Performance and microbial community of the completely autotrophic nitrogen removal over nitrite process with a submerged aerated biological filter

Xiu Yue, Zhuhan Liu, Guangping Yu, Qianhua Li and Jiali Tang

ABSTRACT

Stable performance is a technical problem in the completely autotrophic nitrogen removal over nitrite (CANON) process with one single stage, which needs to be addressed. In the current work, a laboratory-scale submerged aerated biological filter (SABF) with a 3-L working volume was introduced into the CANON process to enhance its stable performance for 290 days under the following conditions: temperature of 30 ± 1 °C and dissolved oxygen (DO) level of 0.2–0.8 mg·L^{-1}. The results showed that the average ammonium nitrogen removal efficiencies (ANRE) and total nitrogen removal efficiencies (TNRE) were 97.4% and 75.7%, respectively. A 16S rRNA gene high-throughput sequencing technology confirmed the phyla Proteobacteria and Planctomycetes as the ammonium oxidizing bacteria (AOB) and anaerobic ammonia-oxidizing bacteria (AnAOB) of this CANON process with SABF, respectively. The major contributor to nitrogen removal was the genus *Candidatus* Brocadia, in Brocadiae. The aim is to present an effective strategy as a reference for the design of full-scale plant for the CANON process.

Key words | ammonium oxidizing bacteria, anaerobic ammonia-oxidizing bacteria, completely autotrophic nitrogen removal over nitrite, nitrogen removal, submerged aerated biological filter

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INTRODUCTION

The completely autotrophic nitrogen removal over nitrite (CANON) process, with a high loss of nitrogen, has been reported in several systems with high ammonium loading and low organic carbon content in the wastewaters (Third *et al.* 2001). In this process, ammonia nitrogen (NH_4^+-N) is partly converted to nitrite nitrogen (NO_2^--N) by aerobic ammonia oxidizing bacteria (AOB) and subsequently, anaerobic ammonia oxidizing bacteria (AnAOB) convert NH_4^+-N with NO_2^--N to dinitrogen gas (N_2) (Olav Sliekers *et al.* 2002). Compared with the nitrification-denitrification process, the CANON process has the advantages of saving 62.5% oxygen consumption, 100% organic carbon source, and 90% sludge production, etc. (Miao *et al.* 2016). However, a major limitation of this process is the slow growth rate of AnAOB (with doubling times of approximately 10–12 days) (Jeanningros *et al.* 2010) and the nitrate nitrogen (NO_3^--N) accumulation attributed to the nitrite oxidizing bacteria (NOB) enrichment, leading to the unstable performance of the CANON process.

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad (1)$$

$$NH_3 + 1.32NO_2^- + H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2H_2O \quad (2)$$

$$NH_3 + 0.85O_2 \rightarrow 0.11NO_3^- + 0.44N_2 + 0.14H^+ + 1.43H_2O \quad (3)$$

To improve the performance of the CANON process, appropriate reactor configurations are vital for rapid enrichment and stable retention of biomass, which are critical to provide a sufficient SRT for the CANON process (Liao *et al.* 2006; Cho *et al.* 2010; Zhang *et al.* 2013). At present, various types of reactors are widely reported to be used to perform the CANON process (Qian *et al.* 2017; Wang *et al.* 2017). However, the different

doi: 10.2166/wst.2018.316

growth demands of the AOB and AnAOB, and effluent NO_3^--N concentration accumulation have negative impacts on the stable performance of the CANON process (Zhang *et al.* 2013; Varas *et al.* 2015).

A submerged aerated biological filter (SABF) equipped with combined carriers may be suitable for the nitrogen-rich wastewater treatment by the CANON process. On the one hand, combined carriers with a large specific area could help in the accumulation of microorganisms with a relatively long doubling time, which is helpful for sludge retention (El-Shafai & Zahid 2013). On the other hand, the dissolved oxygen (DO) concentration gradient on the biofilm can help co-existence between AOB and AnAOB. These advantages obviously have positive effects on the start-up time and performance of the CANON process.

The purpose of this study was to start up and perform a novel laboratory-scale single-stage CANON process in a SABF reactor equipped with combined carriers at 30 ± 1 °C. The nitrogen removal potential and process stability were then investigated for 290 d. Furthermore, the microbial community of the CANON process with a SABF was analyzed by a 16S rRNA gene high-throughput sequencing technology, and the major contributor to nitrogen removal was detected. The aim of this study was to present an effective strategy as a reference for the design of full-scale plant for the CANON process.

