Effect of C/N ratios on nitrogen removal and microbial communities in the anaerobic baffled reactor (ABR) with an anammox-coupling-denitrification process

Chongjun Chen, Min Zhang, Xuliang Yu, Juan Mei, Ying Jiang, Yaoqi Wang and Tian C. Zhang

ABSTRACT

Effects of different C/N (NO_2^-N) ratios on nitrogen removal and microbial community structure were investigated using an anaerobic baffled reactor (ABR). Results indicated that the C/N ratio exerted an important effect on nitrogen removal in the anammox-coupling-denitrification process associated with the ABR. When the C/N ratio was 1.29, the ABR could achieve the highest total nitrogen (TN) removal efficiency of 99.9%. Most of TN was removed in the 1st and 2nd compartment, accounting for about 81.0–97.6% of total TN removal. The nitrogen removal resulted from the interaction among anammox, heterotrophic denitrificans, and other microbes within the ABR. The contribution of anammox to nitrogen removal varied from 6.8% to 32.4%. High-throughput MiSeq sequencing analyses revealed that the C/N ratio was one of the most important factors regulating the microbial community structure, and the predominant phylum changed from Proteobacteria to Chloroflexi with the elevated C/N ratio. In addition, the *Candidatus* Brocadia was the major anammox bacterium, and its percentage varied from 1.0–2.9% at day 9 to 2.8–9.1% at day 46.

Key words | anaerobic baffled reactor (ABR), anammox, C/N ratio, denitrification, microbial community structure, MiSeq sequencing analysis

Chongjun Chen (corresponding author)
Min Zhang
Xuliang Yu
Juan Mei
Ying Jiang
Yaoqi Wang
School of Environmental Science and Engineering,
Suzhou University of Science and Technology,
Suzhou 215009,
China
E-mail: *chongjunchen@163.com*

Chongjun Chen
Juan Mei
Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment,
Suzhou 215009,
China

Chongjun Chen
Jiangsu Key Laboratory of Anaerobic Biotechnology,
Jiangnan University,
Wuxi 214122,
China

Tian C. Zhang
Civil Engineering Dept.,
University of Nebraska-Lincoln (Omaha Campus),
Omaha, NE 68182-0178,
USA

INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a lithoautotrophic biological conversion process to remove nitrogen by oxidizing ammonium to N_2 using nitrite as the electron acceptor and use CO_2 as carbon source under the anoxic condition (Lackner *et al.* 2014; Tang *et al.* 2017). Compared with the traditional nitrification/denitrification process, the anammox process combined with partial nitrification can save 50–60% aeration, 100% cost for adding organic carbon, and 90% operation cost (Volcke *et al.* 2005; Tal *et al.* 2006). However, being lithoautotrophic, anammox bacteria grow slowly (the maximum specific growth rate = 0.0027 h^{-1} and doubling time = 10–12 days) (Jetten *et al.* 1998; Wr *et al.* 2007), and often are outcompeted by heterotrophic denitrifiers (Wang & Kang 2005; Dapena-Mora *et al.* 2006; Molinuevo *et al.* 2009). In addition, the anammox process could only remove about 90% of the influent nitrogen as ammonia/nitrite and left about 11% of nitrogen as nitrate in the effluent (Zhang *et al.* 2017).

Up to now, several processes have been developed to achieve nitrogen and chemical oxygen demand (COD) removal synchronously (Tang *et al.* 2014; Hien *et al.* 2017), such as partial nitrification-anammox processes, completely autotrophic nitrogen removal over nitrite (CANON), single stage nitrogen removal using the anammox and partial nitritation (SNAP), simultaneous partial nitrification anammox and denitrification (SNAD) and oxygen-limited autotrophic nitrification/denitrification (OLAND). However, these combined systems may be difficult to operate, because several

doi: 10.2166/wst.2018.516

factors such as pH, oxidation-reduction potential (ORP), dissolved oxygen (DO) need to be controlled (Bi *et al.* 2015; Zhang *et al.* 2017) while anammox bacteria and heterotrophic denitrifiers often compete for substrate in the reactor. Therefore, the current knowledge gap concerning the improvement of anammox-based systems is how to reduce mutual competition-induced negative influence while simultaneously promoting synergetic performance between anammox and denitrification bacteria. It is hypothesized that such an improvement can be achieved by controlling the growth of anammox bacteria and heterotrophic denitrifiers in different spaces (zones) of a single reactor. To prove this hypothesis, we conducted this work with an anaerobic baffled reactor (ABR), and evaluated the effects of C/N ratios on the microbial communities and performance of different zones of the ABR.

Historically, the ABR was initially developed at Stanford University by McCarty and his co-workers to treat high strength wastewater (Bachmann *et al.* 1985; Barber & Stuckey 1999). Conceptually, the ABR has several advantages over well-established systems such as the up-flow anaerobic sludge blanket (UASB) and the anaerobic filter, including better resilience to hydraulic and organic shock loadings, longer biomass retention times, lower sludge yields, and the ability to partially separate between the various phases of anaerobic catabolism (Barber & Stuckey 1999). A typical ABR is usually divided into four or five equal compartments (Jin *et al.* 2012). Bacteria within the reactor settle because of gas production and flow characteristics (Sponza & DemiRden 2010). More importantly, the ABR can partially separate different trophies with different compartments (e.g. zones for digestion, or anammox or denitrification) while it makes the entire system function for removal of organic carbon and nitrogen. In other words, if one controls the different microorganisms in each of ABR's compartments and makes them function in sequence for nitrite denitrification + anammox + nitrate denitrification, nitrogen could be removed by an anammox-coupling-denitrification process within the ABR.

Examining the microbial community structures of the novel process is quite necessary to understand the complex interactions occurring in the ABR. Because wastewater with different C/N ratios would significantly influence the environment (e.g. aerobic or anaerobic) and different microbial communities in different compartments, we used pyrosequencing to provide significant insights into the evolution of microbial community structure in the ABR system. Pyrosequencing developed by illumine MiSeq sequencing is a high-throughput method that can generate a huge amount of DNA reads through a massively parallel sequencing-by-synthesis approach (Hu *et al.* 2012). The method has been used to identify the key microorganisms involved in complex sludge samples of laboratory-scale nitritation–anammox sequencing batch reactor (SBR) (Liang *et al.* 2014), denitrification and anammox reactor (SFDA) (Wang *et al.* 2016), ABR anammox reactor (Chen *et al.* 2016) and suspended-growth anoxic anammox reactor (Liu *et al.* 2017).

Based on these arguments, the objectives of this study were to: (1) evaluate the performance of the ABR anammox reactor under different nitrite and nitrate strength with various COD/NO_2^-N ratios; (2) assess the stability of the anammox and denitrification process in different compartments under different COD/NO_2^-N ratios; and (3) analyze the contribution of different kinds of functional bacteria to nitrogen and COD removal using MiSeq pyrosequencing.

