2013)
SBR	–	–	100	– 100	Strous et al. (1999)
Serum flasks	Batch	(30 ± 2)/(7.1–7.2)	53/100 ^a	– 50/– 100	Carvajal-Arroyo et al. (2014a)
Serum flasks	Batch	(30 ± 2)/(7.1–7.2)	384/500	– 50/– 100	Carvajal-Arroyo et al. (2014a)
Serum vials	Batch	37/7.8	224	– 50	Oshiki et al. (2011)
UBF	Continuous	(30 ± 1)/–	420	No inhibition	Jin et al. (2013)
EGSB	Continuous	(35 ± 1)/(6.8–7.0)	768	– 24	Chen et al. (2011)
SBR	Batch	35/(7.6–7.7)	500	– 39 ^b	Scaglione et al. (2012)
SBR	Batch	35/(7.6–7.7)	< 175	– 50 ^c	Scaglione et al. (2012)
Serum flasks	Batch	(30 ± 2)/7.2	100	– 100 ^a	Carvajal-Arroyo et al. (2014b)
Serum flasks	Batch	(30 ± 2)/7.5	100	Mild inhibition	Carvajal-Arroyo et al. (2014b)
Serum flasks	Batch	35/7.9	> 300 ^d	Inhibition	Zhang et al. (2016)
Up-flow fixed-bed	Continuous	34/	500 ^e	No inhibition	Aktan et al. (2012)
Serum bottles	Batch	37/–	> 250 ^f	Inhibition	Cho et al. (2010)
Serum bottles	Batch	37/–	< 400 ^g	No inhibition	Cho et al. (2010)
SBR	Continuous	35/7.5	289	– 50	Present research

UASB upflow anaerobic sludge blanket reactor, UBF up-flow biofilm reactor, ABF anaerobic biological filtrated reactor, RBC rotating biological contactor, SBR sequencing batch reactor

^aAnAOB was pre-exposed to nitrite in absence of ammonium

^bShort exposure time was 3–4 h

^cExposure time was prolonged to 24 h

^dThe inhibitory threshold concentration

^eThe threshold concentration of NH_4^+ -N inhibition

^fThe granular anammox biomass

^gThe homogenized biomass

inhibition was reversible process, which was in agreement with previous reports (Carvajal-Arroyo et al. 2014b; Fernandez et al. 2012; Jin et al. 2013). However, some other scholars still thought that nitrite inhibition on anammox process was irreversible (Egli et al. 2001; Jetten et al. 2005). As reported by Lotti

et al. (2012), the difference was resulted from inhibition degree on AnAOB. Reversible inhibition meant a reversible decrease of catabolic activity in presence of nitrite, while irreversible inhibition referred to physical damage to cell. As a result, the physical characteristics of AnAOB in present research

did not changed and the activity could quickly restore with low $\text{NO}_2^- \text{-N}/\text{NH}_4^+ \text{-N}$.

With regard to the mechanism result from nitrite inhibition on anammox activity, there was no unified result. Philips et al. (2002) stated that formation of toxic nitrite derivative occurred in reactor. The reactive derivatives (e.g. NO , NO_2 and ONOO^-) were toxic to enzyme proteins, lipids and DNA (Mehl et al. 1999). Apart from byproducts, inorganic carbon limitation and function inhibition of cell organelle were also regarded as reasons for anammox activity loss (Lotti et al. 2012). In addition, many studies considered that free nitrous acid (FNA) was the key inhibitor (Egli et al. 2001; Jaroszynski et al. 2011). FNA could act as a protonophore at high concentration, which caused inhibition through interfering trans-membrane proton gradient in AnAOB (Carvajal-Arroyo et al. 2014b). Besides, it could pass through membrane via passive diffusion due to hydrophobic group (Almeida et al. 1995). However, recent study revealed that nitrite concentration regulated inhibition level and FNA was not responsible for inhibition (Lotti et al. 2012).