MATERIALS AND METHODS

Reactor system

As shown in Figure 1(a), this experiment was conducted with a laboratory-scale SABF made of organic glass with a 3-L working volume and hydraulic retention time (HRT) of 24 h. Water insulation was used to warm the reactor. The combined carriers fixed in the SABF, shown in Figure 1(b), which was used to enhance the attachment of microorganisms to provide an excellent adsorption capability. The temperature was maintained at 30 ± 1 °C by a temperature controller (ST504, Wenzhou Shangtong Instruments Co., Ltd, China). The influent was pumped from the bottom of the reactor by a constant flow pump (HL-Z, Shanghai Huxi Analysis Instrument Factory Co., Ltd, China). DO was provided from the bottom of the reactor by air pressure pump (LP20, Shenzhen Xing Risheng Industrial Co., Ltd, China), and controlled at 0.2–0.8 mg·L^{-1} by an air rotameter (LZB-3WB, Changzhou Shuanghuan Thermo-Technical Instrument Co., Ltd, China).

Synthetic wastewater and sludge

In this experiment, seeding sludge was obtained from Nansha wastewater sewage (Guangzhou, China). After aeration for 3 days, the detailed parameters of this sludge were

Figure 1 | Schematic diagram of the SABF system.

shown in Table 1. The compositions of the synthetic wastewater and trace element solution are shown in Tables 2 and 3. Furthermore, the qualities of the influent in this experiment are shown in Table 4. The existing influent NO_3^--N and COD were attributed to the tap water that was used to make the synthetic wastewater with chemical agents.

The microbial communities of sludge samples were analyzed by Guangzhou RiboBio Co., Ltd using a 16S rRNA gene high-throughput sequencing technology. The detection process is provided in Supplementary material A (available with the online version of this paper).

Analytical methods

The concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, MLSS and MLVSS were measured using the procedures described in the APHA *Standard Methods* (APHA/AWWA/WEF 1998). The effluent samples were filtered by qualitative filter papers before analysis.

Table 1 | Parameters of the inoculums in this experiment

Item	Unit	Value
pH	–	7.0–7.2
Mixed liquor suspended solids (MLSS)	$mg{\cdot}L^{-1}$	14,000–14,500
Mixed liquor volatile suspended solids (MLVSS)	$mg{\cdot}L^{-1}$	8,000–8,500

Table 2 | Ingredients of synthetic wastewater sample in this experiment

Item	Unit	Value
NH_4Cl	$g{\cdot}L^{-1}$	0.33–0.44
KH_2PO_4	$g{\cdot}L^{-1}$	0.03
$MgSO_4$	$g{\cdot}L^{-1}$	0.01
$CaCl_2$	$g{\cdot}L^{-1}$	0.02
$NaHCO_3$	$g{\cdot}L^{-1}$	1.00
Trace element solution	$ml{\cdot}L^{-1}$	0.35

Table 3 | Ingredients of trace element solution in this experiment

Item	Unit	Value
$FeCl_3{\cdot}6H_2O$	$g{\cdot}L^{-1}$	3.52
$MnCl_2{\cdot}4H_2O$	$g{\cdot}L^{-1}$	0.36
$CuSO_4{\cdot}5H_2O$	$g{\cdot}L^{-1}$	0.08
$ZnSO_4{\cdot}7H_2O$	$g{\cdot}L^{-1}$	0.30
$CoCl_2{\cdot}6H_2O$	$g{\cdot}L^{-1}$	0.38

Table 4 | Key water quality of the influent

Item	Unit	Value
NH_4^+-N	$mg{\cdot}L^{-1}$	82.5–113.5
NO_2^--N	$mg{\cdot}L^{-1}$	0.0
NO_3^--N	$mg{\cdot}L^{-1}$	2.0–2.3
COD	$mg{\cdot}L^{-1}$	0–14
pH	–	7.9–8.3

The TN_{inf} and TN_{eff} concentrations, ANRE, TNRE, ΔNO_3^--N concentration, and the ratio of ΔNO_3^--N /ΔNH_4^+-N were calculated according to Supplementary material B (available with the online version of this paper). Where NH_4^+-N_{inf}, NO_2^--N_{inf}, NO_3^--N_{inf} and TN_{inf} were the ammonium, nitrite, nitrate and total nitrogen concentrations of the influent, respectively. NH_4^+-N_{eff}, NO_2^--N_{eff}, NO_3^--N_{efff} and TN_{eff} were the ammonium, nitrite, nitrate and total nitrogen concentrations of the effluent, respectively.