MATERIALS AND METHODS

Chemicals and reagents

Glucose ($C_6H_{12}O_6$, 98%, CAS: 50-99-7), ammonium sulphate ($(NH_4)_2SO_4$, 98%, CAS: 7783-20-2) and sodium nitrite ($NaNO_2$, 98%, CAS: 7632-00-0) used in the this study were all purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. The substrate was weighed and dissolved in distilled water for synthetic wastewater.

Experimental setup and operation

Reactor design

An ABR (Figure 1) made of PVC was used in this study. It had a length of 37.5 cm, a width of 8 cm, a height of 33 cm and a water level of 26.5 cm with the working volume of 6.36 L. The ABR was divided into five equal-volume compartments, and had an up-flow area to down-flow area of 4:1 and a baffle angle of 45° near the bottom of the reactor. The ABR was sealed to ensure the anaerobic environment. Gas generated in every compartment was collected and passed through a water-sealed bottle for exhaustion. The whole reactor was covered with black plastic films to evade the light. The flow rate of the influent was precisely controlled by peristaltic pump (BT101S, Longer, Baoding, China). The reactor was kept in a water bath to maintain the temperature at 30 ± 1 °C (Graaf 1996).

In order to ensure the sequential occurrence of 'nitrite denitrification, anammox and nitrate denitrification' in five

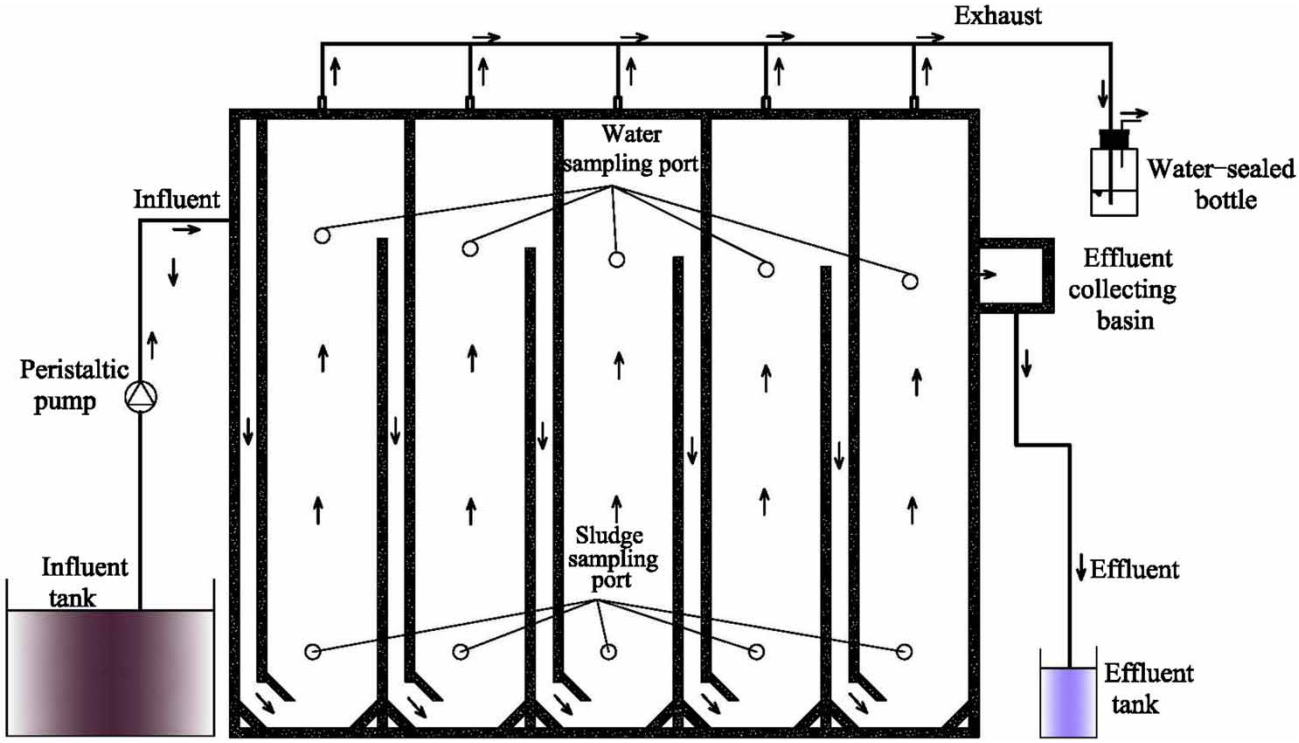

Figure 1 | Schematic diagram of the ABR system. The height of sampling ports in the upper part of the reactoris the same as that of the baffles along the direction of water flow.

compartments of the ABR, anaerobic sludge collected from a municipal wastewater treatment plant (WWTP) was inoculated into the 1st and 5th compartment, and the laboratory cultured anammox sludge was inoculated into the 2nd, 3rd and 4th compartment. The sludge in each compartment of the ABR had a mixed liquid suspended solids (MLSS) of $8.9 \pm 2.7\ g \cdot L^{-1}$ and mixed liquor volatile suspended solids (MLVSS) of $8.3 \pm 3.1\ g \cdot L^{-1}$.

Synthetic wastewater and experimental procedure

Initially, the ABR was fed with synthetic wastewater with COD, nitrite and ammonium (made with $C_6H_{12}O_6$, $(NH_4)_2SO_4$ and $NaNO_2$) together with $1\ mg \cdot L^{-1}$ trace element solutions I and II (Table 1) (Graaf 1996). As shown in Table 1, the process operation was divided into five phases. During the whole experimental period, the COD concentration in the influent was maintained at $180\ mg \cdot L^{-1}$. The total nitrogen (TN) (by adding nitrite and ammonium) was $180\ mg \cdot L^{-1}$, but the ratio of nitrite to ammonium varied in the five phases, leading to the C/N(COD/NO_2^-N) ratios of 1.64, 1.50, 1.38, 1.29 and 1.20 in phases I–V, respectively.

Table 1 | Characteristics of feed solution during the entire experimental period

Experimental phase days (d)	I 1–10	II 11–22	III 23–34	IV 35–46	V 47–57
NH_4^+-N ($mg\ L^{-1}$)	70	60	50	40	30
NO_2^-N ($mg\ L^{-1}$)	110	120	130	140	150
COD ($mg\ L^{-1}$)	180	180	180	180	180
C/N(NO_2^-N)	1.64	1.50	1.38	1.29	1.20
Trace element solution I[a]	$1\ mL\ L^{-1}$				
Trace element solution II[b]	$1\ mL\ L^{-1}$				

[a]Trace element solution I = EDTA $15\ g\ L^{-1}$ and $FeSO_4$ $5\ g\ L^{-1}$.
[b]Trace element solution II = EDTA $15\ g\ L^{-1}$, $ZnSO_4 \cdot 7H_2O$ $0.43\ g\ L^{-1}$, $CoCl_2 \cdot 6H_2O$ $0.24\ g\ L^{-1}$, $MnCl_2 \cdot 4H_2O$ $0.99\ g\ L^{-1}$, $CuSO_4 \cdot 5H_2O$ $0.25\ g\ L^{-1}$, $Na_2MoO_4 \cdot 2H_2O$ $0.22\ g\ L^{-1}$, $NiCl_2 \cdot 6H_2O$ $0.19\ g\ L^{-1}$, $Na_2SeO_4 \cdot 10H_2O$ $0.21\ g\ L^{-1}$, H_3BO_4 $0.014\ g\ L^{-1}$[23].

