Upgrading of the symbiosis of *Nitrosomonas* and anammox bacteria in a novel single-stage partial nitrification–anammox system: Nitrogen removal potential and Microbial characterization

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ABSTRACT

A novel single-stage partial nitrification–anammox process equipped with porous functional suspended carriers was developed at 25 °C in a CSTR by controlling dissolved oxygen < 0.3 mg/L. The nitrogen removal performance was almost unchanged over a nitrogen loading rate ranging from 0.5 to 2.5 kg NH₄⁺-N/m³/d with a high nitrogen removal efficiency of 81.1%. The specific activity of AOB and anammox bacteria was of 3.00 g-N/g-MLVSS/d (the suspended sludge), 3.56 g-N/g-MLVSS/d (the biofilm sludge), respectively. The results of pyrosequencing revealed that *Nitrosomonas* (5.66%) and *Candidatus_Kuenenia* (4.95%) were symbiotic in carriers while *Nitrosomonas* (40.70%) was predominant in the suspended flocs. Besides, two specific types of heterotrophic filamentous bacteria in the suspended flocs (*Haliscomenobacter*) and the functional carrier biofilm (*Longilinea*) were shown to confer structural integrity to the aggregates. The novel single-stage partial nitrification–anammox process equipped with functional suspended carriers was shown to have good potential for the nitrogen-rich wastewater treatment.

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1. Introduction

Another alternative nitrogen removal route to traditional that of the more conventional nitrification/denitrification process (N/DN) is the anaerobic ammonium oxidation (anammox) process (Strous et al., 1999). In this process, ammonium is utilized as the electron donor for the reduction of nitrite, yielding nitrogen gas and nitrate as the final products. The drawback of this process, however, is its high dependency on the availability of nitrite, which is not common in real wastewaters. When the anammox is preceded by partial nitrification using a pretreatment process with only 57% of ammonium oxidation to nitrite by ammonia-oxidizing bacteria (AOB) (Liu et al., 2014), not only are the shortcomings of the anammox process overcome, but the environmental impact of wastewater treatment processes is significantly reduced. The organic carbon and aeration requirements have been reported to decrease by 100% and 60%, respectively (van Loosdrecht and Salem, 2006). Since the shortest doubling time of anammox bacteria was reported to be a long 3.3 days (Lotti et al., 2014), sludge production may be reduced by as much as 90% due to this extremely low growth yield of anammox bacteria and also of AOB. Moreover, The N_2O emission from the full-scale partial nitrification–anammox process was reported to be lower than that from the activated sludge system (Kampschreur et al., 2009). Over the last decade, improvements to the partial nitrification–anammox process have resulted in its application to the full-scale treatment of high-strength ammonium wastewater with low C/N ratios and elevated temperatures. Among the over 100 operating installations worldwide (Lackner et al., 2014), 88% proceed as a single-stage process with both AOB and anammox bacteria in a single reactor. The completely autotrophic nitrogen removal over nitrite (C-ANON) is an example of the many examples of a single-stage nitrification and anammox processes, another example is oxygen limited autotrophic nitrification and denitrification (OLAND), and yet another is the Deammon system used in Hattingen, Germany (Ling, 2009; Rosenwinkel and Cornelius, 2005; Third et al., 2001; Vlaeminck et al., 2009).

The challenges in the application of the single-stage partial nitrification and anammox process are that distinct habitats are required for the AOB and anammox bacteria for the aerobic and anaerobic activities and that nitrite oxidizing bacteria (NOB) competes with AOB or anammox bacteria for the substrate. It has been reported that the difficulties could be overcome by immobilizing bacteria and washing out NOB under a low dissolved oxygen (DO) concentration (< 1.0 mg/L) (Lin et al., 2014). The schemes of immobilizing bacteria include forming a granular biomass (Niu et al., 2016; Wang et al., 2017), the entrapment of biomass into gel pellets (Isaka et al., 2013; Magrí et al., 2012) and attaching a carrier forming biofilm on the surface of (Chen et al., 2009; Daverey et al., 2015, 2013). Symbiosis and the enrichment of AOB and anammox bacteria has been shown in the aerobic outer-layer and anaerobic inner-layer of the granular, gels, and in the biofilm, with a low risk of biomass wash-out (Magrí et al., 2012). Of the three schemes, Ren et al. (2014) reported that the biofilm is much simpler and more effective due to the improved ability to retain biomass in the reactor and the large contribution towards total reactor activity. Numerous reports have been reported focused on the various carriers which can be used in the biofilm single-stage process, such as the Kaldnes K1 carrier, a nonwoven carrier, and a ceramics-grain carrier (Daverey et al., 2015; Liu et al., 2017; Ren et al., 2014). Various reactor configurations have been employed in the single-stage partial nitrification and anammox process, including the rotating biological reactor (Rosenwinkel and Cornelius, 2005), the sequencing batch reactor (Chu et al., 2015; Daverey et al., 2015), and the membrane bioreactor (van der Star et al., 2008).

