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Shortcut biological nitrogen removal in continuous-flow anoxic/aerobic process for treating low-strength ammonium wastewater

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ABSTRACT

The aim of this study was to achieve shortcut biological nitrogen removal (BNR) in a laboratory-scale continuous-flow anoxic/aerobic process. A BNR reactor and a hybrid biofilm nitrogen removal (HBNR) reactor fed with low-strength ammonium wastewater were employed. The pathway of nitrogen conversion in both reactors was also evaluated. Results showed that effective biological nitrogen removal could be reliably achieved in both reactors, with over 69% total nitrogen (TN) and 95% NH_4^+-N removal efficiencies. HBNR improved the TN removal efficiency by over 15% relative to conventional BNR. Based on nitrogen mass balance, the fates of nitrogen in the HBNR and BNR differed, with most nitrogen removal occurring by SBNR via nitrite in the anoxic tank of the BNR and by simultaneous nitrification–denitrification via nitrite in the aerobic tank of the HBNR. The specific oxygen utilization rate test and real-time quantitative polymerase chain reaction indicated that ammonia-oxidizing bacteria were dominant in activated sludge and biofilm, and low dissolved oxygen selected against nitrite-oxidizing bacteria (NOB), even if NOB was not completely washed out from the reactors.

Keywords: Shortcut biological nitrogen removal (SBNR); Anoxic/aerobic process; Low ammonium concentration; Wastewater; Hybrid biofilm reactor; Real-time quantitative PCR (qPCR)

1. Introduction

Many existing municipal wastewater treatment plants need to be upgraded to meet the increasingly stringent standards for total phosphorus and nitrogen in discharged wastewater. Traditional biological nitrogen removal (BNR) processes such as anoxic/aerobic process require relatively long aeration periods and high oxygen concentrations during nitrification, while the denitrification process is limited by the quantity of organic carbon, which is insufficient in municipal wastewater treatment plants [1]. Therefore, many novel biological nutrient removal processes have been adopted, including shortcut biological nitrogen removal (SBNR), simultaneous nitrification–denitrification (SND), and anammox and denitrifying phosphorus removal processes. For example, when compared with the traditional BNR process, the SBNR process resulted in a saving of 25% of oxygen supply for

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nitrification while reducing the carbon source requirement for denitrification by 40% [2]. Owing to these advantages, this process has attracted a great deal of attention for its potential to treat various types of wastewater [3,4]. However, nitrite accumulation is difficult to attain because nitrite-oxidizing bacteria (NOB) generally have higher substrate utilization rates than ammonia-oxidizing bacteria (AOB) [5].

In recent years, most successful applications of SBNR have been associated with wastewater containing high ammonium concentrations, such as anaerobic sludge digestion liquor and landfill leachate [6,7]. It has been suggested that the key factors for inhibition and elimination of NOB in such systems are free ammonia (FA) and free nitrous acid (FNA). However, for domestic wastewater, which often has a NH_4^+-N concentration lower than 80 mg L^{-1} and a low C/N ratio, the inhibitory effect of FA and FNA on NOB is often unavailable [8]. Accordingly, low dissolved oxygen (DO) concentration [9], high pH [10], and high temperature [11] are thought to be important factors to accomplish partial nitrification to nitrite during treatment of low-strength ammonium wastewater. C/N ratio is one of the most critical parameters for nitrogen removal because it directly influences the growth of nitrifiers and denitrifiers.

Two types of systems are usually used for BNR from wastewater: sequencing batch reactors (SBRs) and continuous-flow systems. Although SBNR is recognized as a cost-effective and sustainable BNR process, nitrite accumulation has been difficult to achieve in continuous-flow processes treating domestic wastewater [4]. Most successful scenarios regarding SBNR have been conducted in SBRs [12,13]. Low DO is regarded as a key factor to achieve nitrogen removal via nitrite pathway in continuous-flow systems for treatment of low-strength ammonium wastewater [14]. This is mainly because AOB and NOB have significantly different oxygen affinities. Specifically, NOB is thought to be more sensitive to low DO concentration than AOB [15]; therefore, NOB would be eliminated under the long-term effects of low DO. However, low DO concentration might lead to sludge bulking and low nitrification rates [16].

There has been increasing interest in biofilm processes for BNR from wastewater. A hybrid shortcut biological nutrient removal reactor was applied to the conventional anoxic/aerobic process to achieve partial nitrification–denitrification for the successful treatment of ammonium-rich wastewater [17]. In this process, the aerobic tank was filled with polyvinyl alcohol sponge media to maximize the hold-up and solids retention time (SRT) of AOB, and a significant amount of chemical oxygen demand (COD) was removed by shortcut denitrification of nitrite in the anoxic tank. However, information regarding use of the hybrid biofilm reactor in continuous-flow mode during the treatment of low-strength ammonium wastewater through the partial nitrification pathway is still sparse [18]. In this study, a hybrid biofilm nitrogen removal (HBNR) reactor was adopted to retain AOB. The objectives of the study were (1) to achieve shortcut BNR from wastewater with a low ammonium concentration (60 mg/L); (2) to evaluate the performance of hybrid biofilm reactor during continuous-flow anoxic/aerobic process and compare their nitrogen removal with that of a conventional BNR reactor under low DO; and (3) to discuss the mechanisms responsible for cost-effective BNR.

2. Materials and methods

2.1. Experimental set-up and operational conditions

Two different laboratory-scale continuous-flow anoxic/aerobic process systems (BNR reactor and HBNR reactor) containing three compartments with a working volume of 12 L were used (Fig. 1). Both reactors consisted of a 5 L anoxic tank with a lid, a mechanical stirrer, a 5 L aerobic tank, and a 2 L settling tank. Strings of sponge carriers (1.00 cm^3 at 20%, v/v) were fixed in the aerobic tank of the HBNR to

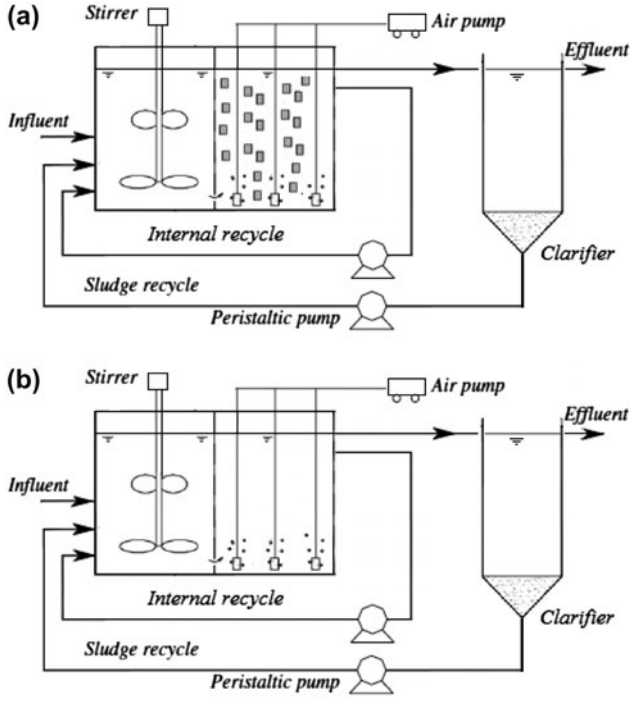

Fig. 1. Schematics of HBNR (a) and BNR (b) reactors.

attach and retain AOB. There was no carrier in the aerobic tank of the BNR. The DO concentration in the aerobic tank was maintained by aeration with an air pump through porous stone diffusers. The aeration strength was adjusted using rotameters. Influent flow, sludge return, and internal recycling were all controlled by peristaltic pumps (Lange Co., Ltd., China).