Analytical methods

Water sampling and analytical methods

The influent and effluent samples of the ABR were collected every two days. COD, ammonium, nitrate, and nitrite nitrogen were analyzed according to the standard methods (APHA 2012) using ultraviolet spectrophotometer (DR6000, Hach, USA). TN concentration was the sum of ammonium, nitrate and nitrite concentration. All the analyzes were

performed in duplication and the average of the analytical results were reported.

Mass balance calculations

Ammonium was barely removed by anammox process. The ratio of nitrite consumption, ammonium consumption to nitrate production was 1.32:1:0.26. The equation for contribution of anammox process to nitrogen removal θ_A is:

$$\theta_A = \frac{(1 + 1.32 - 0.26)\ \text{Ammonium}_{\text{removal}}}{TN_{removal}} \quad (1)$$

where $\text{Ammonium}_{\text{removal}}$ and $\text{TN}_{\text{removal}}$ are the average removal amount of ammonium and TN in the phase, respectively. The contribution of denitrification process to nitrogen removal θ_D is:

$$\theta_D = 1 - \theta_A \quad (2)$$

DNA extraction and polymerase chain reaction (PCR)

In this study, microbial community structure was determined on days 9 (Phase I) and 46 (Phase IV) using 16S rRNA gene Illumina MiSeq. The sludge samples (A for day 9 and B for day 46) were collected from the sludge sampling port of each compartment in the ABR. All the samples were stored at −70 °C until DNA extraction. Microbial DNA was extracted from sludge samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols. The V3-V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s and a final extension at 72 °C for 10 min) using primers 338F (5′-ACTCCTACGGGAGGCAG-CAG-3′) and 806R (5′-GGACTACHVGGGTWTC TAAT-3′), where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA.

Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using Quanti Fluor™-ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols at Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China).

Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.9.1) with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (ii) exact barcode matching, two nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; and (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded.

Operational units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimerical sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU123)16S rRNA database using a confidence threshold of 70% (Amato *et al.* 2013). Analyses for rarefaction curves, and calculation of richness estimators (Ace and Chao1) and diversity indices (Shannon and Simpson) were performed using the MOTHUR program (Schloss *et al.* 2009). All the raw reads have been deposited at NCBI Sequence Read Archive (SRA) database under the accession number of SRR131988.

RESULTS AND DISCUSSION

Reactor performance

Overall nitrogen and COD removal

Figure 2 shows the variations of nitrogen concentrations in the influent and effluent. Figure 3 shows the COD removal efficiency in the ABR. In Phases I–IV, the nitrite removal efficiency was always higher than 98% at C/N ratios about 1.64–1.29. However, the effluent nitrite concentration increased to 18 $mg \cdot L^{-1}$, and the removal efficiency decreased to 87.3 ± 0.3% in Phase V at the C/N ratio of 1.20. On the contrary, the removal efficiency of ammonium increased from 39.6 ± 14.4% in Phase I to 87.6 ± 6.8%, 94.2 ± 4.7%, 100% and 100% in Phases II–V, respectively. The average ammonium concentration of the effluent was 44.5 $mg \cdot L^{-1}$, 6.8 $mg \cdot L^{-1}$ and 2.8 $mg \cdot L^{-1}$ in Phases II and III, and was nearly zero in Phases IV and V. Unfortunately,

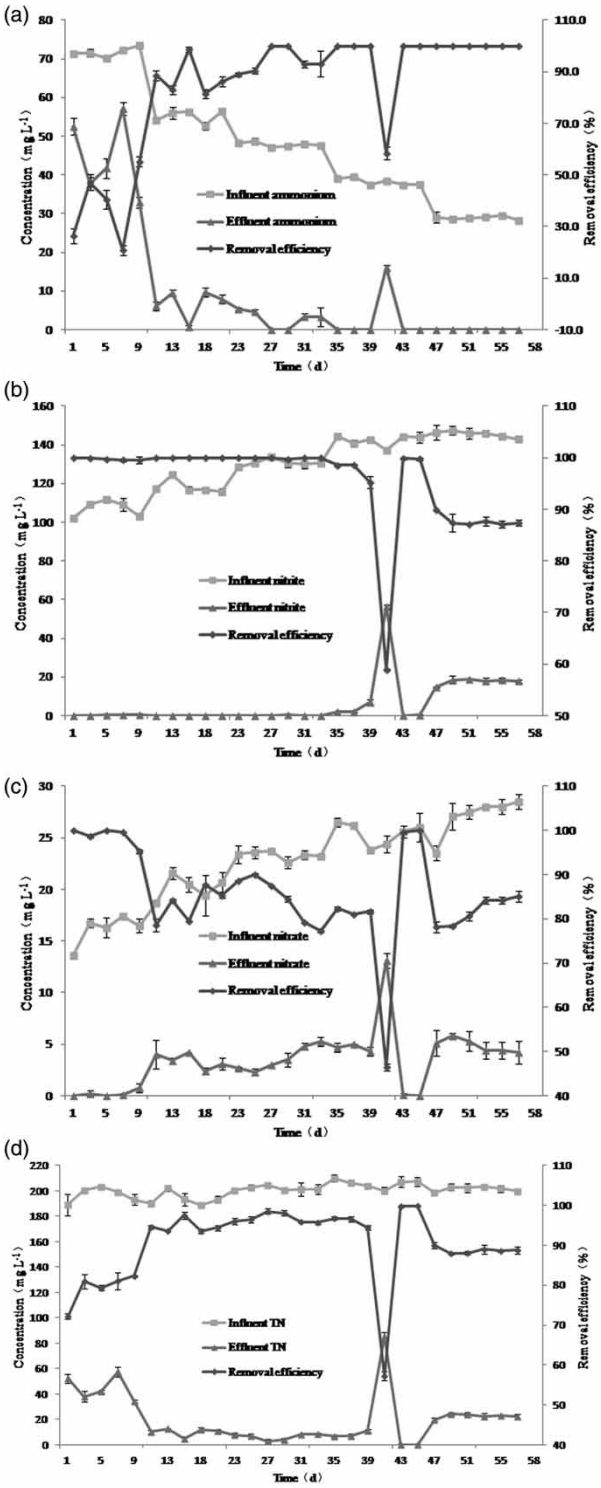

Figure 2 | Variations of influent and effluent nitrogen concentrations in the ABR under different operational phases: (a) ammonium; (b) nitrite; (c) nitrate; and (d) TN.