However, the reported affordable nitrogen loading rate in the previous single-stage partial nitrification–anammox biofilm systems was relatively low (0.03 – 1.5 kg-N/ m^3 /d) (Chu et al., 2015; Daverey et al., 2015, 2013; Zhang et al., 2015a,b; Zheng et al., 2016), which may make

it difficult for the need for nitrogen rich wastewater treatment to be met. The continuous stirred tank reactor (CSTR) equipped with functional suspended carriers may be suitable for the nitrogen rich wastewater treatment by single-stage partial nitrification–anammox. On one hand, a CSTR reactor could provide a completely mixed state of flocs and a carrier biofilm with a substrate, without the risk of excess substrate spatially, which is contribute to enduring a high loading rate. On the other hand, porous functional suspended carriers with a large specific area could help in the accumulation of anammox bacteria with a relatively long doubling time, which is helpful for sludge retention.

The purpose of this study was to set up a lab-scale novel single-stage partial nitrification–anammox process in a CSTR reactor equipped with porous functional suspended carriers at 25°C . The nitrogen removal potential and process stability was then investigated for gradual increases in the nitrogen loading rate (NLR) (0.5 – 2.5 kg NH_4^+ -N/ m^3 /d). Finally, the functional bacteria activity in the reactor was assessed from a macro aspect and the bacterial diversity and abundance of prevalent bacteria was characterized by pyrosequencing for a better understanding of the effect of functional carriers on the process performance as a reference for the design of full-scale plants.

2. Material and method

2.1. Functional suspended carrier

The functional suspended carrier applied in this study was a hollow cylinder made of hydrophobic polypropylene (pp) resin with good adherence to the microorganism assured by special porous foam. The carrier was $4\text{ mm } \Phi \times 4\text{ mm L}$, with a true specific gravity of 0.98 g/cm^3 and a specific surface area of $1500\text{ m}^2/\text{m}^3$. Before the reactor was dosed with the bio-carriers, they were immersed in a neutral cleaning solution for 24 h, to promote hydrophilic properties and thereby enhance both the flow of the carrier in the reactor and the contact between the carrier and the liquid phase.

2.2. Experimental design and setup

As shown in Fig. 1, the single-stage partial-anammox process was carried out in a lab-scale CSTR reactor made of transparent polyvinyl chloride (PVC) plastic with a working volume of 5 L and a 1 L clarifier (Hojo et al., 2016). The temperature in the reactor was set and kept in the range of $25 \pm 1^\circ\text{C}$ by a heater with temperature adjuster set in the reaction zone. Influent was introduced into the reaction zone with a peristaltic pump. Air was supplied using an air pump through a glass tube, serving both to mix and oxygenate the sewage. The porous polypropylene carriers (packing ratio: 30 v%) in the reactor were in a continuous movement with horizontal and vertical mixing due to the air supplied through a glass tube inset into the bottom of the reactor. The aeration rate (AR) was adjusted by a glass rotameter in pace with NLR variations. Sensors for temperature, pH, oxidation reduction potential (ORP) (HORIBA D-72, Japan), and DO (HORIBA OM-70, Japan) were installed in the reactor for the on-line monitoring of pH, ORP and DO, respectively.

The reactor was operated in seven stages (Table 1). During the first four stages, the NLR were stepwise increased from 0.5 to 1.0 kg/ m^3 /d by shortening the HRT from 12 h to 6 h with a constant substrate concentration of NH_4^+ -N to 250.0 mg/L. In the last three stages, the NLR was gradually increased to 2.5 kg/ m^3 /d by increasing the substrate concentration of NH_4^+ -N to 625.0 mg/L with a constant HRT of 6 h.

2.3. Influent wastewater composition

During the whole operation period, the reactor was continuously fed with a synthetic influent, containing ammonium as the single nitrogen source, mineral salts, and no organic carbon in order to minimize denitrification effects. NH_4^+ -N was supplemented in the form of

Fig. 1. Schematic drawing of single-stage partial nitritation and anammox system (biofilm carriers packing ratio: 30 v%).

Table 1
Operation conditions in reactor

Stage	Duration (d)	NLR (kg/m ³ /d)	NH ₄ ⁺ -N (mg/L)	HRT (h)
I	1–193	0.5	250.0	12
II	194–377	1.0	250.0	6
III	378–402	1.5	375.0	6
IV	403–422	2.0	500.0	6
V	424–434	2.5	625.0	6
VI	435–460	2.0	500.0	6
VII	461–470	2.5	625.0	6

NH₄HCO₃. The ratio of HCO₃[−] to NH₄⁺ of synthetic influent was fixed at 2.0 by adding approximately equal amounts of NaHCO₃ to NH₄HCO₃. The mineral medium of synthetic influent was made as follows: 0.081 g/L KH₂PO₄, 0.214 g/L Na₂HPO₄·12H₂O, 0.036 g/L CaCl₂·2H₂O, 0.051 g/L MgCl₂·6H₂O, and 1 mL/L trace element (8.304 g/L Na₂EDTA·2H₂O, 5.000 g/L FeSO₄·7H₂O, 0.215 g/L ZnSO₄·7H₂O, 0.120 g/L CoCl₂·6H₂O, 0.495 g/L MnCl₂·4H₂O, 0.125 g/L CuSO₄·5H₂O, 0.110 g/L Na₂MoO₄·2H₂O, 0.095 g/L NiCl₂·6H₂O, 0.078 g/L Na₂SeO₃, 0.007 g/L H₃BO₄). The pH in the substrate ranged between 7.8 and 8.7.

