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Effects of HRT and nitrite/ammonia ratio on anammox discovered in a sequencing batch biofilm reactor

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Abstract

There are three key aspects of substrate effect on anaerobic ammonia oxidizing (anammox) bacteria:(1) substrate concentration - based nitrogen loading rate (NLR), (2) hydraulic retention time (HRT)-based NLR and (3) Nitrite/ammonia ratio. The first part has been fully investigated in the past while the latter two are still lack of deep understanding. In this study, two types of substrate effect (HRT-based NLR and nitrite/ammonia ratio) were experimentally proved based on a 226-day operation of a sequencing batch biofilm reactor (SBBR) that was dominated by anammox bacteria. A modified first-order substrate removal kinetic model was developed, which fit well to the experimental results. Decreasing HRTs from 72h to 6h were applied to the SBBR and the HRT=6h was proven to be optimal, when the highest nitrogen removal rate (NRR) occurred (1.62kg-N•m^{-3}•d^{-1} and the total nitrogen removal efficiency>90%). In addition, the influent nitrite/ammonia ratio of 1.2 benefitted a stable and effective operation of anammox SBBR with an improved ammonia removal efficiency (by 17%) and an enhanced NRR (from 0.93 kg-N•m^{-3}•d^{-1} to 1.14 kg-N•m^{-3}•d^{-1}).

Key words: Anammox; HRT; nitrite /ammonia ratio; biofilm; substrate removal kinetic

Introduction

Anaerobic ammonium oxidation (anammox) is an efficient and environmentally benign process for nitrogen-rich wastewater treatment, such as landfill leachate, rejects water, sludge digester liquids and dry-spun acrylic fiber wastewater [1-3]. Anammox bacteria are able to utilize nitrite (NO_2^-) as alternative terminal electron acceptors along with ammonia (NH_4^+) being oxidized into nitrogen (N_2), which is principally different to conventional denitrification that employs nitrate (NO_3^-) as electron acceptors [4]. Compared to conventional nitrification-denitrification technologies, anammox process saves a huge amount of energy consumption from less use of aeration, carbon source and alkali, and reduces production of excess sludge [5, 6].

Anammox bacteria are strictly anaerobic chemolithoautotrophs with extremely low growth rate and hence they are difficult to be enriched. An effective reactor configuration can play a critical role to solve this difficulty. Previous work based on the batch or pilot-scale reactors have proven that biofilm-based bioreactors are ecologically feasible and beneficial to slow growing anammox bacteria [7-9]. Granular biomass reactors can work successfully on anammox under a certain range of hydraulic retention times (HRTs), but the possibility of granules being washed out could be high if HRT was lower than 3 hours [10]. Carrier-based biofilm reactors have higher sludge retention capacity and can run under short HRTs without negative influence of biomass washout [10, 11]. Those properties are beneficial to culture anammox biomass because the biofilms growing on a substratum can provide anammox bacteria with fine anaerobic micro-environments, where aerobic bacteria more likely grow on the outer layer as a barrier to oxygen and inhibitory substances [12-16]. Recently, sequencing batch biofilm reactors (SBBRs) that contain PVC mesh medium have been proven of high surface area and so regarded as an efficient design for enriching anammox bacteria [17-19].

HRT, influent nitrite/ammonia ratio and other factors are important factors ruling substrate effect and thus critical to anammox process [20-24]. A practical purpose when applying anammox is to pursue a shorter HRT for higher nitrogen loading rate (NLR), which is, for most cases, a sole way to enhance NLR. Although increasing nitrite concentrations can also bring higher NLR, for practical considerations, nitrite concentrations are always required to be within safe ranges in case of inhibition effect [25, 26].

The purpose of this study is to comprehensively evaluate substrate effect on anammox bacteria, i.e. HRT and nitrite/ammonia ratio, in a SBBR reactor. A substrate removal kinetic modeling were built to investigate the effect of nitrite/ammonia ratio on NRR, to find the optimal HRT and nitrite/ammonia ratio, and eventually to suggest a doable way to keep a stable and efficient anammox process.