the removal efficiency of ammonium and nitrite decreased to 58.3% and 58.9% on day 41 at the C/N ratio of 1.29 because of accidental damage of the heating device. The anammox bacteria was suitable to cultivate at $30 \pm 1\,^{\circ}C$, the sudden decrease of temperature caused a sharp drop in anammox activity (Graaf 1996). The effluent nitrate was below detection limit in Phase I, and increased to 2–5 $mg \cdot L^{-1}$ in Phases II–V. However, the effluent nitrate concentration increased dramatically to 13.1 $mg \cdot L^{-1}$ on day 41. From Phase I to Phase II, the average TN removal efficiency of the ABR increased from 79.2% to 94.7%, and became stable above 96.8% in Phases III and IV. In addition, the TN removal efficiency decreased to 88.5% in Phase V at the C/N ratio of 1.2. When the C/N ratio was 1.29 in Phase IV, the ABR could achieve the highest TN removal efficiency at 97.5%.

The influent COD concentration was controlled at 180 $mg \cdot L^{-1}$, and the effluent COD concentration was always below 12 $mg \cdot L^{-1}$ with the removal efficiency always above 97% during the whole experimental period. Meanwhile, the COD removal efficiency increased slowly from 97.0% to 99.6% from Phase I to Phase IV, and achieved the highest in Phase IV. Same as the TN removal trend, the COD removal efficiency decreased dramatically to 84.9% on day 41. There were two main causes for these results: (1) the low temperature (under 15°C) significantly decreased the activity of denitrifying bacteria (the optimum temperature is 30 °C) (Angar *et al.* 2016); and (2) the low temperature (under 15°C) significantly decreased the activity of anammox bacteria, which caused a lower nitrate production from anammox process (Ren *et al.* 2014). However, the COD removal was associated with the nitrate production from anammox process by nitrate type denitrification.

Nitrogen removal performance of each compartment in the ABR

The variations of nitrogen in the flow path of the ABR in response to different C/N ratios were analyzed to investigate the nitrogen removal performance in the ABR anammox system (Figure 4). Most of TN was removed in the 1st and 2nd compartment, accounting for about 81.0–97.6% of total TN removal. Nitrite was removed in the 1st and 2nd compartments, while ammonium was almost completely removed in the 2nd compartment. However, almost all the nitrate in influent was removed in the 1st compartment. With a decrease in the C/N ratio, the nitrite removal position was alerted from the 1st compartment, leading to the 1st or 2nd compartment interaction, while the ammonium removal still occurred in the 2nd compartment. The nitrite concentration was lower for anammox in the 2nd compartment at a higher C/N ratio when most of nitrite was removed by nitrite type denitrification in the 1st compartment. However,

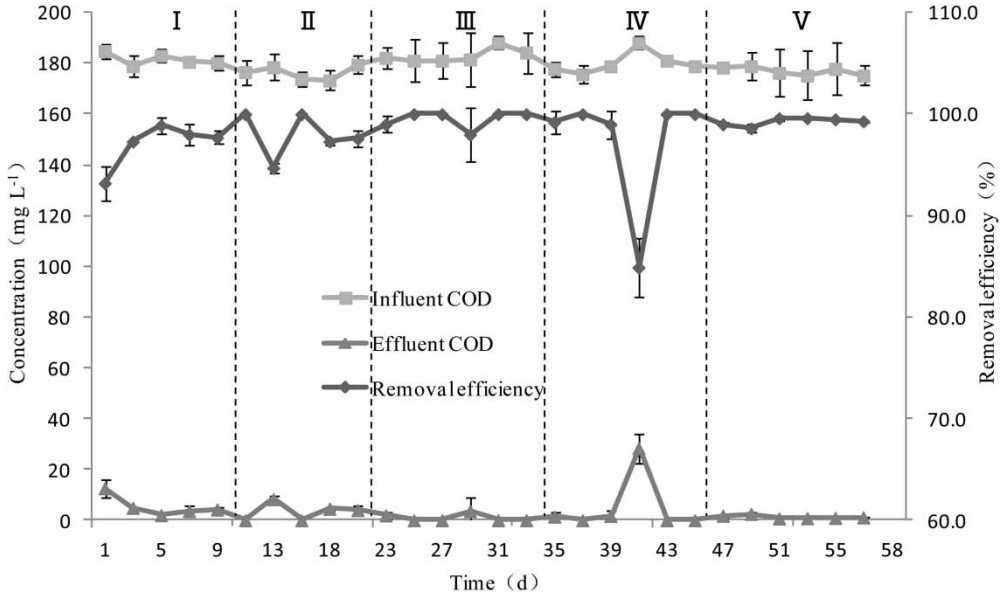

Figure 3 | COD removal efficiencies in ABR under different operational phases.

the influent nitrite nitrogen was also suitable for the anammox (nitrite: ammonium = 1.32) in the 2nd compartment after the denitrification in the 1st compartment while the C/N ratio was lower, resulting in the simultaneous removal of nitrite and ammonium nitrogen by the anammox-coupling-denitrification process in the 1st and 2nd compartments (Kumar & Lin 2010; Kindaichi *et al.* 2016).

Mass balance calculations of nitrogen removal in ABR

With the variation of C/N ratios, the nitrogen removal capacity and the contribution of different phases varied greatly. The denitrification process made a major contribution to the nitrogen removal in the whole experiment (Figure 5). The nitrogen removal contribution of denitrification decreased from 93.2% in Phase I to 57.9% in Phase III, and increased from 63.9% in Phase IV to 67.6% in Phase V. Contrary to the denitrification contribution, the nitrogen removal contribution by anammox was 6.8%, 45.6%, 42.1%, 36.1% and 32.4% in Phases I–IV, respectively. Therefore, the anammox contribution was highest in Phase II (C/N ratio of 1.50) and decreased from Phase II to Phase V (C/N ratio from 1.50 to 1.20). In Phase I, 180 mg/L of COD was added to the anammox system and the nitrogen removal contribution of anammox reaction was low. With the gradual adaptation of organic carbon source in ABR reactor, the anammox reaction gradually enhanced with the removal efficiency up to 45.6%. However, the ratio of nitrite to ammonium was increased from 2.00 in Phase II to 5.00 in Phase V, and ammonium concentration of influent was deceased. The limitation of ammonium led to the gradual decline of anammox contribution for nitrogen removal.