2.4. Inoculum sludge

The seed sludge was obtained from a partial nitritation reactor maintained long-term for over 450 days at low DO concentration (0.1–0.8 mg/L) (Liu et al., 2014) with a nitrogen removal efficiency of about 50%. The initial mixed liquor suspended solid (MLSS) concentration of the reactor was set at 2000 mg/L.

2.5. Activity tests

Activity tests of and the AOB, NOB and anammox bacteria, of the biomass from biofilm and suspended were performed. 50 mL of suspended or 100 pieces of carrier were collected from the continuous reactor at stage II (Day 373).

The specific activity of AOB and NOB (SAOA, SNOA) were performed in 200 mL Erlenmeyer flasks at a temperature of 30 ± 1 °C (Kristensen et al., 1992). The composition of the nutrient solution was

the same as the synthetic wastewater in the continuous reactor, but the initial concentration of NH₄⁺-N or NO₂[−]-N was 150 mg/L in all Erlenmeyer flasks. Non-O₂-limiting conditions (DO concentration, > 2 mg/L) were used. The mixed liquor was sampled at 0.5 h intervals, filtered through a 0.45 μm polyether sulfone filter, and analyzed for NH₄⁺-N, NO₂[−]-N, NO₃[−]-N. The MLSS and mixed liquor volatile suspended solids (MLVSS) of all the flasks were determined at the end of the tests.

The specific anammox activity (SAA) experiments were carried out using 120 mL serum bottles (Niu et al., 2016). A substrate consisting of NH₄⁺-N (70 mg/L) and NO₂[−]-N (90 mg/L) was added along with other chemicals to match the composition of synthetic wastewater in the continuous experiment. After the liquid phase and carriers were added, the serum bottles were closed and flushed with high-purity nitrogen gas. Then, all the bottles were placed in a water shaking bath (70 ± 5 rpm) at 33 ± 1 °C (BT 100, Yamato, Japan). The liquor samples were taken at regular intervals and concentrations of NH₄⁺-N, NO₂[−]-N, NO₃[−]-N in samples were analyzed. Finally, the concentration of protein in the biomass was measured to calculate the MLVSS of each activity test. In accordance with the reported protein content in waste activated sludge, at 40–63% of VSS (Li and Noike, 1987; Sears et al., 2005), the protein concentration divided by 0.5 is considered to be equal to the MLVSS concentration.

2.6. Microbial community analysis

The samples (suspended floc and carrier biomass) were collected from the reactor during stage II and were immediately frozen at −20 °C until DNA extraction. Microbial DNA was directly extracted from the samples using an Ultra Clean Soil DNA Isolation Kit (MO-BIO) according to the manufacturer's instructions. For pyrosequencing, the isolated DNA were amplified with the modified forward primer 5'-CCTAYGGGRBGCASCAG-3' and a revise primer of 3'-GGACTACHVGGGTHTCTAAK-5'. The polymerase chain reaction (PCR) amplifications were run under the following cycling conditions: 5 min initial denaturation at 95 °C, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and were completed with a final extension at 72 °C for 10 min. The composition of PCR productions of V3–V4 regions of 16S rRNA was

Fig. 2. Variation of nitrogen compounds concentrations and evolution of AOR, NOR, ANR, NRE and ratio of $\text{NO}_3^- \text{-N} / \Delta \text{NH}_4^+ \text{-N}$.

determined by pyrosequencing using the Roche 454 FLX Titanium sequencer (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA). The raw reads were all treated with the 454 Pyrosequencing SOP, compared with Sliva (silva_v123 as proved by official website) and clustered by the Ribosomal Database Project (RDP) (Claesson et al., 2009). The selected reads were all treated with chimera removal and linguine removal. Sequences were denoised using the 'pre.cluster' command in the Mothur platform to remove the sequences likely to be pyrosequencing errors (Huse et al., 2010; Roeselers et al., 2011). Eventually, the numbers of high quality sequences were 6047 and 4664 in samples from suspended floc and carrier biomass with an average length of 500 bp. To facilitate the comparison between different samples, the numbers of sequences were normalized to the same sequencing depths of 4664 by Mother program (Roeselers et al., 2011). Then a taxonomic classification of the bacterial sequences was carried out using the RDP Classifier. The effective sequences were clustered into operational taxonomic units (OTUs) at 97% using the standard operation procedure of the Mothur and Qiime programs (Roeselers et al., 2011). Based on the grouping of unique OTUs, the frequency data for each distance level was used to construct rarefaction curves and diversity indices.

2.7. Chemical analysis

Samples were taken from both the influent and effluent every other day. Concentrations of $\text{NH}_4^+ \text{-N}$, $\text{NO}_2^- \text{-N}$, $\text{NO}_3^- \text{-N}$ were analyzed by capillary electrophoresis (Agilent 7100) (Liu et al., 2014) after being filtered through a 0.45 μm polyether sulfone syringe filter. The MLSS and MLVSS were determined according to the Standard Methods (APHA, 1998). Protein was determined by the Lowry procedure (Lowry et al., 1951). The morphology and microstructure of the biomass on the surface of the carriers were characterized by scanning electron microscopy (SEM) (JSM-6500F, Japan Electron Optics Laboratory Co., Japan). The samples was washed three times with 0.1 M phosphate buffer, fixed with 2.5% glutaraldehyde solution for 4 h at 4 $^\circ\text{C}$, and then washed 6 times with 0.1 M phosphate buffer. Samples were gradient dehydrated by 50%, 60%, 70%, 80%, 90%, 95%, and 100% ethanol (10 min per step) and air dried. Samples were sputter coated with gold and their surface morphology and structural characteristics were observed by SEM (Zhen et al., 2014).