Materials and methods

Reactor setup and operation

The SBBR had a total exchange volume of one liter. Temperature was kept 35±1°C by a water jacket. A magnetic stirrer was equipped at the bottom of the reactor (Figure 1). The HRT was gradually shortened from 3 days to 6 h. The synthetic wastewater (stored in a dark and cool container and pH kept around 7.0by adding $KHCO_3$) was batch fed into the reactor after periodically sparging nitrogen gas (10 minutes sparging before feeding), in order to minimize the growth potential of aerobic microorganisms in SBBR. The reactor was operated sequentially in cycles and each cycle contained feeding (10 min), settling (20 min), discharging (10 min) and mixing (for the time left). Different carriers (ring-style and sheet-style) were placed inside the

SBBR with a packing rate of about 40%. The ring carriers are mainly made by high-density polyethylene (Dalian Yu Du Environmental Engineering Technology Co., Ltd，China) with a diameter of 10 mm, a specific surface area of 3 m^2/g and a specificy density of 965-968 kg/m^3. Besides of the ring carriers, some sheet-style carriers (diameter of 3 cm and thickness of 1 mm) were placed in the top, middle and bottom part of SBBR for close observation of attachment. The reactor was covered by an opaque cloth to avoid the growth of algae and photosynthetic bacteria.

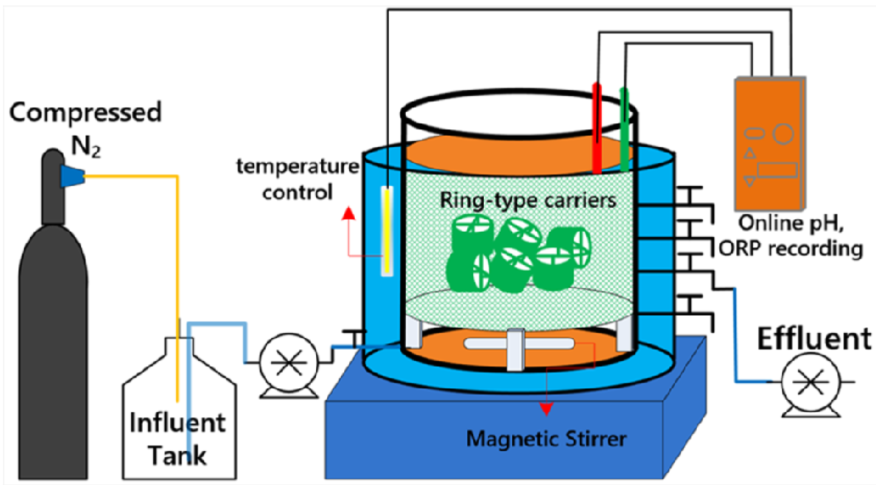

Figure 1 Experimental setup of the SBBR

Inoculating sludge and wastewater

The SBBR was inoculated by two sources of seeding sludge (in total 6.25g VSS): (1) a bench-scale sequencing batch reactor (SBR) treating synthetic ammonia-rich wastewater under ambient temperature (5g VSS biomass) [27]. (2) a pilot-scale (17 m^3) anammox reactor treating synthetic ammonia-rich wastewater (1.25g VSS biomass). The SBBR used in this study was fed with synthetic medium (Table1) with addition of 1.25mL/L trace elements [4, 5, 28].