Composition of microbial community

The ABR could achieve the highest TN removal efficiency when the C/N ratio was 1.29 in Phase IV. Therefore, the sludge from each compartment of the ABR in Phases I and IV was collected. To investigate the community structure of total bacteria, Illumina MiSeq sequencing was analyzed, yielding 20,990–37,329 clustered operational taxonomic units (OTUs) at a dissimilarity level of 0.03 (Table 2). To compare the bacterial diversity among different samples, Chao1 estimator and Shannon's diversity indices were calculated. Chao1 estimator is defined as a theoretical lower bound of species richness under a commonly used multinomial model (Chao *et al.* 2015). Shannon's diversity indices are combined for both bacterial richness and evenness, and are responsive to rare species in terms of species richness (Strous *et al.* 1999; Murphy *et al.* 2017). Chao1 values showed that the variation of C/N ratios decreased the richness in the 1st compartment while increasing the richness in the other four compartments. However, Shannon index showed that the richness in all the compartments decreased with the variation of C/N ratios.

The effective bacterial sequences in each sample were assigned to different taxa levels using RDP classifier at 85% threshold. The abundances of different phyla in

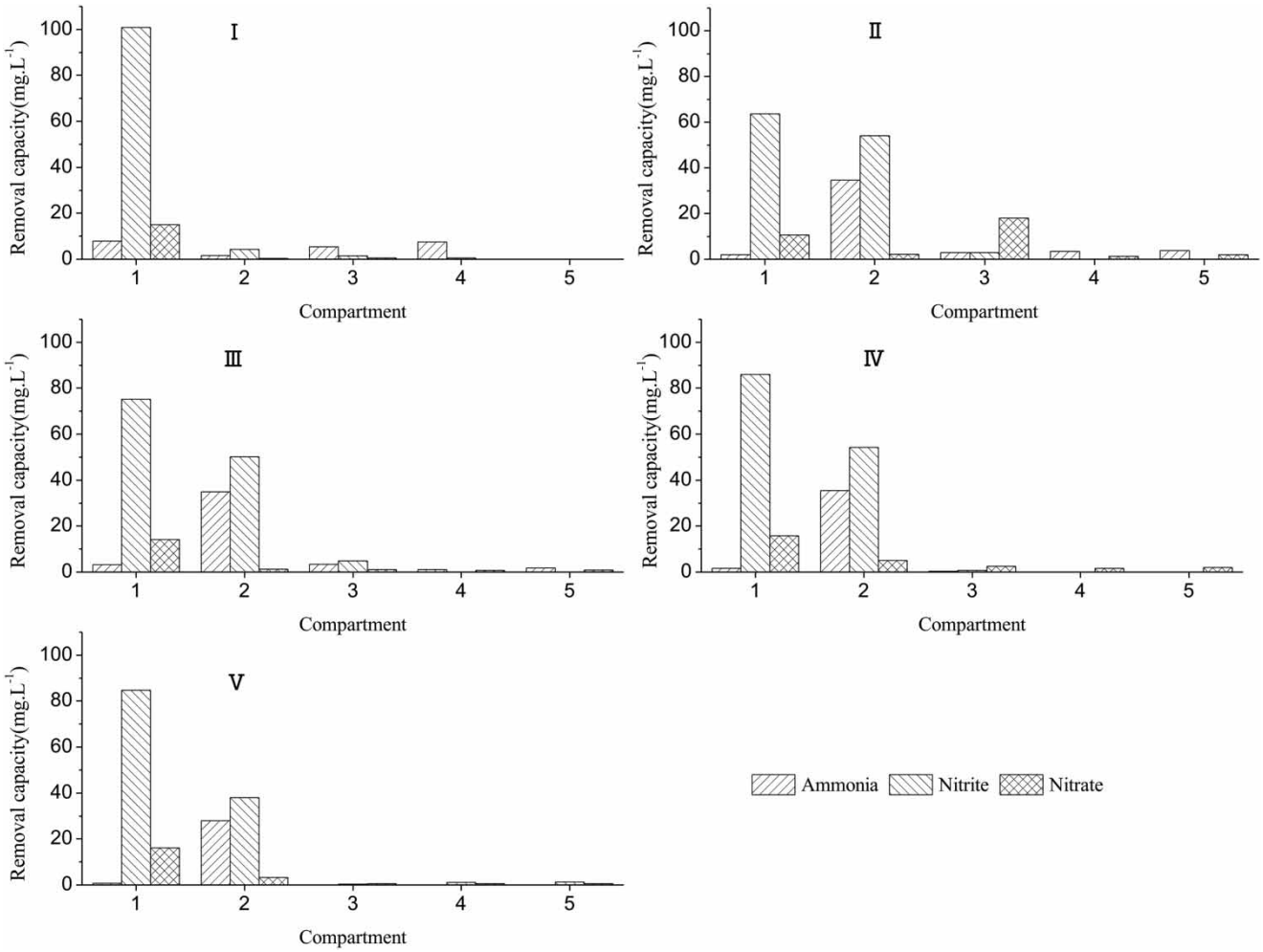

Figure 4 | Distribution of nitrogen removal in each compartment of the ABR reactor.

samples from different compartments of the ABR are shown in Figure 6. In the samples A1 to A5 from Phase I, the phylum Proteobacteria (22.8–59.7%) was predominant in all samples, followed by Chloroflexi (15.1–22.1%) and Chlorobi (2.5–24.0%). However, the phylum Chloroflexi (28.0–54.0%) replaced the Proteobacteria (9.5–21.5%) as the dominant population in the ABR in Phase IV, and the phylum Chloroflexi accounted for 3.7–25.1%. The Chloroflexi bacteria were facultative anaerobes frequently found in anaerobic reactors which may be important for granulation and present in a highly enriched way in anammox biomass (Cho *et al.* 2010). In addition, the Chloroflexi bacteria were also found to be able to consume organic matter in a methane fermentation system (Qiao *et al.* 2008). However, the role of Chloroflexi bacteria in the ABR is unknown. The phylum Proteobacteria was highly represented in samples A1 and B1, which were collected from the 1st compartment of the ABR. This result indicated that denitrification was always important in the 1st compartment for nitrite and COD removal. The Planctomycetes, which was related to anammox bacteria, was more enriched in samples A2 (3.5%), A3 (5.1%) and A4 (2.6%), and increased to 6.6–43.1% in Phase IV, B2 (6.6%), B3 (10.2%), B4 (14.9%) and B5 (43.1%), respectively. It was worth mentioning that the Planctomycetes in sample B5 was dominant, but the underlying reason needs to be further investigated. Meanwhile, the change of microorganisms in each compartment was consistent with the profile of nitrogen removal. The denitrification bacteria always existed and decreased with a decrease in C/N ratio. However, the anammox bacteria existed in the 2nd, 3rd and 4th compartment and increased as the C/N ratio decreased.