When nitrite was excessive in wastewater, strategies to alleviate inhibition were needed. Firstly, addition of anammox intermediates [e.g. hydrazine (N_2H_4) or hydroxylamine ($\text{NH}_2\text{-OH}$)] was proved to be effective method to restore anammox activity (Bettazzi et al. 2010; Carvajal-Arroyo et al. 2013). Secondly, sufficient inorganic carbon source in reaction system was another strategy (Yang et al. 2011). The addition of inorganic carbon source would accelerate removal rate of $\text{NH}_2\text{-OH}$ which led to accumulation of N_2H_4 . Thirdly, embedding AnAOB into gel carriers would enhance the stress resistance of it (Kimura et al. 2010). Besides, nitrite inhibition could be greatly relieved by bio-film (Cho et al. 2010). Fourthly, AnAOB could be reactivated by addition of exogenous $\text{NO}_3^- \text{-N}$ if it was severely stressed by $\text{NO}_2^- \text{-N}$ (Li et al. 2016). Exogenous $\text{NO}_3^- \text{-N}$ could promote a secondary transport system used by AnAOB to alleviate $\text{NO}_2^- \text{-N}$ inhibition. Fifthly, as reported by Cho et al. (2010), the granular anammox biomass would tolerate higher nitrite concentration (400 mg/L) than homogenized biomass (200 mg/L). In addition, the mildly alkaline pH value was beneficial for AnAOB to tolerate nitrite inhibition (Carvajal-Arroyo et al. 2014b). Cells were severely affected

when exposed to nitrite at acidic pH value, and it could tolerate nitrite at the pH range of 7.4–7.8.

Influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$

Nitrogen removal performance of R2 was presented in Fig. 3. With increasing $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ (from 1/1.32 to 5.0), nitrite removal rate (NRR) was stabilized at 0.41 kg/($\text{m}^3 \text{ d}$). $\text{NH}_4^+ \text{-N}$ was over-consumed in R2 when excessive $\text{NH}_4^+ \text{-N}$ was added. Previous studies reported that more $\text{NH}_4^+ \text{-N}$ would be converted to N_2H_4 when influent $\text{NH}_4^+ \text{-N}$ was excessive (Jin et al. 2013; Strous et al. 1999). The maximum ARR occurred at influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ of 4.0, peaking at 0.36 kg/($\text{m}^3 \text{ d}$). With $\text{NH}_4^+ \text{-N}$ added further, both ARR and NRR decreased. When the ratio approached to 7.0, they were 0.15 and 0.14 kg/($\text{m}^3 \text{ d}$), respectively. It demonstrated that anammox activity was inhibited when influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ was higher than 5.0.

Through the non-competition inhibition model, IC_{50} value of 786 mg/L was acquired. It was similar with the result reported by Dapena-Mora et al. (2007). The anammox activity comparison resulting from $\text{NH}_4^+ \text{-N}$ effect was presented in Table 2. The inhibition degree was different from previous studies. Different pH and temperature not only affected AnAOB activity directly, but also inhibited anammox process indirectly through changing FA concentration. Anammox system in this work was more sensitive to ammonium than previous reports (Aktan et al. 2012; Tang et al. 2010). Different reactor configurations could result in it. SBR was used in this work and H^+ was consumed during the process. As a result, pH would increase within a cycle and high pH inhibited anammox activity.