Table 1 The composition of the synthetic wastewater

Nutrient medium	Unit（mg/L）	Trace elements	Unit（mg/L）
NH_4Cl	134-749	$ZnSO_4 \cdot 7H2O$	430
$NaNO_2$	173-1160	$CuSO_4 \cdot 5H2O$	250
$KHCO_3$	500	$MnCl_2 \cdot 4H_2O$	990
KH_2PO_4	10	$NiCl_2 \cdot 6H_2O$,	190
$MgSO_4 \cdot 7H_2O$	60	$CoCl_2 \cdot 6H_2O$	240
$CaCl_2 \cdot 2H_2O$	5	H_3BO_4	14
$FeSO_4$	6.25	$NaSeO_4 \cdot 10H_2O$	210
EDTA	6.25	$NaMoO_4 \cdot 10H_2O$	220

Analysis

Measurement of ammonia, nitrite, nitrate were done according to standard methods[29]. Briefly, ammonia was determined with the Nessler spectrophotometric method. Nitrite was measured using the N-(1-naphthyl)-ethylenediamine spectrophotometry. Nitrate was analyzed with the nitrate electrode. DO, pH and temperatures were measured by a WTW (pH/Oxi 340i, Germany) portable multi-parameter test set. Total nitrogen was analyzed by a TN analyzer (TOC-VCPN-6000, Shimadzu, Japan) [30].

Fluorescence in situ hybridization analysis (FISH) and scanning electron microscope (SEM)

During the days around 102, the SBBR entered the stable stage, which was characterized that anammox populations became dominant and the reactor performance (in nitrogen removal rate) was stable as well. Under such period, a mature anammox community in the SBBR can be characterized by FISH and SEM. Fresh biofilms were collected and fixed in paraformaldehyde and stored in 98% ethanol under -25°C for further FISH test. The probe Amx 820 that is specific for

Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis*) was purchased from TaKaRa, Dalian, China and was labeled with Cy3[31]. The hybridizations with fluorescent probes were performed according to a previous protocol [27].The samples were counterstained by DAPI. A confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (488 nm) and He-Ne laser (543 nm) was used for observation.

The biofilm samples for SEM test were firstly washed with a phosphate buffer and fixed with 2% glutaraldehyde overnight at 4°C, followed by a series of processes including successive dehydration, drying and gold coating according to previous method [27]. A Hitachi S-4700 (Japan) scanning electron microscope was used to capture micrographs.

First-order substrate removal model

Following the online recording data, we compared and screened the fitting results of various models, and then established a first-order substrate removal model to simulate the SBBR performance, which was simple and capable of properly matching the observations. Within the first-order substrate removal model, the change rate of substrate concentration in a complete mixed system can be expressed as [32, 33]:

$$-\frac{ds}{dt} = \frac{QSi}{\mathrm{V}} - \frac{QSe}{\mathrm{V}} - kSe \qquad (1)$$

Some assumptions of the SBBR system are: (1) it keeps a pseudo-steady-state condition, (2) the influent filling is instantaneous, and (3) there is no diffusion limitation within the biofilms [34]. Since the change rate (*-ds/dt*) was negligible, the equation can be transitioned as:

$$\frac{\mathrm{QSi}}{\mathrm{V}} - \frac{QSe}{\mathrm{V}} = \mathrm{k}Se \qquad (2)$$

Further described as:

$$\frac{\mathrm{Si} - \mathrm{Se}}{\mathrm{HRT}} = \mathrm{k}Se \qquad (3)$$

Where Q and V are the inflow rate (L/h) and the reactor volume (L), *Si*and *Se* are influent and effluent substrate (ammonia and nitrite) concentrations (mg/L), *k* is the first-order substrate removal rate constant (1/h), HRT is the hydraulic retention time (h).

The HRT can be considered as the reaction time (*t*) for each batch. To solve the equation closer to the actual situation of the reactor, the first-order substrate removal constant *b* was used to modify in the equation and so the equation can be derived as:

$$\frac{Si - Se}{t} = kSe + b \qquad (4)$$

Then *b* is the first-order substrate removal constant.