Table 1 shows the operational conditions of two reactors during three periods. The internal and sludge recycle ratios were 2 and 1, respectively. The temperature and pH in both reactors were 28 ± 2℃ and 7.7 ± 0.1. In the aerobic tank, the DO concentration was maintained at about 4.0 mg L^{-1} during period I (acclimatization phase, day 1–12), after which it was decreased to about 1.5 mg L^{-1} during period II (day 13–64), and then to below 1 mg L^{-1} by lowering the aeration rate. In the anoxic tank, the DO concentration was kept at about 0.4 ± 0.1 mg L^{-1}. SRT was controlled by the intermittent discharge of waste sludge. The average SRT in the HBNR reactor and BNR reactor was calculated as $V(X_a + X_s)/Q_wX_w$ and VX_s/Q_wX_w and the average SRT in the BNR reactor. X_a, X_s, and X_w were the biomass concentrations attached to the media, in suspension and in the wasted biomass, respectively. Q_w was the time-averaged waste-flow rate. V_w was the volume of waste sludge. The influent flow rate Q ranged from 20 to 24 L d^{-1}, resulting in a hydraulic retention time (HRT) in the bioreactor of 0.5–0.6 d.

2.2. Synthetic wastewater and seed sludge

Our investigation of the water quality of domestic wastewater in Zhejiang Province, China revealed that it is characterized by low organic and phosphorus levels, high nitrogen concentration, a C/N ratio of approximately 6.1 and a maximum NH_4^+-N concentration of about 60 mg L^{-1}. In this study, synthetic wastewater was made of starch, acetate, $(NH_4)_2SO_4$, KH_2PO_4, and trace elements. The compositions of the wastewater influents are shown in Table 2. The composition of the trace element solution (g L^{-1}) was $ZnSO_4{\cdot}7H_2O$, 0.43; H_3BO_4, 0.014; $MnCl_2.4H_2O$, 0.99; $Na_2MoO_4{\cdot}2H_2O$, 0.22; $CuSO_4{\cdot}5H_2O$, 0.25; $CoCl_2{\cdot}6H_2O$, 0.24; $NiCl_2{\cdot}6H_2O$, 0.19; and EDTA, 10.0. The C/N ratio of the influent was about 6.0 throughout the experiment. The initial concentration of alkalinity was 558 mg L^{-1} (as $CaCO_3$), and the pH of the influent was not adjusted. The seed sludge was activated sludge taken from a secondary clarifier of the Qige Wastewater Treatment Plant in Hangzhou, China. The initial mixed liquor suspended solids (MLSS) concentration of sludge in the BNR and HBNR was about 3,000 mg L^{-1}.

Table 2
The composition of synthetic wastewater

Compounds	Concentration (mg·L^{-1})
$(NH_4)_2SO_4$	60 (as N)
$NaHCO_3$	600
Sodium acetate and soluble starch	360 (as COD)
NaCl	585
KH_2PO_4	54.4
$MgSO_4{\cdot}7H_2O$	98.4
Trace element solution	1 mL·L^{-1}

2.3. Specific oxygen uptake rate

Specific oxygen utilization rate (SOUR) was determined using the method described by Chung et al. [17]. Measurements were conducted using a 1 L closed respirometric cell made of glass and equipped with an oxygen electrode (Mettler Toledo, FiveGo) connected to a recorder. The O_2 concentration in the cell was measured over time to determine the rates of uptake, which were compared with the control. The solution for the SOUR test for AOB consisted of 30 mg $L^{-1}NH_4^+$-N and other trace elements (Table 2), with no organic compounds or nitrite. The solution for NOB was composed of 30 mg N $L^{-1}NO_2^-$-N and

Table 1
Overview of operational conditions for HBNR and BNR

Periods	Period I (1–12 d)	Period II (13–64 d)	Period III (65–120 d)
HRT (d)	0.5	0.5/0.6	0.6
SRT (d)	25	15–20	40
DO in anoxic tank (mg L^{-1})	0.5	0.4	0.3
DO in aerobic tank (mg L^{-1})	~4.0	~1.5	~1.0
Nitrogen loading rate (mg m^{-3} d^{-1})	144	144/120	120

other trace elements (Table 2), with no organic compounds or ammonium. A control test was conducted using solution that contained only trace elements. A mixed liquor sample or a detached biofilm suspension from the reactor was saturated with oxygen by aeration, after which the cell was carefully closed without leaving air bubbles and the cell suspension was then stirred with a magnetic stirrer at 150 rpm and 28°C, respectively. The initial pH value was controlled at 7.6–7.8 and all tests were conducted in triplicate. The SOUR (mg O_2 g^{-1}VSS h^{-1}) of each sample was equal to the slope of DO depletion vs. time divided by the VSS in the cell.

2.4. Analytical methods

Samples were collected from the aerobic tank, anoxic tank and settling tank and then immediately filtered through a 0.22 μm hydrophilic membrane filter. Total organic carbon (TOC) was measured by a TOC analyzer (TOC-L, Shimadzu, Japan). NH_4^+-N, NO_2^--N, and NO_3^--N, alkalinity, MLSS, and mixed liquor volatile suspended solids were all determined according to the standard methods (APHA, 1998). pH and temperature were monitored using a pH electrode (Mettler Toledo, FiveEasy, Switzerland). DO concentration was detected using a DO electrode (Mettler Toledo, FiveGo, Switzerland). All samples were measured in triplicate.

2.5. DNA extraction and real-time quantitative PCR

Genomic DNA was extracted using the fast DNA spin kit for soil (BioTeke Corporation) according to the manufacturer's protocols. DNA samples were stored at −20°C for subsequent assays. We confirmed the intact DNA by 1% agarose gel electrophoresis and DNA concentrations were determined using a NanoDrop 2000 spectrophotometer.