With regard to stoichiometric analysis, $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ slightly decreased with increasing $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$. When influent $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ was 7.0, $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ decreased to 0.92. As reported by Tsushima et al. (2007), AnAOB could convert some $\text{NH}_4^+ \text{-N}$ to $\text{NO}_2^- \text{-N}$, which devoted to dropping $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$. However, the result did not agree with Aktan et al. (2012) who indicated $\Delta\text{NO}_2^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$ did not change with increasing $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$ in an up-flow fixed-bed reactor. In terms of $\Delta\text{NO}_3^- \text{-N}/\Delta\text{NH}_4^+ \text{-N}$, it slightly increased with dropping $\text{NH}_4^+ \text{-N}/\text{NO}_2^- \text{-N}$. This was also consistent with previous report from Lotti et al. (2012) who found that

Fig. 3 Nitrogen removal performance with different influent $\text{NH}_4^+\text{-N}/\text{NO}_2^-\text{-N}$: **a** concentrations of three nitrogen species in influent and effluent; **b** nitrogen removal efficiency; **c** loading and removal rates of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$; **d** stoichiometric ratio. (Color figure online)

$\Delta\text{NO}_3^-\text{-N}/\Delta\text{NH}_4^+\text{-N}$ increased upon exposure to high $\text{NH}_4^+\text{-N}$ concentration. Decreasing influent $\text{NH}_4^+\text{-N}/\text{NO}_2^-\text{-N}$ back to 1/1.32, anammox activity completely recovered with 7 cycles' operation (data not shown). This indicated $\text{NH}_4^+\text{-N}$ inhibition on anammox was reversible process and high $\text{NH}_4^+\text{-N}$ content would

not damage the internal structure of AnAOB. It was also consistent with previous report that the inhibition was reversible though it was time-consuming (Zhang et al. 2016).

Many scholars had thought that the actual inhibitor to anammox was FA (Aktan et al. 2012; Dapena-Mora

Table 2 Comparison of anammox activity resulting from NH_4^+ -N effect

Reactor	Operation mode	T(°C)/pH	NH_4^+ -N/(mg/L)	Inhibition/%	References
SBR	–	–	1000	No inhibition	Strous et al. (1999)
Vials	Batch	30/7.8	770	– 50	Dapena-Mora et al. (2007)
UASB	Continuous	(35 ± 1)/6.8	1000	No inhibition	Tang et al. (2010)
Serum flasks	Batch	35/7.9	> 500 ^a	Inhibition	Zhang et al. (2016)
Serum flasks	Batch	(30 ± 2)/(7.4–7.5)	616	– 16 ^b	Carvajal-Arroyo et al. (2013)
Serum flasks	Batch	(30 ± 2)/(7.4–7.5)	616	– 34 ^c	Carvajal-Arroyo et al. (2013)
Up-flow fixed-bed	Continuous	34/	1500 ^d	No inhibition	Aktan et al. (2012)
SBR	Continuous	35/7.5	786	– 50	Present research

^aThe inhibitory threshold concentration^bThe granular anammox enrichment culture (GEC)^cThe suspended anammox enrichment culture (SEC)^dThe threshold concentration of NH_4^+ -N inhibition

et al. 2007; Fernandez et al. 2012). However, the threshold of FA concentration was variable. FA could penetrate through AnAOB cell membrane, which was toxic to anammox activity (Gallert and Winter 1997). In addition, FA could also neutralize membrane potential and resulted in cell death (Jaroszynski et al. 2012). Sludge concentration was also a key factor nitrogen removal performance. High biomass concentration in reactor could strengthen tolerance to NH_4^+ -N inhibition. In addition, controlling pH at a relatively low value could also beneficial for tolerance resulting from NH_4^+ -N inhibition. With high NH_4^+ -N/ NO_2^- -N, the excess of NH_4^+ -N and the deficiency of nitrite resulted in poor nitrogen removal performance. Partial nitrification/anammox process was much better than single anammox process. The excessive ammonium could be oxidized to nitrite at partial nitrification process. Not only NH_4^+ -N concentration decreased, but also NO_2^- -N was produced for further anammox process.