At the genus level, the *Candidatus* Brocadia, *Candidatus* Jettenia, *Candidatus* Kuenenia were detected in the

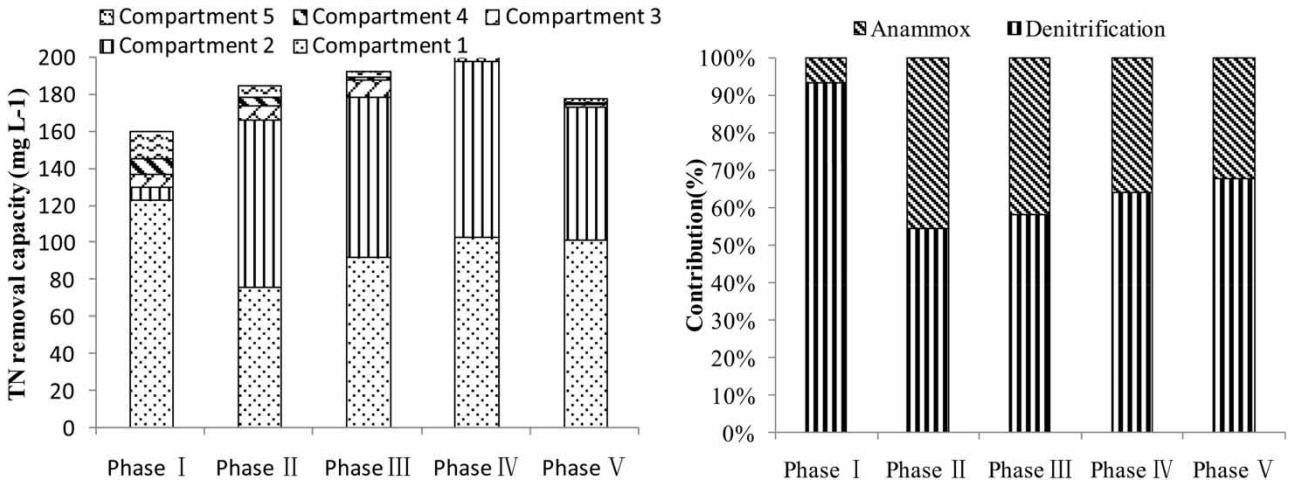

Figure 5 | Total nitrogen removal capacity and contribution rate in different phase.

Table 2 | Richness and diversity estimators of microbial communities in the ABR

Sample*	Sequence	Chao1	ACE	Shannon	Simpson
A-1	22,959	431	412	3.75	0.061
A-2	21,077	485	501	4.02	0.048
A-3	23,226	573	558	4.19	0.047
A-4	20,990	633	631	4.53	0.033
A-5	21,483	542	548	4.44	0.035
B-1	23,824	355	358	2.62	0.283
B-2	32,022	568	576	3.88	0.078
B-3	23,159	661	642	3.97	0.073
B-4	37,329	668	672	4.42	0.041
B-5	22,020	586	573	2.74	0.228

*$\alpha = 0.03$. Sample A was obtained at day 7 and Sample B at day 46. A-1 means sample A from the 1st compartment, while B-5 means from sample B from the 5th compartment.

samples (Figure 7), which was consistent with other sludge samples from a suspended-growth anoxic anammox reactor treating low-strength wastewater at low temperatures (Liu *et al.* 2017). The *Candidatus* Brocadia displayed a low abundance of 1.0–2.9% in samples A1–A3 in Phase I (day 9), and increased to 2.8–9.1% in samples B1–B3 in Phase IV (day 46). As the anammox process is mediated by bacteria belonging to the family Brocadiaceae (Kuenen 2008), the anammox activity could be indicated by the abundance of Brocadiaceae. In a recent study, the *Candidatus* Brocadia was detected in a denitrification–anammox synergistic reactor (Peng *et al.* 2017), an anammox-UASB reactor with low-strength wastewater treatment (Reino & Carrera 2016), an anammox biofilm reactor (Zekker *et al.* 2016) and so on. However, the *Candidatus* Jettenia dramatically increased to 40.4% of all the genus level bacteria in sample B5 in Phase IV. The exact reason for this sudden increase is unknown at this stage.

Implications

In this study, the ABR with phase separation was used to achieve the carbon and nitrogen removal synchronously via an anammox-coupling-denitrification process. Based on the suitable C/N ratio of influent, the highest TN removal efficiency could achieve up to 99.9%. In this ABR, the inhibition effect of organic compounds on anammox was reduced, while the removal rate of TN was improved by differentiating treatment processes in different compartments. Therefore, results of this study provide a new way to maintain the high activity of anammox and achieve higher TN removal even if the reactor is under constraint of high organic matter strength. However, future studies should be carried out for clarifying the carbon and nitrogen removal of each compartment and for optimizing the ABR's structure.

CONCLUSIONS

The C/N (NO_2^-N) ratio exerted an important effect on nitrogen removal through the anammox-coupling-denitrification process. When the C/N ratio was 1.29, the ABR could achieve the highest TN removal efficiency of 99.9%. Most of TN was removed in the 1st and 2nd compartments, accounting for about 81.0–97.6% of total TN removal. Based on the nitrogen balance, the nitrogen removal resulted from the interaction associated with the

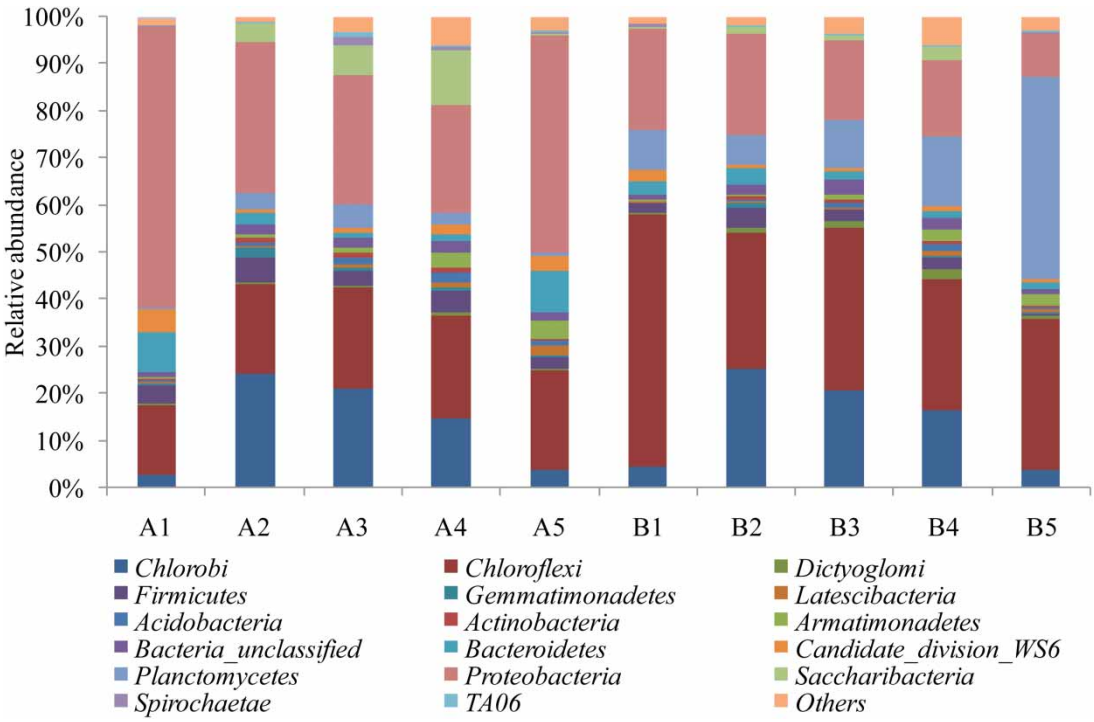

Figure 6 | Abundances of different phyla in samples from different compartments of ABR. Relative abundance is defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Phyla with their maximum abundance less than 1% in any sample are regarded as minor phyla.