The ammonia oxidation rate by AOB (AOR), nitrite oxidation rate (NOR), nitrogen removal rate by anammox (ANR) and nitrogen removal efficiency (NRE) were calculated based on nitrogen balance and

stoichiometry according to the Eqs. (1)–(5) (Wang et al., 2017):

$$[\text{TN}]_{\text{Eff}}(\text{mg/L}) = (\text{NH}_4^+-\text{N}) + [\text{NO}_2^--\text{N}] + [\text{NO}_3^--\text{N}]_{\text{Eff}} \quad (1)$$

$$[\Delta\text{N}](\text{mg/L}) = [\text{NH}_4^+-\text{N}]_{\text{Inf}} - [\text{TN}]_{\text{Eff}} \quad (2)$$

$$\text{AOR}(\text{kg-N/m}^3/\text{d}) = \frac{[\text{NH}_4^+-\text{N}]_{\text{Inf}} - [\text{NH}_4^+-\text{N}]_{\text{Eff}} - [\Delta\text{N}]/2.04}{\text{HRT}} \quad (3)$$

$$\text{NOR}(\text{kg-N/m}^3/\text{d}) = \frac{[\text{NO}_3^--\text{N}]_{\text{Eff}} - \frac{0.26\Delta\text{N}}{2.04}}{\text{HRT}} \quad (4)$$

$$\text{ANR}(\text{kg-N/m}^3/\text{d}) = \frac{[\Delta\text{N}]}{\text{HRT}} \quad (5)$$

$$\text{NRE}(\%) = \frac{[\Delta\text{N}]}{[\text{NH}_4^+-\text{N}]_{\text{Inf}} - [\Delta\text{N}]} \quad (6)$$

where $[\text{TN}]_{\text{Eff}}$ is the TN concentration in the effluent (mg-N/L); ΔN is the difference of TN concentrations between the influent and effluent; $[\text{NH}_4^+-\text{N}]_{\text{Inf}}$ is the ammonia concentration in the influent (mg-N/L); $[\text{NH}_4^+-\text{N}]_{\text{Eff}}$, $[\text{NO}_2^--\text{N}]_{\text{Eff}}$, $[\text{NO}_3^--\text{N}]_{\text{Eff}}$ are the concentrations of ammonia, nitrite and nitrate in the effluent (mg-N/L), respectively.

3. Results and discussion

3.1. Overall performance of the single-stage partial nitrification and anammox reactor

The efficiency of the single-stage partial nitrification–anammox reactor was estimated by determining the concentrations of NH_4^+-N , NO_2^--N and NO_3^--N in the effluent every other day. The entire operation period (470 days) was divided into 7 stages: the start-up period (stage I), the NLR increasing period (stages II–V), and the NLR repeating test period (stages VI and VII).

3.1.1. Start-up period (day 1–76)

The concentrations of nitrogen compounds in the influent and effluent and evolution of AOR, NOR, ANR, NRE and ratio of $\text{NO}_3^--\text{N}/\Delta\text{NH}_4^+-\text{N}$ in the single-stage partial nitrification–anammox reactor are shown in Fig. 2. The operation parameters (the DO, AR, ORP, pH, free ammonia (FA) and free nitrous acid (FNA)) of the reactor are shown in supplementary materials. During the start-up period, the DO concentration was controlled and kept lower than 0.15 mg/L. During the first 40 days of stage I, the nitrate concentration in the effluent sincerely was higher than samples without the addition of any carrier under an NLR condition of 0.5 kg $\text{NH}_4^+-\text{N}/\text{m}^3/\text{d}$ (Fig. 2a). Since the oxygen half-saturation constants in the Monod kinetics of AOB (0.3 mg/L) was higher than the nitrite-oxidizing bacteria (NOB) (1.1 mg/L) (Wiesmann,

1994), by keeping the AR of 1.0 L/min, with the DO concentration lower than 0.15 mg/L in this period, NOB activity was suppressed: the results in Fig. 2b suggest that the NOR was close to zero. While the ammonia concentration gradually decreased as the nitrite concentration in the effluent increased, with no influence of the AOR, the decrease in the ANR due to the input of the carrier into the reactor led to the gradual decrease of NRE from original 55% to 15%. NH_4^+-N and NO_2^--N were dominant among the surviving nitrogen compounds. From 100 d onwards, the TN concentration in the effluent remained stable.

The deterioration of NRE during the first 40 days could be attributed to one of the following two reasons. Due to the sudden strong shearing force caused by the vertical and horizontal carrier motion, the sludge volume index (SVIs) increased from 31.7 mL/g (day 1) to 238.1 mL/g (day 33). Thereafter, the floc of AOB and anammox bacteria disintegrated, exposing the anammox bacteria inside the flocs to oxygen. That is, the oxygen in the reactor affected the performance of the reactor by slowing down the activity of anammox bacteria, which led to a reduction in the ANR. On the other hand, as a result of the worsening ANR, the transformation from ammonia to nitrite was excessive, and this led to an accumulation of concentrated FNA to over 0.005 mg/L. According to the results of Strous et al. (1999), the inhibition threshold of FNA to anammox bacteria is over 0.004 mg/L; this could explain the inhibition in anammox activity during the first 40 days while the good AOR during the first 40 days may be attributed to the long-term acclimation. The seed sludge in the reactor had been exposed to a partial nitrification environment for over 450 days where the FNA concentration ranges between 0.003 and 0.035 mg/L before it was introduced to the single-stage partial nitrification–anammox process (Liu et al., 2014). The AOB in the reactor became accustomed to the FNA concentration (< 0.035 mg/L), which was the same level as that of the single-stage partial nitrification–anammox process.