Influent NO_3^- -N/ NH_4^+ -N

Nitrate was the product of anammox process, and it was necessary to explore nitrate effect on anammox process. Nitrogen removal performance of R3 was presented in Fig. 4. When influent NO_3^- -N/ NH_4^+ -N was 0.5–1.5, nitrogen removal rates slightly increased. With influent NO_3^- -N/ NH_4^+ -N of 2.5–6.5, ARR and NRR were stabilized at 0.33 and 0.40 kg/(m³ d), respectively. Low influent NO_3^- -N/ NH_4^+ -N did not

inhibit anammox process. However, when influent NO_3^- -N/ NH_4^+ -N was 9.5, ARR and NRR decreased to 0.12 and 0.14 kg/(m³ d), respectively. Influent NO_3^- -N/ NH_4^+ -N higher than 6.5 would adversely affect nitrogen removal performance.

IC₅₀ of 685 mg/L was achieved through the non-competition inhibition model. The result was in agreement with Dapena-Mora et al. (2007) who found IC₅₀ of NO_3^- -N inhibition was 630 mg/L. The anammox activity comparison resulting from NO_3^- -N inhibition was presented in Table 3. Although the results were not consistent due to different operation condition, they all suggested that AnAOB had high tolerance to NO_3^- -N than NO_2^- -N. Decreasing influent NO_3^- -N/ NH_4^+ -N back to 0, anammox activity could completely recover with 20 cycles' operation (data not shown). The recovery period was much longer than those of NO_2^- -N and NH_4^+ -N inhibition. The reversibility of nitrogen removal performance indicated that AnAOB structure was not damaged.

With regard to stoichiometric ratio, ΔNO_3^- -N/ ΔNH_4^+ -N kept stable throughout the whole process. However, ΔNO_2^- -N/ ΔNH_4^+ -N slightly decreased at high NO_3^- -N/ NH_4^+ -N. As reported by Kartal et al. (2007), the produced NO_3^- -N could be transformed into NO_2^- -N by AnAOB since they had the ability to reduce NO_3^- -N to NO_2^- -N. The osmotic pressure variation resulting from high salt concentration (KNO₃) might weaken nitrogen removal performance (Li et al. 2016). In view of low nitrogen removal at high influent NO_3^- -N/ NH_4^+ -N, single anammox

Fig. 4 Nitrogen removal performance with different influent $\text{NO}_3^-/\text{NH}_4^+$ -N: **a** concentrations of three nitrogen species in influent and effluent; **b** nitrogen removal efficiency; **c** loading and removal rates of NH_4^+ -N and NO_2^- -N; **d** stoichiometric ratio. (Color figure online)

Table 3 Comparison of anammox activity resulting from NO_3^- -N effect

Reactor	Operation mode	T(°C)/pH	NO_3^- -N/(mg/L)	Inhibition/%	References
SBR	–	–	1000	No inhibition	Strous et al. (1999)
Serum flasks	Batch	$(30 \pm 2)/(7.4\text{--}7.5)$	77–287	– (40–50) ^a	Carvajal-Arroyo et al. (2013)
Serum flasks	Batch	$(30 \pm 2)/(7.4\text{--}7.5)$	77–287	– (15–20) ^b	Carvajal-Arroyo et al. (2013)
Serum bottles	Batch	(30 ± 2) –	99.4	– 25	Li et al. (2016)
Vials	Batch	30/7.8	630	– 50	Dapena-Mora et al. (2007)
SBR	Continuous	35/7.5	685	– 50	Present research

^aThe granular anammox enrichment culture (GEC)^bThe suspended anammox enrichment culture (SEC)

technology was not suitable to treat the wastewater. Simultaneous denitrification/anammox process could be a better choice.

Nitrogen removal performance with nitrogen loading shock

To evaluate the nitrogen removal performance with substrate loading rate, influent NO_2^- -N and NH_4^+ -N were simultaneously added with the ratio of 1.32. The results were presented in Fig. 5. Both NRR and ARR increased when influent NO_2^- -N and NH_4^+ -N were not more than 145 and 110 mg/L, respectively. TNRR arrived at 0.9 kg/(m³ d). With NO_2^- -N and NH_4^+ -N were added further, both NRR and ARR decreased. However, they recovered with 10 cycles' operation. When influent NH_4^+ -N was 170 mg/L, ARR was about 0.57 kg/(m³ d) which was 63% higher than that at 85 mg/L NH_4^+ -N. AnAOB could gradually adapt to high nitrogen loading rate.