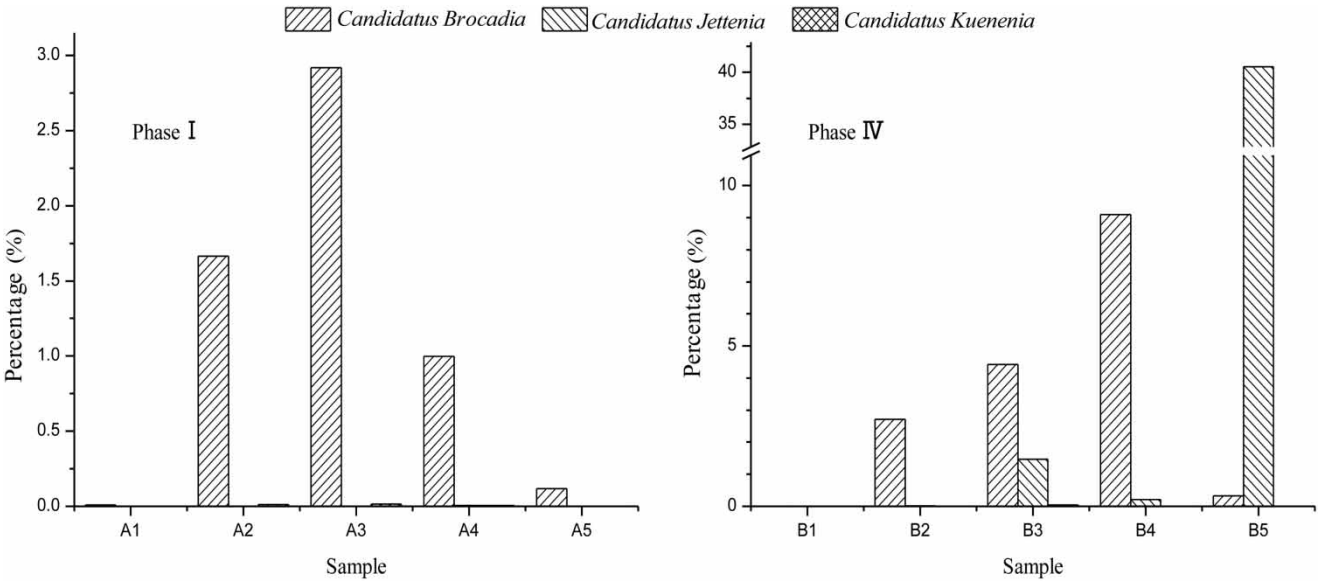

Figure 7 | Distribution of different anammox bacteria in samples from different compartments of ABR.

anammox-coupling-denitrification process. The nitrogen removal contributed by anammox varied from 6.8% to 32.4%. High-throughput MiSeq sequencing analyses revealed that the C/N ratio was an important factor regulating the bacterial community structure, and the predominant phylum changed from Proteobacteria to Chloroflexi with the elevated C/N ratio. In addition, the *Candidatus* Brocadia was the major anammox bacteria

and its percentage varied from 1.0–2.9% on day 9 to 2.8–9.1% on day 46.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (No. 51508366, No. 51508367, No. 51578353), Natural Science Foundation of Jiangsu Province (No. BK20140282) and Opening Fund of Jiangsu Key Laboratory of Anaerobic Biotechnology (Jiangnan University) (KFLAB201701).

REFERENCES

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Gaskins, H. R., Stumpf, R. M., Yildirim, S. & Torralba, M. 2013 Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *The ISME Journal* **7** (7), 1344–1353.

Angar, Y., Kebbouche-Gana, S., Djelali, N. E. & Khemili-Talbi, S. 2016 Novel approach for the ammonium removal by simultaneous heterotrophic nitrification and denitrification using a novel bacterial species co-culture. *World Journal of Microbiology and Biotechnology* **32** (3), 36.

APHA 2012 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC, USA.

Bachmann, A., Beard, V. L. & McCarty, P. L. 1985 Performance characteristics of the anaerobic baffled reactor. *Water Research* **19** (1), 99–106.

Barber, W. P. & Stuckey, D. C. 1999 The use of the anaerobic baffled reactor (ABR) for wastewater treatment: a review. *Water Research* **33** (7), 1559–1578.

Bi, Z., Takekawa, M., Park, G., Soda, S., Zhou, J., Qiao, S. & Ike, M. 2015 Effects of the C/N ratio and bacterial populations on nitrogen removal in the simultaneous anammox and heterotrophic denitrification process: mathematic modeling and batch experiments. *Chemical Engineering Journal* **280**, 606–613.

Chao, A., Chiu, C. H., Hsieh, T. C., Davis, T., Nipperess, D. A. & Faith, D. P. 2015 Rarefaction and extrapolation of phylogenetic diversity. *Methods in Ecology and Evolution* **6** (4), 380–388.

Chen, C. J., Sun, F. Q., Zhang, H. Q., Wang, J. F., Shen, Y. L. & Liang, X. Q. 2016 Evaluation of COD effect on anammox process and microbial communities in the anaerobic baffled reactor (ABR). *Bioresource Technology* **216**, 571–578.

Cho, S., Takahashi, Y., Fujii, N., Yamada, Y., Satoh, H. & Okabe, S. 2010 Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. *Chemosphere* **78** (9), 1129–1135.

Dapena-Mora, A., Fernández, I., Campos, J. L., Mosquera-Corral, A., Méndez, R. & Jetten, M. S. M. 2006 Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology* **40** (4), 859–865.

Graaf, A. A. V. D. 1996 Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology* **142** (8), 2187–2196.

Hien, N. N., Tuan, D. V., Nhat, P. T., Van, T. T. T., Tam, N. V., Que, V. O. N. X. & Dan, N. P. 2017 Application of oxygen limited autotrophic nitritation/denitrification (OLAND) for anaerobic latex processing wastewater treatment. *International Biodeterioration & Biodegradation* **124**, 45–55.

Hu, M., Wang, X. H., Wen, X. H. & Xia, Y. 2012 Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. *Bioresource Technology* **117** (10), 72–79.