Results and discussion

Observation of anammox bacteria

A mature anammox community was observed after about 100 days and during such period the reactor performance was stable as well. Clear and large area of red fluorescence that was corresponding to anammox bacteria was observed by CLSM (Figure 2A-C), indicating high abundance of anammox bacteria existing in the biofilms. The SEM proves that the heterogeneous surface of the carriers (Figure 2D) helped to harbor biofilms and the round shape anammox bacterial cells (Figure 2E) can be clearly seen in the biofilms. All these proofs indicate a suitable period to do the online monitor and build the first-order substrate removal model and eventually to evaluate and predict the SBBR performance.

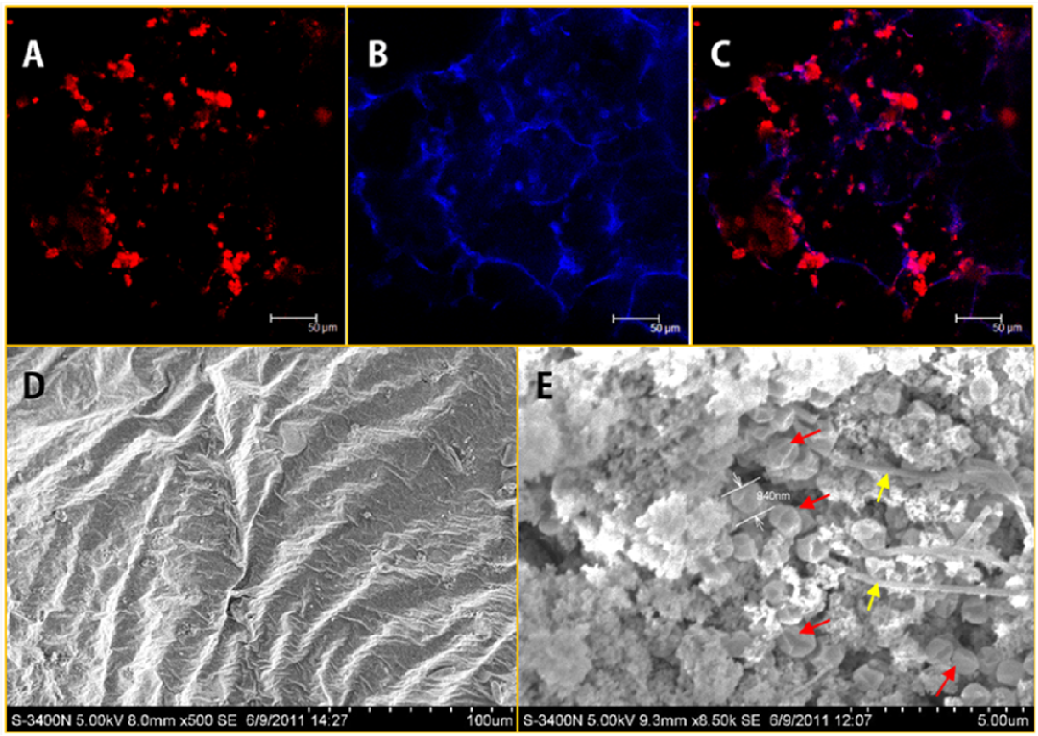

Figure 2 Molecular and microscopic evidences of anammox bacterial cells in the SBBR. A, Fluorescence in situ hybridization (FISH) micrograph of Cy3-labeled Amx820 (targeting two anammox bacterial species *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis*). B, FISH micrograph of DAPI stained sample (targeting total bacteria). C, FISH micrograph of Cy3-labeled Amx820 (targeting anammox bacteria) and conterstained with DAPI. The dominancy of anammox bacterial community can be seen in this figure based on the percentage of the red fluorecence among the blue one. D, Scanning electron microscopy (SEM) of the surface of a virgin carrier; E, SEM of a mature biofilm growing on the surface of a carrier. Heterogeneous surface of the carriers (Figure 2D) help to harbour biofilms (Figure 2E) and the round shape anammox bacterial cells (pointed by red arrows) can be clearly seen in the biofilms.