Anammox performance was unstable at high nitrogen loading rate, and sludge floatation occurred. Similar result was also observed by previous studies (Chen et al. 2010; Strous et al. 1997). The sludge floatation not only decreased sludge retention time but also damaged the system stability. It became an important problem when nitrogen loading rate was enhanced. The granular floatation caused washout of AnAOB and deteriorate anammox process. However, stoichiometric ratios were kept stable throughout the whole operation. Anammox process still dominated the reactor. Decreasing the loading rate back to initial state, seven cycles were needed to alleviate sludge floating.

Sludge size was also analyzed and the result was presented in Fig. 6. The diameter of sludge (sampled on 5th cycle) was 0–4.5 mm, and the mean diameter was 1.58 mm. However, the diameter of sludge (sampled on 35th cycle) was larger and the mean diameter was 2.25 mm. The larger granular sludge formed at high nitrogen loading rate was easier to float. Chen et al. (2010) indicated that the entrapped gas bubble was real reason for granule floatation. Mechanical breakage of granular sludge might be an effective method to solve sludge floatation (Chen et al. 2010; Yoda and Nishimura, 1997). The floating sludge in this work occurred at loading rate of 1.45 ± 0.02 kg/(m³ d) which was much lower than Chen et al. (2010). This might be attributed to low inorganic carbon source (KHCO_3) and shortage of other nutrients.

Kinetic analysis

The inhibitory threshold concentration of NO_2^- -N was lower than NH_4^+ -N and NO_3^- -N. AnAOB was more sensitive to NO_2^- -N inhibition than NH_4^+ -N and NO_3^- -N. In order to describe and predict anammox performance, NO_2^- -N inhibition characteristics were analyzed by kinetic models. The experimental data and simulated results were presented in Fig. 7 and the parameters predicted by different models were listed in Table 4. In terms of R^2 , Han-Levenspiel model, Luong model and Tseng-Wayman model could effectively simulated NO_2^- -N inhibition on anammox process. Although the Haldane model, Aiba model and Edwards model were widely used in substrate inhibition (Chen et al. 2011; Jin et al. 2013; De Prá et al. 2016), they were not suitable to use in this work. The

Fig. 5 Nitrogen removal performance with different substrate loading rate: **a** concentrations of three nitrogen species in influent and effluent; **b** nitrogen removal efficiency; **c** loading and removal rates of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$; **d** stoichiometric ratio. (Color figure online)

low R^2 values of Haldane model and Aiba model also occurred in previous report (Niu et al. 2016). With regard to Tseng-Wayman model, the predicted $r_{\max}$ was only 0.16. It was not consistent with the

experimental data in present research. As a result, the Han-Levenspiel model and Luong model were more suitable to simulate nitrite inhibition than others.

Fig. 6 Sludge particle size distribution: **a** sludge sampled on 5th cycle; **b** sludge sampled on 35th cycle

Fig. 7 Kinetic simulation by different models. (Color figure online)

AIC was an effective measurement to evaluate model appropriateness (Akaike 1974). It estimated the quality of each model by relative value, and the lowest AIC value suggested the most appropriate model. The AIC also dealt with the trade-off between appropriateness of model and complexity of model (assessed by parameter number) (De Prá et al. 2016). The analysis of AIC for different models used in nitrite inhibition was presented in Fig. 8. The AIC values of Han-Levenspiel model and Luong model were -50 and -46 , respectively. This indicated that the Han-Levenspiel model was more accurate than the Luong model. In addition, the F-Value was about 700, which suggested appropriateness in describing nitrogen removal performance. Based on the analyses above, the Han-Levenspiel model was the best one to describe the nitrite inhibition on anammox process.