Jetten, M. S. M., Strous, M., Pas-Schoonen, K. T. V. D., Schalk, J., Dongen, U. G. J. M. V., Graaf, A. A. V. D., Logemann, S., Muyzer, G., Loosdrecht, M. C. M. V. & Kuenen, J. G. 1998 The anaerobic oxidation of ammonium. *FEMS Microbiology Reviews* **22** (5), 421–437.

Jin, R. C., Yu, J. J., Ma, C., Yang, G. F., Hu, B. L. & Zheng, P. 2012 Performance and robustness of an ANAMMOX anaerobic baffled reactor subjected to transient shock loads. *Bioresource Technology* **114** (3), 126–136.

Kindaichi, T., Awata, T., Mugimoto, Y., Rathnayake, R. M. L. D., Kasahara, S. & Satoh, H. 2016 Effects of organic matter in livestock manure digester liquid on microbial community structure and in situ activity of anammox granules. *Chemosphere* **159**, 300–307.

Kuenen, J. G. 2008 Anammox bacteria: from discovery to application. *Nature Reviews Microbiology* **6** (4), 320–326.

Kumar, M. & Lin, J. G. 2010 Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal–Strategies and issues. *Journal of Hazardous Materials* **178**, 1–9.

Lackner, S., Gilbert, E. M., Vlaeminck, S. E., Joss, A., Horn, H. & van Loosdrecht, M. C. M. 2014 Full-scale partial nitritation/anammox experiences–an application survey. *Water Research* **55** (10), 292–303.

Liang, Y. H., Li, D., Zhang, X. J., Zeng, H. P., Yang, Z. & Zhang, J. 2014 Microbial characteristics and nitrogen removal of simultaneous partial nitrification, anammox and denitrification (SNAD) process treating low C/N ratio sewage. *Bioresource Technology* **169** (5), 103–109.

Liu, W. R., Yang, D. H., Chen, W. J. & Gu, X. 2017 High-throughput sequencing-based microbial characterization of size fractionated biomass in an anoxic anammox reactor for low-strength wastewater at low temperatures. *Bioresource Technology* **213**, 45–52.

Molinuevo, B., García, M. C., Karakashev, D. & Angelidaki, I. 2009 Anammox for ammonia removal from pig manure effluents: effect of organic matter content on process performance. *Bioresource Technology* **100** (7), 2171–2175.

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C. A., Dempsey, E. M., O'Toole, P. W., Ross, R. P., Ryan, C. A. &

Stanton, C. 2017 The composition of human milk and infant faecal microbiota over the first three months of life: a pilot study. *Scientific Reports* **7**, 40597.

Peng, S., Zhang, L., Zhang, D. J., Lu, P., Zhang, X. & Qiang, H. 2017 Denitrification synergized with ANAMMOX for the anaerobic degradation of benzene: performance and microbial community structure. *Applied Microbiology and Biotechnology* **101**, 4315–4325.

Qiao, S., Kawakubo, Y., Cheng, Y., Nishiyama, T., Fujii, T. & Furukawa, K. 2008 Anammox process for synthetic and practical wastewater treatment using a novel kind of biomass carriers. *Water Science & Technology* **58** (6), 1335–1341.

Reino, C. & Carrera, J. 2016 Low-strength wastewater treatment in an anammox UASB reactor: effect of the liquid upflow velocity. *Chemical Engineering Journal* **313**, 217–225.

Ren, Y. H., Li, D., Li, X. K., Liu, Y., Ding, A. & Zhang, J. 2014 High-rate nitrogen removal and microbial community of an up-flow anammox reactor with ceramics as biomass carrier. *Chemosphere* **113** (113), 125–131.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H. & Robinson, C. J. 2009 Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied Environmental Microbiology* **75** (23), 7537–7541.

Sponza, D. T. & DemiRden, P. 2010 Relationships between chemical oxygen demand (COD) components and toxicity in a sequential anaerobic baffled reactor/aerobic completely stirred reactor system treating Kemicetine. *Journal of Hazardous Materials* **176** (1–3), 64–75.

Strous, M., Kuenen, J. G. & Jetten, M. S. M. 1999 Key physiology of anaerobic ammonium oxidation. *Applied Environmental Microbiology* **65** (7), 3248–3250.

Tal, Y., Watts, J. E. M. & Schreier, H. J. 2006 Anaerobic ammonium-oxidizing (anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system. *Applied Environmental Microbiology* **72** (4), 2896–2904.

Tang, C. J., Zheng, P., Ding, S. & Lu, H. F. 2014 Enhanced nitrogen removal from ammonium-rich wastewater containing high organic contents by coupling with novel high-rate ANAMMOX granules addition. *Chemical Engineering Journal* **240** (6), 454–461.

Tang, C. J., Duan, C. S., Yu, C., Song, Y. X., Chai, L. Y., Xiao, R., Wei, Z. & Min, X. B. 2017 Removal of nitrogen from wastewaters by anaerobic ammonium oxidation (ANAMMOX) using granules in upflow reactors. *Chemical Engineering Journal* **240** (6), 454–461.

Volcke, E. I., Van Hulle, S. W., Donckels, B. M., van Loosdrecht, M. C. M. & Vanrolleghem, P. A. 2005 Coupling the SHARON process with anammox: model-based scenario analysis with focus on operating costs. *Water Science Technology* **52** (4), 107–115.

Wang, J. L. & Kang, J. 2005 The characteristics of anaerobic ammonium oxidation (ANAMMOX) by granular sludge from an EGSB reactor. *Process Biochemistry* **40** (5), 1973–1978.

Wang, B., Peng, Y. Z., Guo, Y. Y., Zhao, M. Y. & Wang, S. Y. 2016 Illumina MiSeq sequencing reveals the key microorganisms involved in partial nitritation followed by simultaneous sludge fermentation, denitrification and anammox process. *Bioresource Technology* **207**, 118–125.

Wr, V. D. S., Abma, W. R., Blommers, D., Mulder, J. W., Tokutomi, T., Strous, M., Picioreanu, C. & van Loosdrecht, M. C. M. 2007 Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. *Water Research* **41** (18), 4149–4163.

Zekker, I., Rikmann, E., Mandel, A., Kroon, K., Seiman, A., Mihkelson, J., Tenno, T. & Tenno, T. 2016 Step-Wise temperature decreasing cultivates a biofilm with high nitrogen removal rates at 9°C in short- term anammox biofilm tests. *Environmental Technology* **37** (15), 1–43.

Zhang, F. Z., Peng, Y. Z., Miao, L., Wang, Z., Wang, S. Y. & Li, B. K. 2017 A novel simultaneous partial nitrification Anammox and denitrification (SNAD) with intermittent aeration for cost-effective nitrogen removal from mature landfill leachate. *Chemical Engineering Journal* **313**, 619–628.

First received 3 September 2018; accepted in revised form 7 December 2018. Available online 17 December 2018