To investigate the changes in microbial populations during the process performance, quantitative polymerase chain reaction (qPCR) was used to determine the abundance of the functional genes, AOB (*amoA*), AOA (*amoA*), NOB (*nxrA*), and denitrifier (*nirK* and *nirS*), as well as total bacterial 16S rDNA as a reference. The primer and probe sequences are shown in Table 3. All qPCRs were conducted in a mixture with a total volume of 10 μL solution containing 1 μL of template DNA (10 ng), 0.2 μL of each forward and reverse primer, 5 μL of iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), and 3.6 μL of sterile water. The qPCR conditions for AOB, NOB, and the denitrifier were as described by Herrmann et al. [25], Wang et al. [20], and Geets et al. [21], respectively. All qPCR assays were performed in triplicate for each sample and included standards and a control without a DNA template. The specificity of each PCR assay was confirmed by melting curve analysis and agarose gel electrophoresis. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from qPCR experiments [26].

2.6. Calculation

The NH_4^+-N removal efficiency, nitrite accumulation ratio (NAR), and total nitrogen (TN) removal efficiency were calculated as follows:

$$\text{NH}_4^+\text{-N removal efficiency} = \frac{\text{NH}_4^+\text{-N}_{\text{inf}} - \text{NH}_4^+\text{-N}_{\text{eff}}}{\text{NH}_4^+\text{-N}_{\text{inf}}} \times 100\% \quad (1)$$

$$\text{NAR} = \frac{\text{NO}_2^-\text{-N}}{\text{NO}_2^-\text{-N} + \text{NO}_3^-\text{-N}} \times 100\% \quad (2)$$

$$\text{TN removal efficiency} = \frac{\text{TN}_{\text{inf}} - \text{TN}_{\text{eff}}}{\text{TN}_{\text{inf}}} \times 100\% \quad (3)$$

3. Results and discussion

3.1. Effective BNR in HBNR and BNR

Fig. 2 shows the concentration profiles of NH_4^+-N, NO_2^--N, and NO_3^--N in the effluent of HBNR (a) and BNR (b) during the entire course of the experiment. The NAR, NH_4^+-N, and TN removal efficiencies in HBNR (a) and BNR (b) throughout the experiment are given in Fig. 3. In period I (day 1–12), DO concentrations were controlled at about 0.5 and 4.0 mg L^{-1} in the anoxic tank and aerobic tank, respectively, to enrich the nitrifying bacteria. In period II (day 13–64), DO in the aerobic tank was decreased to ~1.5 mg L^{-1} to eliminate or suppress NOB because of the differences in specific growth rates between AOB and NOB under limited aeration. In period III (day 65–120), DO concentrations were switched to 0.3 mg L^{-1} in the anoxic tank and 1.0 mg L^{-1} in the aerobic tank, respectively. Table 4 summarizes the performance of the HBNR and BNR throughout the experiment.

In the HBNR, nearly complete oxidation of ammonia to nitrate (full nitrification) was achieved quickly during period I (Fig. 2), during which time 75.6% of NH_4^+-N was oxidized to NO_3^--N. Additionally, the NO_3^--N concentrations were much higher than the

Table 3
Primers and probe sequences

Species	Primer	Sequence (5′–3′)	Reference
AOB	AmoA-1F	GGGGTTTCTACTGGTGGT	[19]
	AmoA-2R	CCCCTCKGSAAAGCCTTCTTC	[19]
AOA	Arch-amoAF	STAATGGTCTGGCTTAGA CG	[20]
	Arch-amoAR	GCGGCCATCCATCTGTATGT	[20]
NOB	F1370-F1	CAGACCGACGTGTGCGAAAG	[21]
	F2843-R2	TCCACAAGGAACGGAAGGTC	[21]
DNB	NirK-1F	GGMATGGTKCCSTGGCA	[22]
	NirK-5F	GCCTCGATCAGRTTRTGGTT	[22]
	NirS-cd3AF	GTSAACGTSAAGGARACSGG	[23]
	NirS-R3cd	GASTTCGGRTGSGTCTTGA	[23]
16S rRNA	16S-338	CCTACGGGAGGCAGCAG	[24]
	16S-518	ATTACCGCGGCTGCTGG	[24]

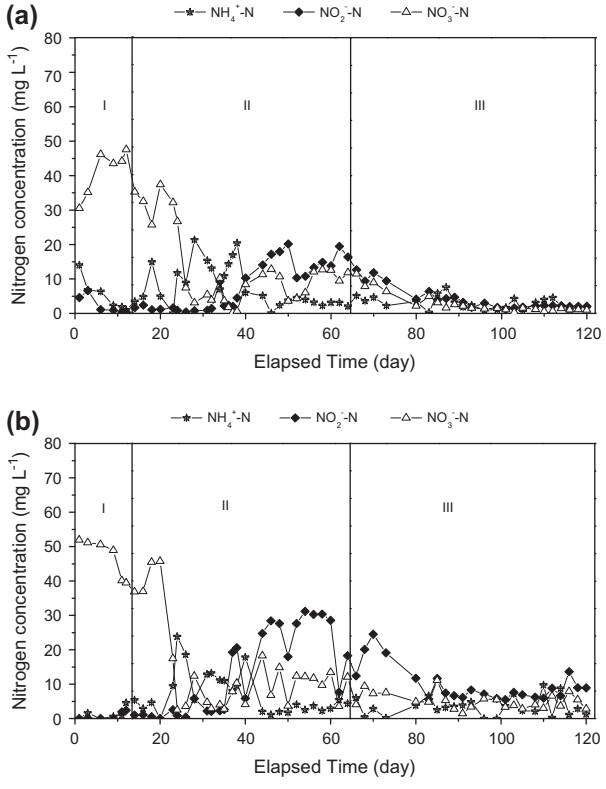

Fig. 2. Concentration profiles of NH_4^+-N, NO_2^--N, and NO_3^--N in the effluent of HBNR (a) and BNR (b).