Kinetics of ammonia and nitrite removal

Selecting a suitable HRT is a key to successful culturing of anammox bacteria. The reactor was tested under different substrate concentrations in order to obtain the optimal HRT and data set for modelling. HRT was decreased stepwise from 3 days to 6 hours. During such process, the reactor went through three stages: period of instability (stage I), transition period (stage II), and robust+stable period (stage III)[27]. The reactor was in a very stable period during HRT of 12 hours and so the substrate concentration was online monitored by a real-time recording mode (Figure 3A, 3B).

Low concentrations (80mg/L) of nitrite and ammonia were initially fed to the reactor. The concentration of ammonia and nitrite decreased to 23mg/L and 0mg/L respectively after 6h, there was no enough nitrite was supplied to anammox in the next 6hour, so initial medium concentration was increased to 140 mg/L (ammonia and nitrite each). The ammonia concentration decreased to 31.8mg/L and nitrite to 8.2mg/L for this time.

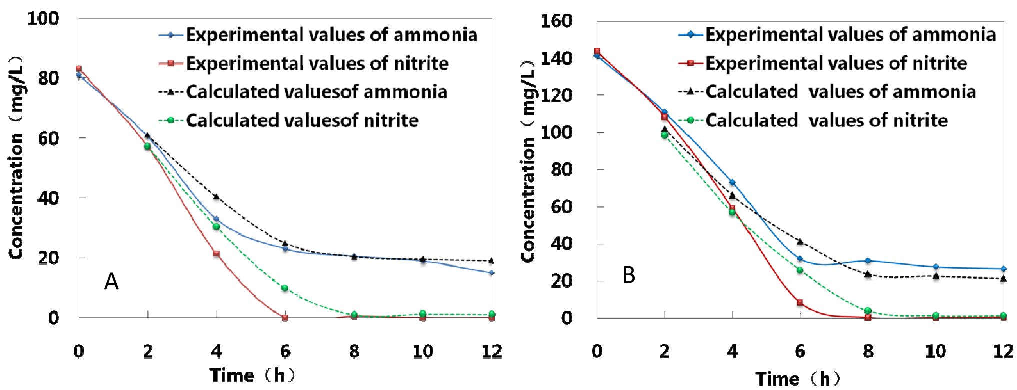

Figure 3 The variation of different substrate concentrations at HRT 12 h (A:80mg/L; B:140mg/L)

The reactor performed in an effective and stable mode on about 100 days after start-up. During this period (HRT 12h), the initial substrate concentration was 70mg/L. In order to clearly express the relationship of removed substrate, the dynamic equation of substrate removal was derived as linear equation. The constituted model fit well to the experimental values under both initial ammonia concentrations of 80mg/L and 140mg/L (the experimental values and calculated values were listed in Figure 3A, 3B), with the r^2 values being 0.962 and 0.965, respectively (Figure 4A, 4B). The model also expressed a fine predictability on nitrite concentration with r^2 of 0.934 and 0.955 (Figure 4C, 4D). The above information demonstrates that the established first-order substrate removal model was suitable to characterize the kinetics for ammonia and nitrite depletion in the anammox SBBR, which can also be applicable to other types of reactors according to previous studies[32, 33].