Table 4 Simulated parameters acquired through different kinetic models

Models	Models parameters						
	$r_{\max}$ [kg/(m ³ d)]	K_S (mg/L)	K_I (S_m) (mg/L)	n	m	R^2	Equations
Aiba	0.43	0.08	384.89	–	–	0.8714	$y = 0.43x/(0.08 + x) \times \exp(-x/384.89)$
Edwards	0.42	0.14	406.82	–	–	0.8421	$y = 0.42 [\exp(-x/406.82) - \exp(-x/0.14)]$
Haldane	0.44	0.08	287.77	–	–	0.7903	$y = 0.44x/(x + 0.08 + x^2/287.77)$
Han-Levenspiel	0.39	0.02	462.35	1.22	0.97	0.9647	$y = 0.39x [1 - (x/462.35)^{1.22}]/[x + 0.06(1 - x/462.35)^{0.97}]$
Luong	0.41	0.06	387.89	0.53	–	0.9468	$y = 0.41x/(x + 0.06) \times (1 - x/387.89)^{0.53}$
Tseng-Wayman	0.16	23.37	333.51	–	0.01	0.9585	$y = 0.16 [x/(23.37 + x)] - 0.01(x - 333.51)$

Fig. 8 AIC analysis of different kinetic models

Concerning the parameters achieved from Han-Levenspiel model, the $r_{\max}$ value was $0.39 \text{ kg}/(\text{m}^3 \text{ d})$, which was lower than previous reports (Jin et al. 2013; Niu et al. 2016). The discrepancy between the results could be attributed to low sludge concentration in this work. The half saturation constant (K_s) could represent bacteria affinity for NO_2^- -N, and it was only 0.02 mg/L . This agreed with Strous et al. (1999) who found the affinity constant for NO_2^- -N was less than 0.1 mg/L . The predicted S_m was 462 mg/L and it suggested the endurance capacity of NO_2^- -N in present research. The higher value of S_m meant the stronger endurance capacity of NO_2^- -N. It indicated that anammox reaction did not occur when NO_2^- -N concentration was higher than 462 mg/L in this work.

Conclusions

SBRs were employed to study the effect of influent substrate ratio on anammox process. With influent NO_2^- -N/ NH_4^+ -N no more than 2.0, TNRR increased whereas ARR stabilized at $0.32 \text{ kg}/(\text{m}^3 \text{ d})$. Anammox activity was inhibited at high NO_2^- -N/ NH_4^+ -N. When NO_2^- -N/ NH_4^+ -N was 4.5, ΔNO_2^- -N/ ΔNH_4^+ -N and ΔNO_3^- -N/ ΔNH_4^+ -N were 1.98 and 0.68, respectively. Both were higher than theoretical value. When influent NH_4^+ -N/ NO_2^- -N was more than 5.0, anammox activity was inhibited. ΔNO_2^- -N/ ΔNH_4^+ -N slightly decreased with increasing NH_4^+ -N/ NO_2^- -N. With influent NO_3^- -N/ NH_4^+ -N of 2.5–6.5, ARR and NRR were stabilized at 0.33 and $0.40 \text{ kg}/(\text{m}^3 \text{ d})$, respectively. Nitrogen removal was worsened when

influent NO_3^- -N/ NH_4^+ -N was higher than 6.5. AnAOB could gradually adapt to high nitrogen loading rate. The inhibitory threshold concentration of NO_2^- -N was lower than NH_4^+ -N and NO_3^- -N. AnAOB was more sensitive to NO_2^- -N than NH_4^+ -N and NO_3^- -N. The Han-Levenspiel and Luong model could be applied to simulate NO_2^- -N inhibition, and the Han-Levenspiel model was better. The predicted S_m was 462 mg/L , which indicated that anammox reaction did not occur when NO_2^- -N concentration was higher than 462 mg/L .

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