NO_2^--N concentrations in the effluent because of the high DO concentration (4.0 mg L^{-1}) in the aerobic tank; therefore, the NH_4^+-N removal efficiency was above 90%, and the average NAR and TN removal efficiency were 5.87% and 14.9%, respectively (Table 4). In period II (day 13–64), the DO concentration in the aerobic tank was decreased to 1.5 mg L^{-1} from day 13 to establish a high nitrite accumulation. In the subsequent days, NH_4^+-N removal was unstable and decreased to 66% on day 38, but the NAR and TN removal efficiency increased gradually to 87 and 57%, respectively, on day 38 (Fig. 3), while nitrate accumulation was reduced significantly from day 26 on (Fig. 2(a)). From day 39, the HRT was changed from 0.5 to 0.6 d to decrease the hydraulic loading rate of the reactor, after which the rate of ammonia oxidation recovered to over 90%. During days 43–64, the NAR and TN removal efficiency reached 61.9 ± 11.2% and 53.0 ± 6.6%, respectively. During period III, the DO concentration was reduced to less than 1 mg L^{-1} in the aerobic tank, resulting in lower effluent concentrations of NO_2^--N and NO_3^--N (Fig. 2). At the end of the experiment, the average effluent concentrations of NO_2^--N and NO_3^--N were approximately 2.0 and 1.0 mg L^{-1}, respectively. On day 80, the TN removal efficiency increased to 84%. This may have occurred because SND in the aerobic tank promoted nitrogen removal (discussed in detail below). Similar to period II, NH_4^+-N removal remained at a high level (95.7 ± 3.2%) during the steady state of period III (day 80–120), NAR ranged from 51 to 81%, and TN removal was stable (88.1 ± 5.1%).

As shown in Fig. 2, there was a similar trend in the concentration profiles of NH_4^+-N, NO_2^--N, and NO_3^--N during period I and period II in the BNR as in the HBNR, but different patterns during period III. During period I, nitrate was the main product of ammonia oxidation, and the NH_4^+-N removal efficiency was greater than 97% (Fig. 3(a)). In period II, NAR increased gradually, reaching more than 60% from day 37, indicating that partial nitrification via nitrite had been achieved (Fig. 3(b)). From day 65 to 120, NH_4^+-N removal was stable, and the NAR was

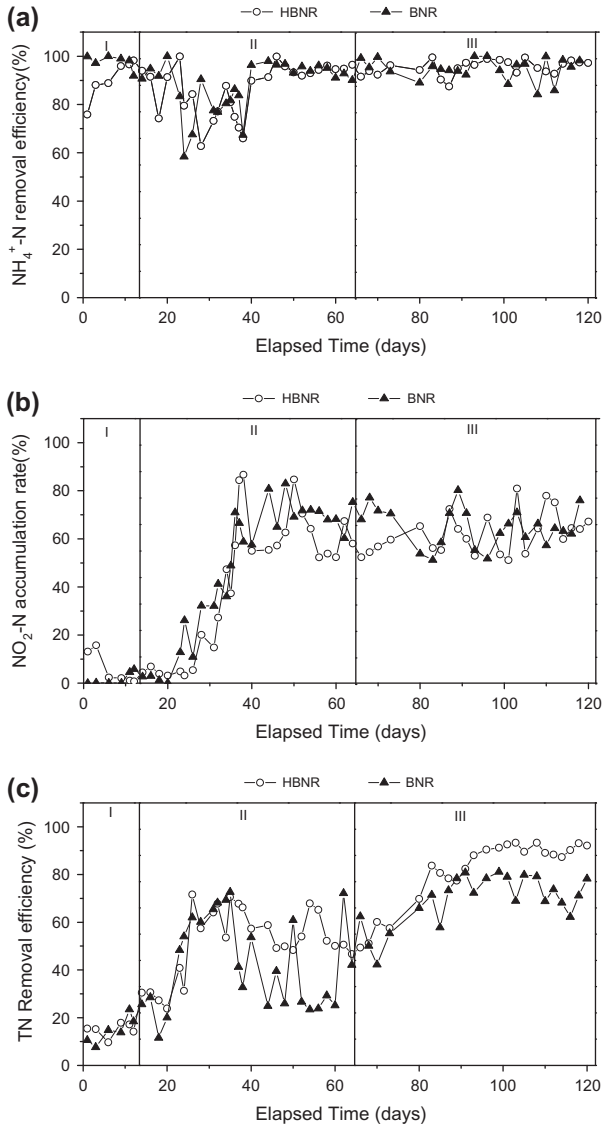

Fig. 3. Profiles of NH_4^+-N removal efficiency (a), NAR (b), and TN removal efficiency (c) in the HBNR and BNR.

higher than that of HBNR, but TN removal was low relative to the HBNR (Table 4).

The above results indicate that shortcut nitrogen removal can be achieved in the continuous-flow anoxic/aerobic process under low DO concentration. The HBNR process improved TN removal by over 15% relative to the conventional BNR process, and had less nitrite and nitrate in the effluent, although NAR was lower than that of the BNR process. In addition, the effluent TN concentration of HBNR in this study reached levels ≤10 mg L^{-1}, which met the class A discharge standards (GB18918–2002, China). These findings are in accordance with those of a study conducted by Gong et al. [18], who found that an integrated anoxic/aerobic biofilm reactor enhanced TN removal with an internal cycling rate of 3.

3.2. Fates of nitrogen and COD in HBNR and BNR

To understand the pathway of nitrogen conversion during the stable effective nitrogen removal process in the HBNR and BNR, the mass balances of nitrogen and COD were investigated. Table 5 summarizes the fates of nitrogen species and COD in both reactors for period III. The calculations were conducted using the method described by Chung et al. [17]. Nitrogen and COD incorporated into biomass were neglected in this analysis because of the long SRT. During period III, HBNR and BNR had different fates for nitrogen, but similar fates for COD. Approximately 94–98% of the NH_4^+-N in the influent was removed by oxidation to NO_x-N in the aerobic tank of both reactors. In the HBNR, only 5% of the N exited as NO_x in the effluent, meaning that 93% was denitrified. Of the denitrified N, 43% was denitrified in the anoxic tank. In the BNR, 20% of the N in the effluent was NO_x, while 74% was denitrified. Most nitrogen removal (about 71%) occurred in the anoxic tank. In the HBNR, 43% of the N was denitrified directly in the aerobic tank, leading to higher nitrogen removal efficiency. Remarkably, about 2.5–7.5% of the N was denitrified in the clarifiers of both reactors. Overall, COD removal was about 92% in both reactors. Approximately 90% of the COD was consumed in the anoxic tank, while only 1.3–1.7% of the COD was removed in the aerobic tank and clarifier by a combination of aerobic oxidation and denitrification (Table 5). These findings indicated that most of the COD in the influent was used to reduce NO_x (denitrification) and bacterial growth in the anoxic tanks of both reactors.