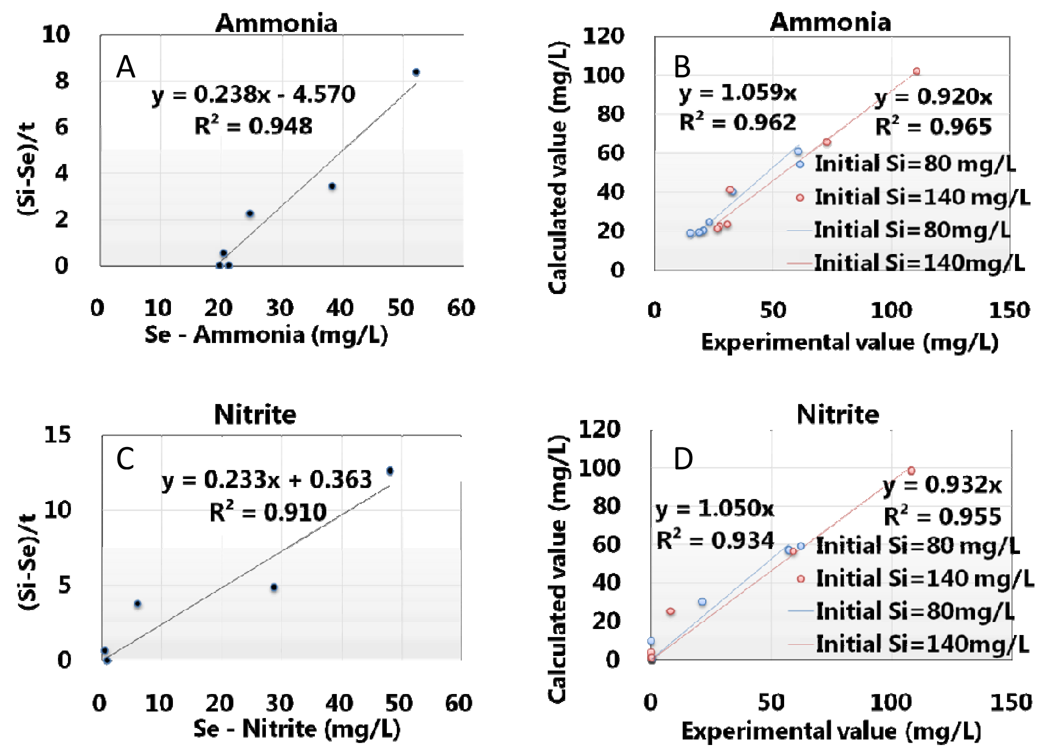

Figure 4 Kinetic characteristic and correlation coefficient. A, kinetic model of ammonia removal; B, correlation coefficient between calculated values and experimental values under different ammonia concentrations (80mg/Land 140mg/L); C, kinetic model of nitrite removal; D, correlation coefficient between calculated values and experimental values under different nitrite concentrations (80mg/L and 140mg/L)

Results showed that the nitrite was 0mg/L and 8.2mg/L after 6h for the groups of initial nitrite of 80mg/L and 140mg/L, respectively (Figure 3). Calculated values of substrate also had similar results (Figure 3). The remaining ammonia and nitrite were not enough anymore to support the growth of anammox bacteria in the following six hours (considering the HRT of 12 h). Consequently, it is necessary to shorten the HRT to 6 h to save half of the time and get a higher nitrogen load. It indicates that the SBBR reactor had an excellent nitrogen removal capacity as well. In addition, it is generally accepted by others that a lower concentration of the substrate mode is superior to the high one under the same HRT conditions [23]. Based on the substrate concentration model, suitable HRT for the reactor and the limitation of substrate to the anammox was also identified as the crucial factors in recent studies. In practice, this model was significant to predict the treatment plant performance and optimize the plant design[33, 35].

Effect of HRT

Anammox was enriched under different HRTs. The initial HRT was 72 h and then shortened step by step from 72 h to 48 h, 24 h, 12 h and 6h. The initial substrate concentration was 70mg/L, the removal efficiency of ammonia and nitrite were be closely observed, in order to promptly increase or decrease the concentration of the substrate. After reactor was start-up 100 days, the reactor stayed in an effective and stable period (HRT was 12h). HRT was mainly discussed in this period. When the HRT decreased from 12 h to 6 h, the ammonia removal efficiency and nitrogen loading rate were both improved. The SBBR reactor performed about 30 days under HRT 12 h, during which period the ammonia and nitrite concentration was increased in stepwise (70mg/L, 84mg/L, 112mg/L, 140mg/L). The ammonia removal efficiency was 77%.When the HRT was set at 6 h; the substrate concentration was elevated from 140 mg/L to 196 mg/L. Accordingly, the ammonia removal efficiency reached to 92% (Figure 5) and the TN removal efficiency increased from 78.6% to 87.1%. The SBBR performed as stable as previously without negative impact. The nitrogen loading rate was increased by four times from 0.28kg-N/m^{3}d^{-1} to1.18kg-N/m^{3}d^{-1} at HRT 6 h (Figure 5). Shortening HRT was an indirect but effective way to improve the anammox efficiency to meet a high nitrogen loading rate, while the increased substrate concentration may stimulate anammox bacteria growth, yielding sufficient biomass to support the increasing loading rate [20]. It is important to note that the medium concentration of nitrite should be carefully controlled since high nitrite (e.g. >210mg/L (15mM)) may result in inhibition to anammox cells [36-38].