As the experiment proceeded, anammox bacteria gradually developed and became attached to the surface of the carriers. Due to the co-existence of oxygen and oxygen-free zones within the biofilm depth, both partial nitrification and anammox processes occurred in a single reactor. The oxygen inhibiting the anammox process was consumed in the outer layer of the biofilm, in effect protecting anammox bacteria from oxygen. From the 40th day onwards, the anammox reaction was promoted, and a good NRE was regained, gradually increasing to 78.2% on the 76th day. The good performance lasted for two weeks during which the ratio of $\text{NO}_3^--\text{N}/\Delta\text{NH}_4^+-\text{N}$ (Fig. 2d) was 0.14 ± 0.01 , in accordance with the theoretical value (0.13) of that by CANON (Third et al., 2001). All of the results suggested that the single-stage partial nitrification–anammox was achieved for no more than 76 days.

Fig. 3. Nitrogen removal performance in each stage.

3.1.2. NLR increasing period (stages II–V)

The nitrogen removal performance at different NLRs is shown in Fig. 3. The NRE in the stable period of stage I (day 123–193) was $70.0 \pm 6.82\%$ with an ANR of $0.34 \pm 0.04 \text{ kg-N/m}^3/\text{day}$ under an NLR at $0.48 \pm 0.02 \text{ kg-N/m}^3/\text{day}$. On day 194, along with the doubling of NLR (stage II), NRE plunged to 46.5% with some residual ammonia in the effluent. Due to the synchronous increase of AR to 1.5 L/min, the NRE rapidly recovered to 68.4% in one week. To further enhance the NRE, the AR was adjusted to 1.60 L/min, and the good nitrogen removal lasted for a long period, with an NRE and ANR of $71.4 \pm 5.53\%$, $0.69 \pm 0.07 \text{ kg-N/m}^3/\text{d}$, respectively.

From day 378 onward, the ammonia concentration in the influent was increased to 375 mg/L to adjust the NLR to $1.5 \text{ kg-N/m}^3/\text{day}$ (stage III). In response to the increase in NLR, the residual concentration of ammonia increased sharply, and the NRE in the first 5 days of stage III dropped from 71.2% to 43.7% gradually due to insufficient AR. Over the next 10 days, because the AR was adjusted to a more appropriate 2.50 L/min, the NRE recovered to 84.1%, which was higher than that of the last two stages. This high level nitrogen removal performance (NRE: $81.1 \pm 3.60\%$), which was slightly lower than the theoretical NRE of 87% achieved in the single-stage partial nitrification–anammox (Third et al., 2001), lasted for 40 HRTs before the NLR was further increased to $2.0 \text{ kg-N/m}^3/\text{day}$.

In stage IV, the ammonia concentration of influent was increased to 500 mg/L. Similar to the results in the previous three stages, the recovery of NRE also took no more than several days due to the help provided by simultaneously adjusting the AR (3.50 L/min). Compared to the results in stage III, the nitrogen removal performance in stage IV was slightly lower and the NRE and ANR were $70.6 \pm 7.46\%$, $1.27 \pm 0.15 \text{ kg-N/m}^3/\text{day}$, respectively.

In stage V, the AR was adjusted to 4.00 L/min even though the ammonia concentration of the influent was increased to 625 mg/L, and the nitrogen removal performance was scarcely affected by the substrate shock. The NRE and ANR were $78.1 \pm 7.25\%$ and $1.90 \pm 0.30 \text{ kg-N/m}^3/\text{day}$, respectively (Fig. 3).

3.1.3. NLR repeating test period (stages VI–VII)

The NLR repeating test (stages VI–VII) was conducted to evaluate the endurance capacity of the novel partial nitrification–anammox system in this study to load fluctuation. It should be noted that when the aeration rate can not be adjusted in time with the nitrogen load fluctuation in the actual wastewater treatment process, both excessive aeration and insufficient aeration will happen. In stage VI, the NLR was decreased gradually from 2.5 to $1.5 \text{ kg-N/m}^3/\text{d}$. As shown in Fig. 2c, even though the AR was kept to 4.00 L/min (excessive aeration), no significant change was noted in the NRE in stage VI (62.3–79.3%) compared to that of stage V under the same AR. While when the NLR was again increased to 2.5, the NRE (59.1–68.5%) did not return to the same level as that of stage V due to insufficient aeration (AR = 3.00 L/min).

The above results suggested that the novel partial nitrification–anammox system in this study had a certain endurance capacity to load fluctuation. The best performance for this system was an NRE of $81.1 \pm 3.60\%$, an ANR of $1.19 \pm 0.15 \text{ kg-N/m}^3/\text{day}$, respectively, at an NLR of $1.5 \text{ kg-N/m}^3/\text{d}$. The maximum affordable NLR was $2.5 \text{ kg-N/m}^3/\text{day}$.