One possible factor contributing to the improved nitrogen removal in the HNBR was the SND process in the aerobic tank under low DO concentration. Fig. 4 shows the typical variations in nitrogen species concentrations along the HBNR and BNR in the three periods during steady state operation. When compared with period I and II, the TN removal efficiency of period III increased significantly in both reactors. However, nitrogen removal primarily occurred in the anoxic tank of the BNR, whereas concentrations of various nitrogen species decreased obviously in the aerobic tank of the HBNR. The contribution of SND efficiency in the aerobic tank of HBNR to nitrogen removal was about 50% during period III. SND was inferred from the observation that TN losses of up to 30% are frequently reported in aerated tanks during

Table 4
Summary of performances of HBNR and BNR during the whole experiment

Reactor	Period	Effluent NH_4^+-N (mg L^{-1})	NH_4^+-N removal efficiency (%)	Effluent NO_2^--N (mg L^{-1})	Effluent NO_3^--N (mg L^{-1})	NAR (%)	TN removal efficiency (%)
HBNR	I (1–12 d)	5.40 ± 4.86	93.5 ± 4.7	2.35 ± 2.61	41.2 ± 6.8	5.87 ± 6.73	14.9 ± 2.9
	II (13–64 d)	7.74 ± 6.18	86.6 ± 10.7	7.83 ± 6.99	12.7 ± 11.2	42.3 ± 28.2	52.0 ± 14.0
	III (65–120 d)	2.93 ± 1.86	95.4 ± 3.1	4.19 ± 3.40	2.89 ± 2.99	62.2 ± 8.5	83.1 ± 12.3
BNR	I (1–12 d)	1.31 ± 1.73	97.7 ± 3.0	0.77 ± 1.11	47.1 ± 5.7	1.81 ± 2.67	14.8 ± 5.6
	II (13–64 d)	7.20 ± 6.09	87.6 ± 10.8	13.1 ± 12.2	13.7 ± 13.0	46.6 ± 27.6	42.6 ± 19.1
	III (65–120 d)	3.38 ± 2.78	94.6 ± 4.6	9.83 ± 5.13	5.09 ± 2.35	65.4 ± 8.3	69.5 ± 10.5

Table 5
The fate of nitrogen and COD in HBNR and BNR for the period III

Average value	HBNR	BNR
Nitrogen, gN d^{-1} (percent of influent mass load)		
(1) NH_4^+-N influent	1.20 (100%)	1.20 (100%)
(2) NH_4^+-N effluent	0.03 (2.5%)	0.07 (5.8%)
(3) NH_4^+-N oxidized to NO_x-N (=(1)–(2))	1.17 (98%)	1.13 (94%)
(4) NO_2^--N in effluent	0.04 (3.3%)	0.15 (13%)
(5) NO_3^--N in effluent	0.02 (1.7%)	0.09 (7.5%)
(6) NO_x-N in effluent (=(4)+(5))	0.06 (5.0%)	0.24 (20%)
(7) Total N in effluent (=(2)+(6))	0.09 (7.5%)	0.31 (26%)
(8) N denitrified and removed as gaseous nitrogen (=(1)–(7))	1.11 (93%)	0.89 (74%)
(9) N denitrified in anoxic tank[a]	0.51 (43%)	0.85 (71%)
(10) N denitrified in aerobic tank	0.51 (43%)	0.01 (0.8%)
(11) N denitrified in clarifier (=(8)–(9)–(10))	0.09 (7.5%)	0.03 (2.5%)
COD, g d^{-1} (percent of influent mass load)		
(12) COD in influent	7.20 (100%)	7.20 (100%)
(13) COD in effluent	0.60 (8.3%)	0.62 (8.6%)
(14) Overall COD removal (=(11)–(12))	6.60 (92%)	6.58 (91%)
(15) COD in effluent from anoxic tank[b]	0.66 (9.2%)	0.66 (9.2%)
(16) COD removed by denitrification in anoxic tank	6.48 (90%)	6.49 (90%)
(17) COD removal in aerobic tank	0.02 (0.3%)	0.04 (0.6%)
(18) COD removal in clarifier (=(14)–(16)–(17))	0.10 (1.4%)	0.05 (0.7%)

[a]This value was obtained by subtracting (NH_4 + NO_x) concentrations in anoxic tank from the combination of (NH_4 + NO_x) recycled from aerobic tank and clarifier and (NH_4 + NO_x) in the influent medium.
[b]This value was obtained by subtracting COD in anoxic tank from the combination of COD recycled from aerobic tank and clarifier and COD in the influent medium.

the activated sludge process [27]. It is assumed that the high SND efficiency was mainly due to the low DO concentration and the existence of biofilm. Low DO levels and the presence of NO_x-N in the early aeration process may result in the occurrence of denitrification [28]. Additionally, it has been shown that low DO concentration has a positive effect on SND removal [29,30]. Indeed, up to 78%, SND was realized under a DO concentration of 0.5 mg L^{-1} using acetate as a carbon source [31]. Biofilm carriers increased the SRT for AOB, while low DO concentration promoted AOB growth and direct denitrification of nitrite in the aerobic tank. Colliver and Stephenson [32] found that, during nitrification, increased NO_2^--N concentrations led to increased denitrification by AOB because of the low oxygen concentration, and some NOB strains were capable of denitrification as well [33]. SND activities increase at low DO concentration in the aerobic tank, as was confirmed in this study. The reduction of nitrite to nitrogenous gas owing to SND activities reduces the energy available for NOB, thereby limiting their growth to some extent. To date, many studies have shown the good performance of SND in biofilm processes [34,35]. SND became a

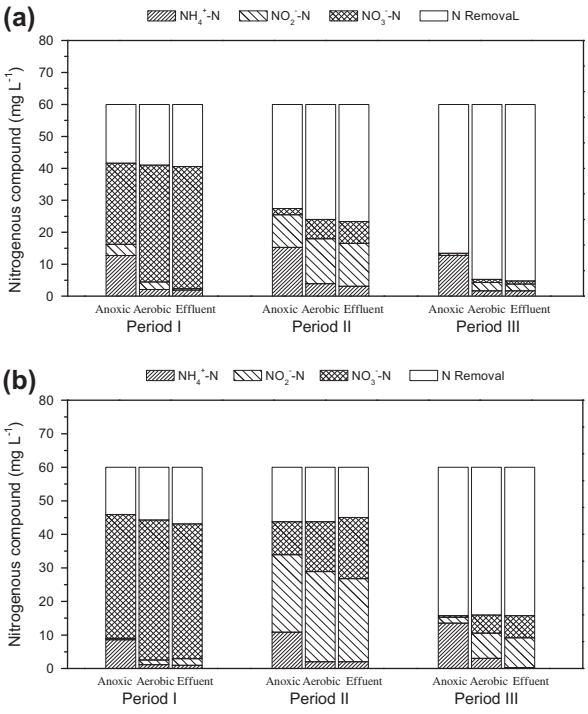

Fig. 4. Typical variation of nitrogen species concentrations along the HBNR (a), and BNR (b) during three periods in stable stage.

promising way of controlling effluent TN in A/O biofilm process [18].