RESULTS AND DISCUSSION

Performance of the CANON process with a SABF

At present, some kinds of ammonia-nitrogen wastewater, such as chemical industry wastewater and slaughter house wastewater, etc., pretreated by physicochemical methods, have characteristics of ammonia nitrogen concentrations of 100–300 mg/L, which is difficult to treat by traditional biological methods. Thus, this research employed an ammonia nitrogen concentration of about 100 mg/L to be treated by the CANON process with a SABF. The performance of this experiment is shown in Figure 2. This was divided into two different phases: the start-up period (phase I, days 1–55) and operational period (phase II, days 56–290). The pH_{inf}, pH_{eff}, pH_{sys}, $temperature_{sys}$, DO_{sys}, COD_{inf}, COD_{eff}, FA_{inf} concentration and ratio of FA_{inf}/NH_4^+-N_{inf} are shown in Supplementary material C (available with the online version of this paper).

In the start-up period, from the 1st day to the 9th day, NO_2^--N accumulation occurred and the nitration reaction was inhibited because of the lower DO concentration of 0.2–0.8 $mg{\cdot}L^{-1}$. From the 10th day to the 37th day, the average ratio of NH_4^+-N_{eff}/NO_2^--N_{eff} was approximately 1.14:1, which signifies that partial nitrification was successfully initiated and a stable anammox-suited effluent was obtained. From the 38th day to the 55th day, the NH_4^+-N_{eff} and NO_2^--N_{eff} concentrations were reduced to 4.5 $mg{\cdot}L^{-1}$ and