The characteristics of single-stage partial nitrification and anammox processes using different kinds of carriers are compared in Table 2. Chen et al. (2009) added non-woven fabric to the non-woven rotating biological contactor (NRBC) to achieve CANON, and reported an NRE of 72% at an NLR of $0.066 \text{ kg/m}^3/\text{d}$. Daverey et al. (2013) suggested that for the fast start-up of a CANON system, the NLR should be $> 0.1 \text{ kg/m}^3/\text{d}$ to avoid the unnecessary growth of NOB, and the start-up period was 51 days at an NLR of $0.12 \text{ kg/m}^3/\text{d}$ (Daverey et al., 2015). Miao et al. (2016) also conducted a fast start-up of a single-stage partial nitrification–anammox (PN/A) system at an NLR of $0.1 \text{ kg/m}^3/\text{d}$ by

introducing enriched granule anammox and partial nitrification sludge within 39 days. In this study, the start-up period was 76 days at an NLR of $0.5 \text{ kg/m}^3/\text{d}$ seeding enriched partial nitrification sludge with “homegrown” anammox bacteria. These results suggest that the faster start-up of single-stage partial nitrification and anammox in different reactors can be attributed to two aspects: (1) the inoculation of enriched partial nitrification and anammox biomass; and (2) appropriate nitrogen loading rates. It should be noted that the majority of investigations into single-stage partial nitrification and anammox processes have been conducted at relatively low NLRs ($0.033\text{--}1.5 \text{ kg/m}^3/\text{d}$) (Zhang et al., 2015a; Zheng et al., 2016). In this study, however, the potential of nitrogen removal was shown to be $2.5 \text{ kg/m}^3/\text{d}$. The up-grading of the nitrogen removal potential for the single-stage partial nitrification and anammox process in this study can be attributed to (1) the great specific surface area ($1500 \text{ m}^2/\text{m}^3$) of the functional porous polypropylene carriers which provides a wide space for the symbiosis of both the AOB and anammox bacteria; (2) the hollow structure of functional carriers which facilitates mass transfer; (3) the relatively high specific activity of AOB and anammox bacteria; (4) and the almost completely mixed state in the CSTR which can help to reduce the opportunity of local substrate shock caused by the increase of the NLR.

3.2. Characteristics of the suspended sludge and biofilm

The development of the biofilm adhered to the carriers and the changes in the suspended flocs is shown in Fig. 4. The color of the suspended sludge changed from carmine (day 0) to a pale brown (day 67), and then became dark brown (day 290). The surface of the carriers gradually changed from white to blood-red, indicating the transform of anammox bacteria from flocs in the suspended sludge to the surface of carriers. After the growth of biofilm for 290 days, the hollow portion of the carrier and surface became packed with abundant blood-red aggregates as the biofilm grew thicker.

The structure of the biofilm attached to carrier (on day 143) was observed by SEM. The SEM images of the surface of carriers are displayed in the supplementary materials. The images showed that the microorganisms were attached to the pores of both inside and outside the carrier surfaces. Meanwhile, the biomass was held together by an abundant number of filaments to form functional bodies shaped as globular and rigid granules, with filamentous bacteria providing the backbone for the stable three-dimensional shapes. The morphological organism observed with typically short rod and spherical-shaped cells were the dominant AOB-like and anammox-like microorganisms (Ni et al., 2010).

3.3. Microbial community activity

SAOA, SNOA and SAA at NLR of $1.0 \text{ kg/m}^3/\text{d}$ (day 373) were investigated and the result of specific activity tests is shown in Fig. 5. The SNOA of the suspended sludge was $3.00 \text{ g-N/g-MLVSS/d}$, while the SAA of suspended sludge was only $0.25 \text{ g-N/g-MLVSS/d}$. In the case of the biofilm sludge, the SAA was $3.56 \text{ g-N/g-MLVSS/d}$, but the SNOA was only $0.07 \text{ g-N/g-MLVSS/d}$. The SNOA was scarcely detected in the suspended sludge and was not detected at all in the biofilm sludge it was well-suppressed by NOB under a low DO concentration. The results of the activity tests indicated that AOB was predominant in the suspended sludge and anammox bacteria was predominant in the biofilm sludge, respectively. Moreover, AOB and anammox bacteria had a symbiotic relationship in the functional biofilm. The SAA of the biofilm sludge was one or two orders of magnitude higher than that ($0.03\text{--}0.6 \text{ g-N/g-MLVSS/d}$) provided in previous studies (Miao et al., 2016; Niu et al., 2016). The results can likely be attributed to two aspects: (1) the functional suspended porous carriers provided anammox bacteria with protective barriers against environmental stress; (2) the existence of a small amount of AOB in the biofilm promoted a mass transfer between AOB and anammox bacteria.

Table 2

Characteristic comparison of single-stage partial nitrification and anammox processes.