3.3. Mechanism of achieving nitrite accumulation in HBNR and BNR

Activities and numbers of AOB and NOB in the HBNR and BNR were investigated by the SOUR test and qPCR, respectively. Many previous studies have shown that AOB were more robust toward low DO than NOB, and their growth rate was about 2.6 times faster than that of NOB when the DO concentration was less than 1.0 mg L^{-1} [36–38]. At low DO concentrations, partial nitrification to nitrite can be achieved in a continuous process without sludge retention by wash out of NOB while retaining AOB [14]. Hence, oxygen limitation has a greater effect on the activity of NOB than AOB. The results of SOUR tests to investigate the activities of AOB and NOB in both reactors are shown in Fig. 5. The biomass was taken from aerobic tanks on day 120. In the HNBR, the SOUR values for AOB (7.0 ± 0.9 mg O_2 $gVSS^{-1}$ h^{-1} for activated sludge and 6.8 ± 1.1 mg O_2 $gVSS^{-1}$ h^{-1} for biofilm) were much higher than those for NOB (1.1 ± 0.1 mg O_2 $gVSS^{-1}$ h^{-1} for activated sludge and 2.0 ± 0.9 mg O_2 $gVSS^{-1}$ h^{-1} for biofilm). The same trend existed in the BNR, with values of 6.9 ± 0.7 mg O_2 $gVSS^{-1}$ h^{-1} for AOB and 1.6 ± 0.2 mg O_2 $gVSS^{-1}$ h^{-1} for NOB being observed. The SOUR results confirmed that AOB had an advantage over NOB in the shortcut BNR process.

Although differences in SOUR for AOB and NOB were observed (Fig. 5), it should be noted that AOB requires three times more oxygen to oxidize ammonia to nitrite than NOB requires that to oxidize nitrite to nitrate. For the biofilm in the HNBR, the AOB (6.8): NOB (2.0) ratio was 3.4:1, but a significant amount of NOB were still in the system and a large portion of nitrite might have been converted to nitrate. The SOUR value for nitrite oxidation of biofilm was higher than that of activated sludge in the HBNR. The biofilm could hold more nitrifiers, including AOB and NOB, and the longer SRT of biofilm led to relatively more NOB being retained in the system. This coincides well with the nitrite accumulation ratio of HBNR, which was lower than that of the BNR.

qPCR was conducted to quantify the relative abundance of nitrification and denitrification functional genes (*amoA*, *nxrA*, *nirK*, and *nirS*), which reflected changes in the population abundance of AOB, AOA, NOB, and DNB in the aerobic and anoxic tank of both reactors throughout the experiment. The relative changes in abundance of the AOB *amoA* gene and NOB *nxrA* gene in the HBNR and BNR during the experiment are shown in Fig. 6. In this study, the AOA *amoA* gene was not detected in any reactors. The qPCR result of biomass collected in period I was as internal control genes. During period II, the abundance of the AOB *amoA* gene was 1.5 times that of period I in anoxic tanks of both reactors. Until period III, the abundance of the AOB *amoA* gene in the

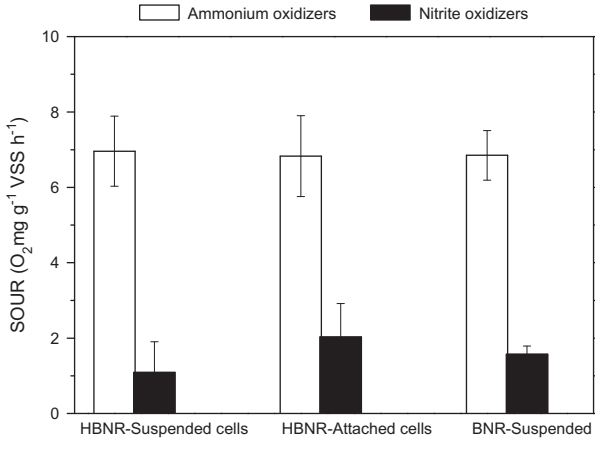

Fig. 5. Results of SOUR test in the aerobic tanks of HBNR and BNR (unit of SOUR is mg O_2 $gVSS^{-1}$ h^{-1}).

biofilm of the aerobic tank of HBNR dramatically increased, and it was 4.5-fold that of period I; however, the abundance of the AOB *amoA* gene in activated sludge showed no evident change compared with period I and II. In the aerobic tank of the BNR, the abundance of the AOB *amoA* gene in the activated sludge was 3.6-fold higher than that in period II. The AOB *amoA* gene abundance decreased in the anoxic tanks of both reactors, probably because of the lower DO concentration (<0.5 mg/L). In addition, NOB were not completely eliminated from the BNR and HBNR, especially in the aerobic tank of the HBNR. When compared with period I, NOB *nxrA* gene abundance in the anoxic tanks of both reactors was obviously decreased by more than 50% during period II and period III. However, the NOB *nxrA* gene density in biofilm and activated sludge from the aerobic tank of the HBNR was higher than that of BNR. It is likely that the biofilm easily retained the biomass, including AOB and NOB. These findings are concordant with the results of the SOUR test. Besides, denitrifying enzymes were detected in the anoxic tank as well as the aerobic tank of the HBNR, and the abundance of the denitrifying *nirS* gene from activated sludge and biofilm in the aerobic tank of HBNR during period III was 4.0 and 3.0 times that during period II, respectively (data not shown). The gene of *nirS* is related to aerobic denitrification [39]. Therefore, in HBNR, biofilm carriers increased the SRT for AOB, while the low DO concentration promoted AOB growth and direct denitrification of nitrite in the aerobic tank.

Microbial analysis demonstrated that the nitrite pathway was established through a substantial increase in the AOB population in aerobic tanks and reduction of the NOB population in the BNR and HBNR. Many studies have shown that in autotrophic partial nitritation process, significant enrichment in the biofilm was observed for AOB [40,41]. Wang et al. [42] also found the abundance of *amoA* gene (AOB) was two orders of magnitude higher than the abundance of *nxrA* gene (NOB) in the membrane-aerated biofilms; however, the *nxrA* gene was always detected during the operation time. Winkler et al. [43] reported that the theoretical NOB/AOB ratio was 0.5 during the nitrification process, but the abundance of NOB compared with AOB is expected to be even lower in systems in which SND is occurring. Thus, the results of SOUR and qPCR conducted in this study support the assumption that HBNR selected more active AOB under low oxygen concentrations and decreased the abundance of NOB to promote SND. This phenomenon could be confirmed by testing the proportions of *Nitrospira*, *Nitrobacter*, and *Nitrosomonas* in future studies.