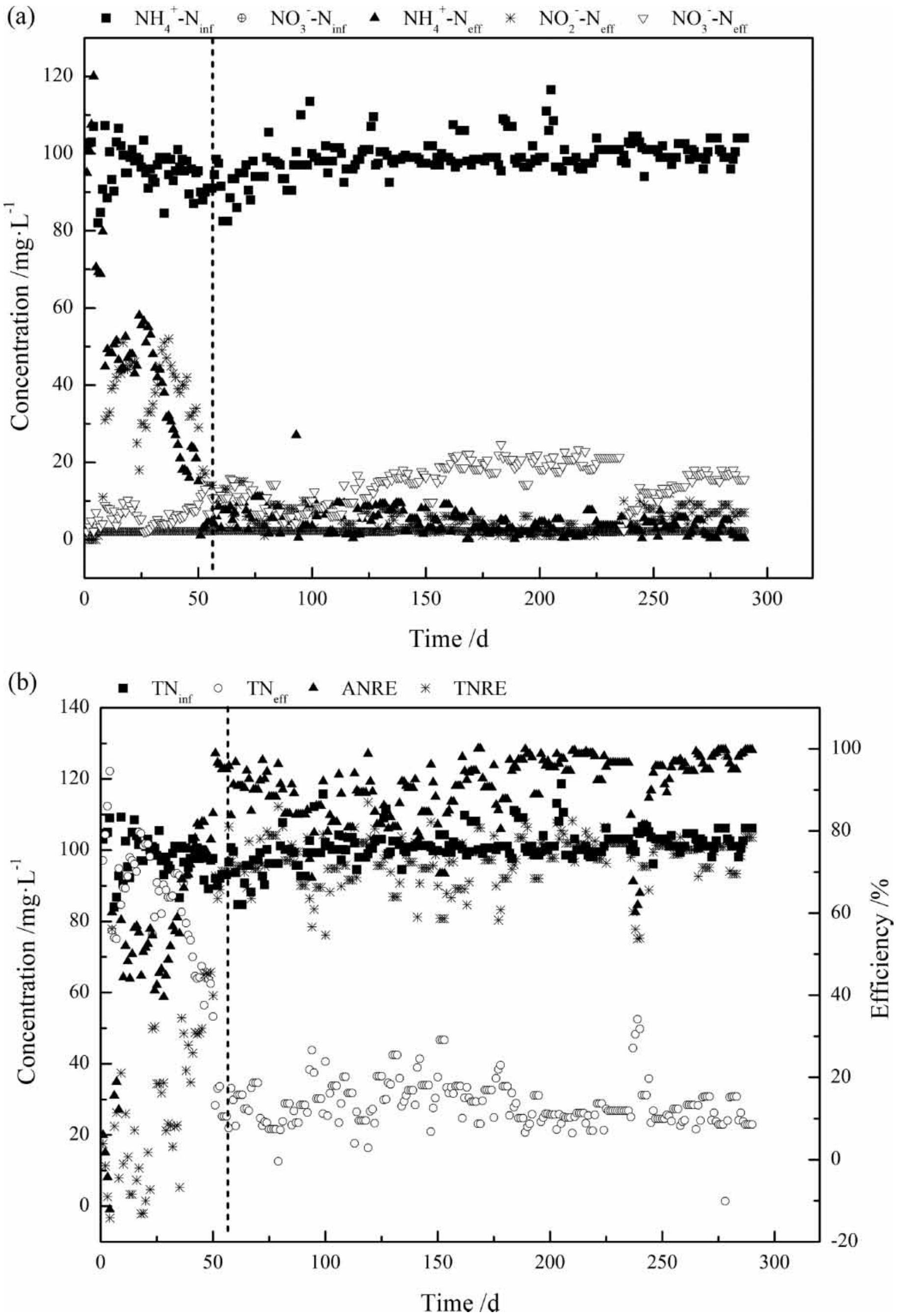

Figure 2 | Performance of the CANON process with a SABF. (*Continued*.)

14.0 mg·L^{-1}, respectively. Meanwhile, the average NO_3^--N_{eff} concentration was maintained at a low value of 6.7 mg·L^{-1}, which demonstrated that the NOB activity was inhibited and the nitrite nitrogen oxidation reaction could be neglected. In the meantime, the ANRE and TNRE were increased to 95.1% and 72.9%, respectively, which is similar to the results from a previous report (Chang *et al.* 2013).

From the 56th day to the 290th day, the CANON process with a SABF was in the operational stage. When the average NH_4^+-N_{inf} concentration was approximately 97.5 mg·L^{-1}, the average NH_4^+-N_{eff} and NO_2^--N_{eff} concentrations were maintained at 2.4 mg·L^{-1} and 10.5 mg·L^{-1}, respectively. Thus, the ANRE and TNRE were maintained at 97.4% and 75.7%, respectively. Furthermore, NOB

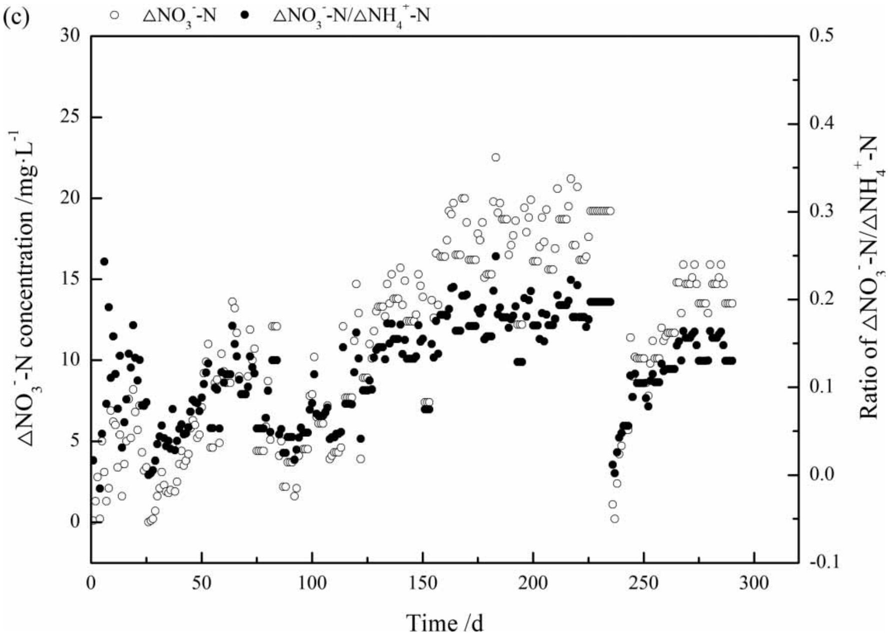

Figure 2 | Continued.

inhibition is important for the stability of CANON because NOB competes with AOB for oxygen and with AnAOB for produced nitrite. Usually, the ratio of ΔNO_3^--N/ΔNH_4^+-N would be the standard for overgrowth of NOB. In this operational period of the CANON process with a SABF, the average ratio of ΔNO_2^--N/ΔNH_4^+-N was 0.10, closer to the theoretical value of 0.11, which signified good performance of this CANON process.