Process	Reactor/carrier		Seed sludge	DO (mg/L)	Start period (d)	Influent (mg-N/L)	NLR (kg/m ³ /d)	NRE (%)	References
CANON	NRBC	Non-woven fabric	A	0.5–0.7	41	200	0.066	72	Chen et al. (2009)
CANON	SBR	Polyurethane spheres	B	< 1.0	164	200	0.033	40–60	Daverey et al. (2013)
SNAD	SBBR	WAS pellet	C	< 0.5	51	200	0.12	95 [*]	Daverey et al. (2015)
Single-stage PN/A	SBR	–	D	Intermittent aeration	39	51.2–67.5	0.10	70	Miao et al. (2016)
Single-stage PN/A	IFAS	Fixed sponge	E	0.3–0.5	20	1500	1.5	80%	Zhang et al. (2015a)
Single-stage PN/A	IFAS	Cubic sponge polyesters	F	–	17	500	1.2	85%	Zheng et al. (2016)
Single-stage PN/A	IFAS	Polypropylene carrier	G	< 0.1	76	250–625	0.5–2.5	70–81	This study

NFBC: non-woven rotating biological contactor; SNAD: simultaneous partial nitrification, anammox and denitrification; PN/A: partial nitrification–anammox.

A: Enriched anammox and partial nitrification biomass; B: SNAD seed sludge; C: Enriched anammox granule and partial nitrification sludge; D: Anammox sludge; E: Nitrifying activated sludge and anammox biofilm; F: Enriched partial nitrification and homegrown anammox sludge; G: Enriched partial nitrification and homegrown anammox sludge.

^{*} Nitrogen removal efficiency by denitrification and single-stage PN/A.

3.4. Microbial community analysis

3.4.1. Microbial community composition of suspended floc and carrier biofilm

The prevalence of bacteria present in the suspended floc and the carrier biofilm samples at NLR of 1.0 kg/m³/d (day 373) was quantified by 454 pyrosequencing. A total of 6047 and 4664 high-quality reads of 16S rRNA were obtained in samples from the suspended floc and carrier biomass. The distribution of bacterial sequences at the phylum and genus level in the suspended floc and carrier biofilm samples, respectively, is shown in Fig. 6.

In the suspended floc samples, a total of 15 bacterial phyla was identified. As shown in Fig. 6a, the microbial community was clearly dominated by the bacterial phylum *Proteobacteria* (50.92%), especially from the beta subgroups (47.20%), 86.23% of which were culture to be *Nitrosomonas* genus. The results are consistent with the analytical results of bacterial communities from 14 sewage treatment plants, in which *Proteobacteria* was the most abundant phylum and accounted for 36–65% of the total effective bacterial sequences (Zhang et al., 2012). The other dominant phyla of suspended in suspended sample were *Bacteroidetes* (32.33%), *Chloroflexi* (9.94%), *Firmicutes* (2.55%), *Acidobacteria* (1.65%) and *Planctomycetes* (1.59%). It is notable that *Haloscomenobacter hydrossis*, which belongs to the *Bacteroidetes* phylum, was the second most dominant genus in the suspended sample. *Haloscomenobacter hydrossis* is a sheathed filamentous bacterium that has been detected worldwide in activated sludge samples because of its morphology makes it easily recognizable: it is a rigid straight filament, extending from the floc surface (thin needle shape appearance) (Eikelboom, 2006).

Fig. 5. Specific activity of AOB, NOB and anammox bacteria in suspended and biofilm sludge.

The 454 pyrosequencing results for the carrier biofilm sample in the reactor are shown in Fig. 6b. A much richer diversity was detected in the carrier biofilm sample than in the suspended sample. A total of 19 identified bacterial phyla were detected, with *Chloroflexi* clearly the dominant phylum, accounting for 30.48% of the total effective bacterial sequences. The above results are consistent with the wide detection of the phylum *Chloroflexi* with anammox bacteria, whose organic products are believed to be scavenged by *Chloroflexi* (Kindaichi et al., 2012). However, the proportion of *Proteobacteria* predominant in the suspended sample was 29.52%, and the other major (relative abundance > 1%) phyla included *Bacteroidetes*, *Planctomycetes*, *Firmicutes*,

Fig. 4. Development of biofilm and appearance changes of suspended floc in the CSTR reactor.

Acidobacteria, Ignavibacteriiae, accounting for 25.45%, 5.66%, 4.23%, 1.07% and 1.02%, respectively.

There was a clear overlap in the suspended floc and carrier biofilm samples with regard to the four major phyla, Chloroflexi, Proteobacteria, Bacteroidetes and Firmicutes, even though the reactor was fed with no

organic carbon substrate. It should be noted that despite the absence of organic matter dosing in this study, the heterotrophic bacteria Chloroflexi, shown to have filamentous representatives by molecular in the sludge (Beer et al., 2002), were predominant (30.5%) in the carrier biofilm sample, and even exceeded the functional autotrophic bacteria

(AOB and anammox bacteria). However, the consequence appeared to be no bulking problem due to the better settling performance of the suspended sludge in the reactor. It was revealed in previous studies that the co-existing uncultured *Chloroflexi* in anammox reactors and autotrophic nitrifying biofilms scavenged organic compounds derived from anammox and nitrifying bacterial cells in activated sludge and membrane bioreactors (Kindaichi et al., 2012; Miura et al., 2007; Okabe et al., 2005). The dominant genus of *Chloroflexi* phylum in both suspended and biofilm samples were *Longilinea* genus, fermenting polysaccharide and protein (Yamada et al., 2006), and they were growing under the strict anaerobic condition; that is, this genus grew in intra-floc locations and at the inner part of biofilm under the low DO concentration condition in this study.