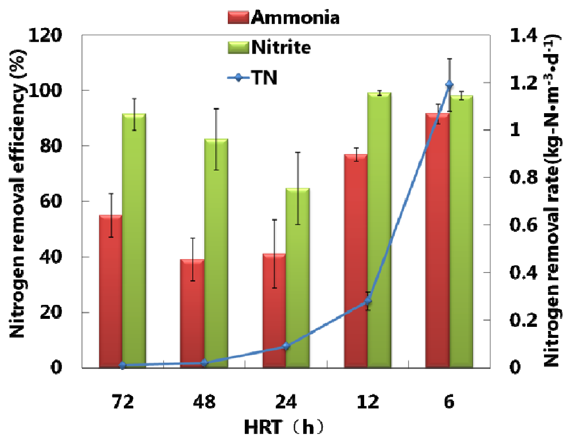

Figure 5 Nitrogen transformation at different HRTs (Left indicates nitrogen removal efficiency(%) and right indicates TN removal rate (kg-N/m^{3}d^{-1})).

The stoichiometry ratios of nitrite/ammonia and nitrate/ammonia are key factors to evaluate the health of an anammox process[39]. The corresponding stoichiometric values 1.32 (nitrite/ammonia) and 0.26 (nitrate/ammonia) have been widely proven and accepted as an indicator to a typical anammox process [6]. In this study, when HRT was decreased from 12 h to 6 h, nitrite/ammonia and nitrate/ammonia ratio reached to 1.26 and 0.26 (Figure 6), respectively, which are close to the theoretical values. However, when the HRT was longer than 12h (period of instability), high accumulated nitrate was observed, which may be result from a strong nitrification or a weak denitrification activity[27]. AOB and NOB were very likely inactive then due to strict control of medium DO and the washout of some loosely attached AOB/NOB from the out layer of biofilm[40]. The real-time experimental results also showed that the linear fitting nitrite/ammonia ratio was 1.25 with the value R^2 of 0.996. According to previous studies, the ratio observed in an upflow biofilter was 1.0±0.171 and 0.2±0.105. The value found in an anammox upflow column reactor was 1.03–1.17[39, 41]. The stoichiometric data strongly indicated a typical anammox process in the SBBR, which was in accordance to the previous molecular biological results that anammox bacteria were dominant with a relative abundance of about 32% [27].

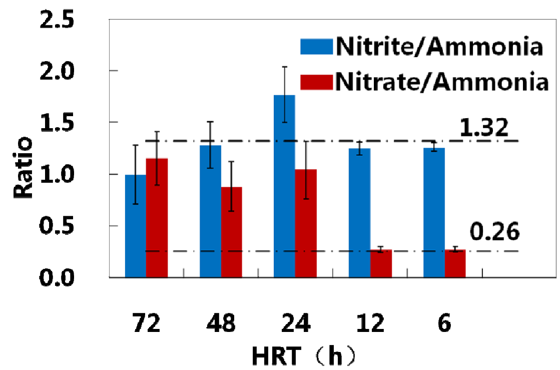

Figure 6 Stoichiometric ratio of nitrite/ammonia and nitrate/ammonia ratio at different HRTs