According to previous studies, some different reactors have been employed to perform the CANON process, as shown in Table 5. Compared with other reactors, the SABF system could avoid membrane fouling (compared with membrane bioreactors, MBR), have a shorter HRT (compared with the upflow anaerobic sludge blanket (UASB) and sequencing batch reactor (SBR)), lower DO consumption (compared with the integrated fixed-film activated sludge reactor, (IFAS)), and more stable performance (compared with the continuous stirred tank reactor (CSTR) and expanded granular sludge bed (EGSB)), and so on. The improvement of the nitrogen removal stability of the CANON process in this study can be attributed to: (1) the large specific surface area (1,500 m^2/m^3) of the combined carriers, which provided a wide space for the ample biomass content of microorganisms; (2) the biofilm, which led to stability and a long retention time of microorganisms; (3) the DO concentration gradient from the inside to the outside of the biofilm in the SABF, which made the AOB live in the outside and the AnAOB live in the inside of the biofilm; (4) proper living conditions with no organic matter added,

Table 5 | Overview of the CANON process with different reactors

Reactor	DO (mg·L^{-1})	pH	HRT (h)	Temperature (°C)	Performance period (d)	NH_4^+-N_{inf} (mg·L^{-1})	TNRE (%)
IFAS reactor [16]	0.3–2.5	7.8–8.1	21.6–24	33–35 °C	300	255–705	80
MBR [17]	0.15	7.6	24	25 ± 0.5	178	80	81
UASB [18]	≤0.5	7–8	36	35 ± 1	285	350	89.4 ± 4.9
SBR [19]	0.2–0.5	7.8–8.7	48–24	33 ± 1	180	500	81.1
CSTR [20]	<0.3	7.8–8.7	12–6	25	76	250–625	81.1
EGSB [21]	<0.6	8.0 ± 1.0	0.83–2.5	32 ± 1	129	270–280	90%

Integrated fixed-film activated sludge reactor, IFAS reactor; Membrane bioreactor, MBR; Upflow anaerobic sludge blanket, UASB; Sequencing batch reactor, SBR; Continuous stirred tank reactor, CSTR; Expanded granular sludge bed, EGSB.

lower DO concentration and appropriate temperature in this system supplied a suitable growth environment between the AOB and AnAOB, but inhibited the NOB and heterotrophic microorganisms. From the above, the CANON process with a SABF reactor was suitable for the enhancement of nitrogen removal in the treatment of low C/N ratio wastewater.

Microbial community of the CANON process with a SABF

A 16S rRNA high-throughput sequencing analysis was conducted to examine the microbial community of the CANON process with a SABF during different performance phases, the results of which are shown in Figure 3.

For the seed sludge, the predominant microorganisms were Proteobacteria, Chloroflexi, Nitrospirae, Planctomycetes, Acidobacteria, Actinobacteria, and Bacteroidetes, which accounted for >83% of all of the microbes detected. Among these microorganisms, the most abundant microorganism was the phylum Proteobacteria, the relative abundance of which was 39.7%. It was detected as the most prominent phylum in WWTPs, and most nitrogen removal related microbes, such as AOB, NOB, and denitrifiers, belong to this phylum. Furthermore, the phylum Nitrospira was recently detected as the dominant and specialized NOB found in most WWTPs.

When the CANON process was built up (b1 and b2), the phyla Proteobacteria and Nitrospirae were reduced because of the elimination of NOB and denitrifiers. The phylum Planctomycetes, affiliated with Anammox bacteria, was obviously enriched and its relative abundances were increased to 16.6% in the upper layer and 19.1% in the bottom layer of the CANON biofilm, respectively. Moreover, the phylum Chloroflexi has often been observed in autotrophic nitrogen removal reactors. It was speculated that these phylum microbes survived because of the SMP and EPS released by autotrophs (AOB and AnAOB). This microbial community led to the start-up of the CANON process with a SABF.

When the CANON process entered the operational stage, the phylum Planctomycetes increased to 30.46% in the upper layer and 35.25% in the bottom layer of the biofilm, respectively. Continuous enrichment of the phylum Planctomycetes had a critical influence on the stable performance of the CANON process with SABF. However, the phylum Proteobacteria was inhibited, and its relative abundance was reduced to 20.8% in the upper layer and 18.9% in the bottom layer of the biofilm, respectively. In all, this special structure provided an appropriate environment for different functional bacteria, which guaranteed a very good nitrogen removal performance.