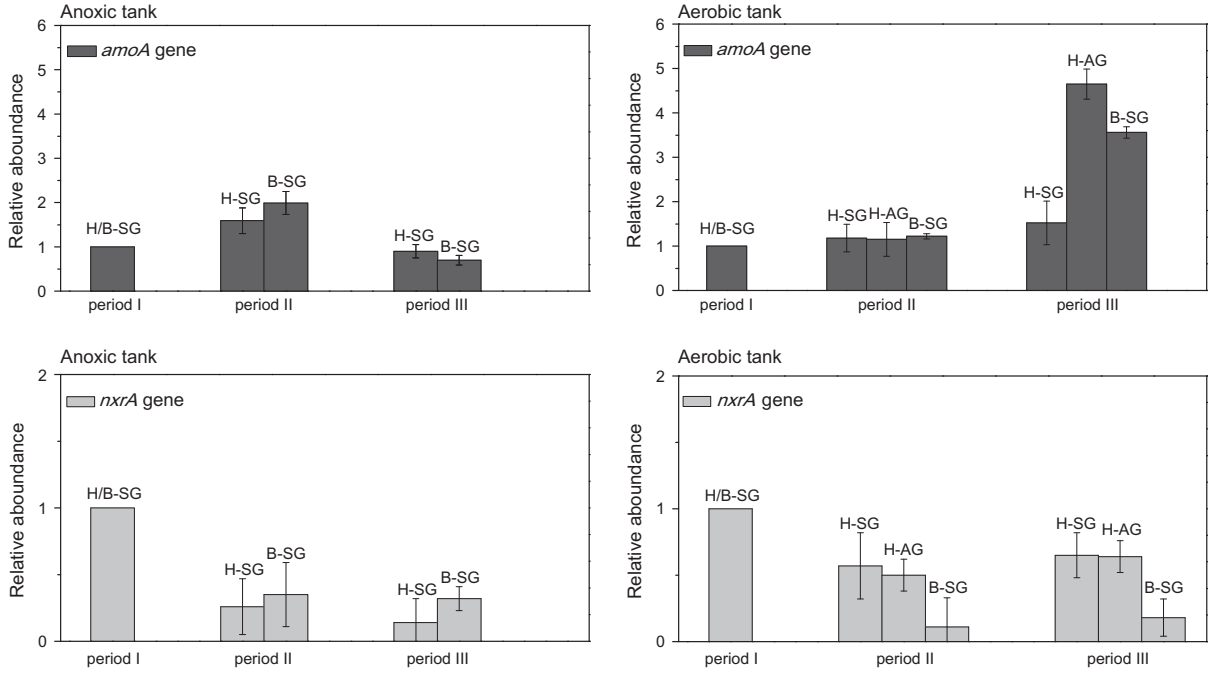

Fig. 6. The relative abundance change of AOB *amoA* gene and NOB *nxrA* gene in aerobic and anoxic tanks of HBNR and BNR during the experiment (H-SG: suspended sludge sample in HBNR; H-AG: attached sludge sample in HBNR; B-SG: suspended sludge sample in BNR).

4. Conclusions

(1) Shortcut nitrogen removal in the continuous-flow anoxic/aerobic process treating low-strength ammonium wastewater was successfully achieved by transition from full nitrification to partial nitrification by controlling the DO concentration in HBNR and BNR reactors.
(2) The TN removal efficiency of HBNR was significantly higher than that of BNR throughout the experiment. The DO concentration was essential to nitrite accumulation and nitrogen removal. Based on nitrogen mass balances, the fates of nitrogen in the HBNR and BNR were distinct from one another. One factor contributing to the improved nitrogen removal in the HNBR was the higher efficiency of the SND process in the aerobic tank due to the low DO concentration and the existence of biofilm.
(3) SOUR and qPCR assays demonstrated that AOB were dominant in activated sludge and biofilm when the SBNR process was established, while low DO concentration promoted AOB growth. NOB was not completely washed out from the reactors, but the abundance of NOB decreased in period III when compared with complete nitrification during period I.

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References

[1] B. Kartal, J.G. Kuenen, M.C.M. van Loosdrecht, Sewage treatment with anammox, Science 328 (2010) 702–703.
[2] A. Pollice, V. Tandoi, C. Lestingi, Influence of aeration and sludge retention time on ammonium oxidation to nitrite and nitrate, Water Res. 36 (2002) 2541–2546.
[3] C. Hellinga, A. Schellen, J.W. Mulder, M.C.M. van Loosdrecht, J.J. Heijnen, The Sharon process: An innovative method for nitrogen removal from ammonium-rich wastewater, Water Sci. Technol. 37 (1998) 135–142.
[4] Y. Ma, Y.Z. Peng, S.Y. Wang, Z.G. Yuan, X.L. Wang, Achieving nitrogen removal via nitrite in a pilot-scale continuous pre-denitrification plant, Water Res. 43 (2009) 563–572.
[5] J.H. Kim, X. Guo, H.S. Park, Comparison study of the effects of temperature and free ammonia concentration on nitrification and nitrite accumulation, Process Biochem. 43 (2008) 154–160.
[6] R. Ganigué, H. López, M. Ruscalleda, M.D. Balaguer, J. Colprim, Operational strategy for a partial nitritation-sequencing batch reactor treating urban landfill leachate to achieve a stable influent for an anammox reactor, J. Chem. Technol. Biotechnol. 83 (2008) 365–371.
[7] A. Miot, K.R. Pagilla, Control of partial nitritation of centrate in a sequencing batch reactor, Water Environ. Res. 82 (2010) 819–829.
[8] Y.Z. Peng, J.H. Guo, H. Horn, X. Yang, S.Y. Wang, Achieving nitrite accumulation in a continuous system treating low-strength domestic wastewater: switchover from batch start-up to continuous operation with process control, Appl. Microbiol. Biotechnol. 94 (2012) 517–526.
[9] H.P. Chuang, A. Ohashi, H. Imachi, M. Tandukar, H. Harada, Effective partial nitrification to nitrite by down-flow hanging sponge reactor under limited oxygen condition, Water Res. 41 (2007) 295–302.
[10] G. Ruiz, D. Jeison, R. Chamy, Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration, Water Res. 37 (2003) 1371–1377.
[11] D. Bougard, N. Bernet, D. Chèneby, J.P. Delgenès, Nitrification of a high-strength wastewater in an inverse turbulent bed reactor: Effect of temperature on nitrite accumulation, Process Biochem. 41 (2006) 106–113.
[12] C. Fux, S. Velten, V. Carozzi, D. Solley, J. Keller, Efficient and stable nitritation and denitritation of ammonium-rich sludge dewatering liquor using an SBR with continuous loading, Water Res. 40 (2006) 2765–2775.
[13] R. Blackburne, Z. Yuan, J. Keller, Demonstration of nitrogen removal via nitrite in a sequencing batch reactor treating domestic wastewater, Water Res. 42 (2008) 2166–2176.
[14] R. Blackburne, Z. Yuan, J. Keller, Partial nitrification to nitrite using low dissolved oxygen concentration as the main selection factor, Biodegradation 19 (2008) 303–312.
[15] H.D. Park, D.R. Noguera, Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge, Water Res. 38 (2004) 3275–3286.
[16] J. Wanner, C. Kragelund, P.H. Nielsen, Microbiology of bulking, in: R. Seviour, P.H. Nielsen (Eds.), Microbial Ecology of Activated Sludge, IWA Publishing, London, 2010, pp. 191–214.
[17] J. Chung, W. Bae, Y.W. Lee, B.E. Rittmann, Shortcut biological nitrogen removal in hybrid biofilm/suspended growth reactors, Process Biochem. 42 (2007) 320–328.
[18] L.X. Gong, L. Jun, Q. Yang, S.Y. Wang, B. Ma, Y.Z. Peng, Biomass characteristics and simultaneous nitrification–denitrification under long sludge retention time in an integrated reactor treating rural domestic sewage, Bioresour. Technol. 119 (2012) 277–284.
[19] S.H. Hong, H.D. Jeong, B. Jung, E.Y. Lee, Analysis and quantification of ammonia-oxidizing bacteria