An instinct advantage of biofilm-based reactors (such as the SBBR in this study) is to maintain a fine-tuned and self-adapted micro-environment, which can benefit both fast growing microorganisms (such as aerobic ones) and slow growers (such as anaerobic ones). In this study, the carrier substratum provided with fine conditions for anammox bacteria to grow and the out-layer biomass played an important role as barriers to oxygen and inhibitory substances, the SBBR reactor used in this study exposed to open air during the entire operation (Figure 1). However, continuous penetration of oxygen did not strongly affect anammox process, nether no inhibition to anammox bacteria. On the contrary, anammox bacteria became dominant after three months. Compare to other reactor configurations such as suspended sludge or granular sludge, SBBR is cost-saving in building and power-saving during practical use as well.

Effect of influent nitrite/ammonia ratio

The effect of influent nitrite/ammonia ratio was investigated under controlled substrate concentrations. Considering the fact that a high concentration of nitrite (e.g.>15mM) may inhibit anammox bacteria and lead to incomplete conversion [42, 43], the SBBR reactor was first fed with nitrite/ammonia ratio of 1:1 (10mM/10mM). The HRT was fixed at 6h. The real-time online results showed that there was not sufficient nitrite to support the growth of anammox after 6 h. Then the ratio was increased to 1.1:1 (11mM/10mM), with the average ammonia removal efficiency increased by 4%. A further increase in nitrite/ammonia ratio to 1.2:1 (12mM/10mM) led to increased ammonia removal efficiency by 17% (Table 2). It is notable that the NRR was improved from 0.93 to 1.14kg-N/m^3d^{-1} during this period under fixed concentration of ammonia but increasing nitrite concentration, meanwhile, the nitrite in effluent was continuously lower than 1mM. The reactor performance was not inhibited by the high nitrite concentration and it is probably attributed to the advantageous biofilm architectures of SBBR carriers [44].

Table 2 -Nitrogen removal efficiencies at different influent ratios of nitrite/ammonia

Nitrite/ammonia ratio	**1**	**1.1**	**1.2**
Ammonia removal（%）	78.9±3.3	88.4±1.8	97.0±2.9
Nitrite removal（%）	99.8±0.4	100.0±0.0	98.6±1.9
TN removal（%）	80.2±1.7	86.6±4.1	89.8±1.9
NRR (kg-N/m^3d^{-1})	0.93±0.04	1.01±0.08	1.14±0.04

It is worthwhile to mention that an even higher mole ratio of nitrite/ammonia (e.g. > 1.5:1) may negatively influence the anammox process. Because it will lead to a higher residual of nitrite, which may promote the growth of NOB and denitrifying bacteria, who can strongly compete with anammox bacteria [45].Previous researchers found that when the influent ratio of nitrite/ammonia increased from 1.5:1 to 1.8:1, the anammox process was severely affected, and most studies conclude that an optimal ratio level should be around 1.2:1[39].

Conclusions

The study demonstrates the co-existence of aerobic bacteria and anammox bacteria was found in the SBBR and anammox bacteria became dominant after three months. The performance of the reactor was also very satisfactory. Compare to other reactor configurations such as suspended sludge or granular sludge, SBBR is cost-saving in building and power-saving.

The HRT and nitrite/ammonia ratio effects on the anammox process were also studied. The results show that an optimal HRT for anammox SBBR is 6 h, under which the highest NRR (1.62kg-N•m^{-3}•d^{-1}) can be reached. The stoichiometric ratio of nitrite/ammonia was proven to be critical to anammox as well and a proper ratio should be 1.2. Kinetic parameters of a first-order substrate (ammonia and nitrite) removal model suitable for SBBR was established and each fits well to the experimental results (r^2=0.962 and 0.965 for ammonia, r^2=0.934 and 0.955 for nitrite). The study demonstrates that the substrate effect, in terms of HRT and stoichiometric ratio of nitrite/ammonia is of great importance to a stable and efficient anammox process.

Acknowledgments

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