From the relative abundance of the filamentous bacteria in this system, it could be deduced that the potential role of the *Chloroflexi* was to provide the stabilizing backbone for the three-dimensional microbial aggregates other than causing bulking (Sezgin et al., 1978). The carbon source of these heterotrophic bacteria in this system may have been the soluble microbial products (SMP) and extracellular polymeric substances (EPS) released by the endogenous metabolism of AOB and anammox bacteria (Barker and Stuckey, 1999). It can be inferred the performance of the single-stage partial nitrification–anammox system in the CSTR reactor is driven not only by functional bacteria (AOB and anammox bacteria) but also by filamentous bacteria, which provide the backbone to the aggregates.

3.4.2. Diversity and abundance of functional bacteria of suspended floc and carrier biofilm

In the suspended flocs sample, a total of 101 genera were identified at the genus level. Of these, the abundance of anammox bacteria genera *Candidatus_Kuenenia* and *Candidatus_Brocadia* was only 1.46%, 0.08%, respectively (Fig. 6a). Two strictly AOB genera, *Nitrosomonas* and *Nitrospira* were detected with 40.70% and 0.41% abundance despite the low DO concentration in the reactor. In the biofilm sample, a total of 91 genera were identified at the genus level, with anammox bacteria genera *Candidatus_Kuenenia* and *Candidatus_Brocadia* representing 4.97% and 0.34%, respectively (Fig. 6b). According to several studies, the genus *Candidatus_Brocadia* exhibited a lower substrate affinity than the genus *Candidatus_Kuenenia* (Puyol et al., 2013; van der Star et al., 2008). In this study, the continuous low level of nitrite in the effluent (Fig. 2a) favored the growth of *Candidatus_Kuenenia* genus. The abundance of two strictly aerobic ammonia-oxidizing genera, *Nitrosomonas* and *Nitrospira* were the same as the anammox bacteria genera, at 5.68% and 0.26%, respectively, despite the anoxic condition in the reactor. The relative abundance of NOB (*Nitrospira*) in the suspended sludge sample was only 0.03%, and no NOB was detected in the biofilm sludge, which was consistent with the results of activity tests and long-term reactor operation and verified the good suppression of NOB in this study.

Pyrosequencing results showed that the coexistence of AOB and anammox bacteria in the reactor played an important role in the nitrogen removal. The results verified the symbiosis of anammox bacteria and AOB, that AOB (*Nitrosomonas*) had a higher abundance in the suspended flocs and that the abundance of two functional bacteria (*Nitrosomonas*, *Candidatus_Kuenenia*) was the same in the biofilm. The segregation of AOB and anammox bacteria was also observed in other floc/granular systems (Wett et al., 2010; Zhang et al., 2015b). The abundance of *Nitrosomonas* (46.4%) in this CSTR with functional suspended carriers was twice as that reported by Chu et al. (2015) in the single-stage nitrification–anammox SBR system. The abundance of anammox bacteria was higher in the biofilm (5.31%) than that in the suspended flocs (1.54%) in this study, which was consistent with the findings of an earlier study on the IFAS (Zhang et al., 2015b). However, the phylogenetic distribution of anammox bacteria between suspended sludge (dominated by *Candidatus_Kuenenia*) and biofilm (dominated by *Candidatus_Brocadia*) reported by Zhang et al. (2015a)

after inoculating an IFAS reactor with nitrifying sludge were completely different from the findings of this study. Chu et al. (2015) inoculated denitrification biomass from anaerobic filter and obtained *Candidatus_Jettenia* (16.8%) as the dominated anammox bacteria in a single-stage nitrification–anammox SBR system. It is assumed that the difference in the dominated anammox bacteria among these studies can be attributed to differences in the seed sludge. Moreover, even though the relative abundance of anammox bacteria (5.31%) of total bacteria in the biofilm was relatively low compared to that reported in other previous studies (16.8%) (Chu et al., 2015), stable nitrogen removal was maintained due to the substantial enrichment and the extremely high activity of anammox bacteria in the functional suspended biofilm (Section 3.3).

In this study, it was shown that AOB preferred to grow in the suspended flocs, where transformation and the diffusion of the substrate and DO were enhanced. The biofilm was shown to have higher resistance to the mass transfer of DO, which led to the sharp quantity and activity drop of AOB in the biofilm sludge. Consequently, the biofilm, which provides a protective barrier for anammox bacteria against environmental stress became a typical reddish color and the anammox bacteria became dominant in the biofilm. It can be inferred that the functional suspended biofilm played a crucial role in the achievement of high nitrogen removal potential in the reactor.

4. Conclusion

A novel single-stage partial nitrification and anammox process was successfully started up at 25 °C in a CSTR reactor equipped with functional suspended carriers. The nitrogen removal efficiency was hardly affected by the NLR (0.5–2.5 kg/m³/d) and was maintained in the range of 70.0–81.1%. The symbiosis of AOB and anammox bacteria in the reactor was enhanced by functional suspended carriers, which clearly ensured performance stability and high nitrogen removal efficiency. Heterotrophic filamentous bacteria conferred structural integrity to the aggregates. The CSTR equipped with functional suspended carriers provided an alternative to nitrogen removal based on anammox.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.07.156>.

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