community with *amoA* gene in sewage treatment plants, J. Microbiol. Biotechnol. 22 (2012) 1193–1193.
[20] R.C. Wang, X.M. Zhang, J.F. Zhao, Nitrifying population dynamics in a redox stratified membrane biofilm reactor (RSMBR) for treating ammonium-rich wastewater, Front. Environ. Sci. Eng. Chin. 5 (2011) 48–56.
[21] J. Geets, M. de Cooman, L. Wittebolle, K. Heylen, B. Vanparys, P.D. Vos, W. Verstraete, N. Boon, Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge, Appl. Microbiol. Biotechnol. 75 (2007) 211–221.
[22] G. Braker, A. Fesefeldt, K.P. Witzel, Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples, Appl. Environ. Microbiol. 64 (1998) 3769–3775.
[23] I.N. Throback, K. Enwall, A. Jarvis, S. Hallin, Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE, FEMS Microbiol. Ecol. 49 (2004) 407–417.
[24] G. Muyzer, E.C. de Waal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.
[25] M. Herrmann, A.M. Saunders, A. Schramm, Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*, Appl. Environ. Microbiol. 74 (2008) 3279–3283.
[26] J.L. Kenneth, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[27] S.P. Sun, C.P. Nàcher, B. Merkey, Q. Zhou, S.Q. Xie, D.H. Yang, J.H. Sun, B.F. Smets, Effective biological nitrogen removal treatment processes for domestic wastewaters with low C/N ratios: A review, Environ. Eng. Sci. 27 (2010) 111–126.
[28] D. Wei, Z.M. Qiao, Y.F. Zhang, L.J. Hao, W. Si, B. Du, Q. Wei, Effect of COD/N ratio on cultivation of aerobic granular sludge in a pilot-scale sequencing batch reactor, Appl. Microbiol. Biotechnol. 97 (2013) 1745–1753.
[29] G. Zhu, Y. Peng, B. Li, J. Guo, Q. Yang, S. Wang, Biological removal of nitrogen from wastewater, Rev. Environ. Contam. Toxicol. 192 (2008) 159–195.
[30] K.A. Third, M. Newland, R. Cord-Ruwisch, The effect of dissolved oxygen on PHB accumulation in activated sludge cultures, Biotechnol. Bioeng. 82 (2003) 238–250.
[31] K.A. Third, N. Burnett, R. Cord-Ruwisch, Simultaneous nitrification and denitrification using stored substrate (phb) as the electron donor in an SBR, Biotechnol. Bioeng. 83 (2003) 706–720.
[32] B.B. Colliver, T. Stephenson, Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers, Biotechnol. Adv. 18 (2000) 219–232.
[33] I. Schmidt, O. Sliekers, M. Schmid, E. Bock, J. Fuerst, J.G. Kuenen, M.S.M. Jetten, M. Strous, New concepts of microbial treatment processes for the nitrogen removal in wastewater, FEMS Microbiol. Rev. 27 (2003) 481–492.
[34] Y. Rahimi, A. Torabian, N. Mehrdadi, B. Shahmoradi, Simultaneous nitrification–denitrification and phosphorus removal in a fixed bed sequencing batch reactor (FBSBR), J. Hazard. Mater. 185 (2011) 852–857.
[35] E. Walters, A. Hille, M. He, C. Ochmann, H. Horn, Simultaneous nitrification/denitrification in a biofilm airlift suspension (BAS) reactor with biodegradable carrier material, Water Res. 43 (2009) 4461–4468.
[36] K. Egli, F. Bosshard, C. Werlen, P. Lais, H. Siegrist, A.J.B. Zehnder, J.R. van de Meer, Microbial composition and structure of a rotating biological contactor biofilm treating ammonium-rich wastewater without organic carbon, Microb. Ecol. 4 (2003) 419–432.
[37] T. Tokutomi, Operation of a nitrite-type airlift reactor at low DO concentration, Water Sci. Technol. 49 (2004) 81–88.
[38] H.D. Park, D.R. Noguera, Characterization of two ammonia oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations, J. Appl. Microbiol. 102 (2007) 1401–1417.
[39] Y. Guo, X.M. Zhou, Y.G. Li, K. Li, C.X. Wang, J.F. Liu, D.J. Yan, Y.L. Liu, D.H. Yang, J.M. Xing, Heterotrophic nitrification and aerobic denitrification by a novel *Halomonas campisalis*, Biotechnol. Lett 35 (2013) 2045–2049.
[40] Z.W. Liang, Z.Y. Han, S.Y. Yang, X.Q. Liang, P. Du, G.F. Liu, Y. Yang, A control strategy of partial nitritation in a fixed bed bioflim reactor, Bioresour. Technol. 102 (2011) 710–715.
[41] H.K. Park, S. Sundar, Y.W. Ma, K. Chandran, Differentiation in the microbial ecology and activity of suspended and attached bacteria in a nitritation-anammox process, Biotechnol. Bioeng. 112 (2015) 272–279.
[42] R.C. Wang, X.M. Zhan, Y.L. Zhang, J.F. Zhao, Nitrifying population dynamics in a redox stratified membrane biofilm reactor (RSMBR) for treating ammonium-rich wastewater, Front. Environ. Sci. Eng. Chin. 5 (2011) 48–56.
[43] M.K.H. Winkler, J.P. Bassin, R. Kleerebezem, D.Y. Sorokin, M.C.M. van Loosdrecht, Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge, Appl. Microbiol. Biotechnol. 94 (2012) 1657–1666.