Functional microorganisms of the CANON process with a SABF at a genus level

In the seed sludge, as Figure 4 shows, the genus *Planctomycetes* was the dominant microorganism and its relative abundance was 1.3%, except for an unnamed

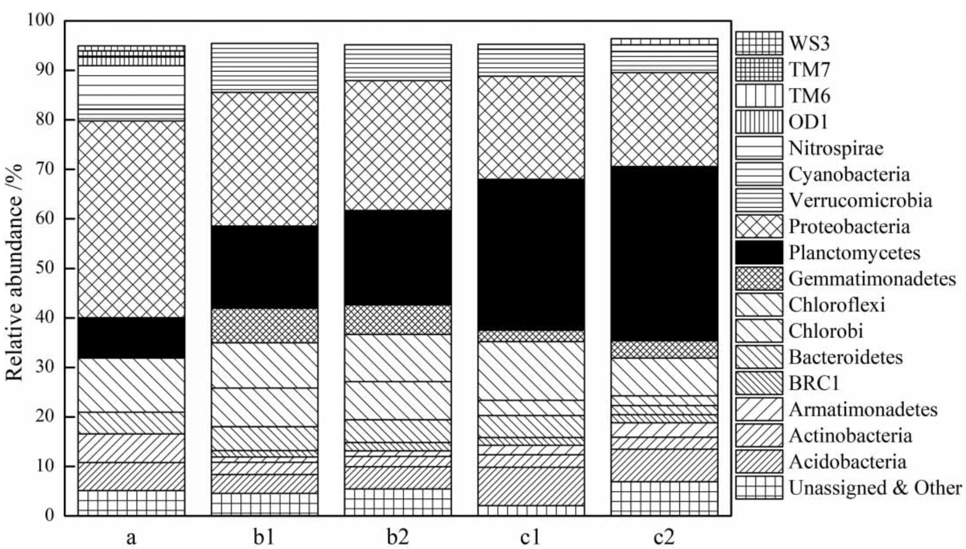

Figure 3 | Microbial community of the CANON process with a SABF at a phylum level (a: seed sludge; b1: the upper layer of CANON biofilm during start-up period; b2: the bottom layer of CANON biofilm during start-up period; c1: the upper layer of CANON biofilm during operational period; c2: the bottom layer of CANON biofilm during operational period).

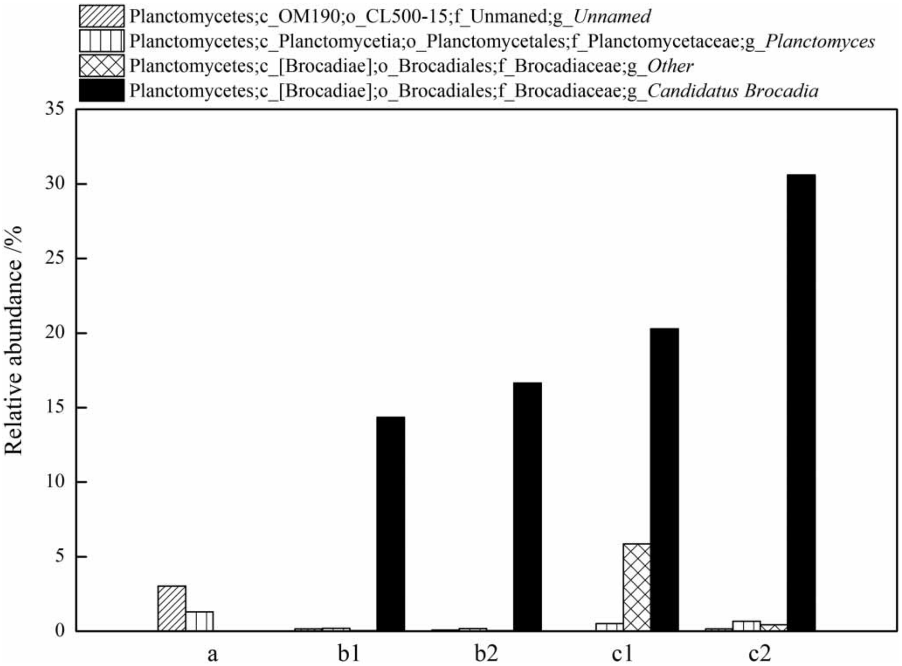

Figure 4 | Functional microorganisms of the CANON process with a SABF at a genus level (a: seed sludge; b1: the upper layer of CANON biofilm during start-up period; b2: the bottom layer of CANON biofilm during start-up period; c1: the upper layer of CANON biofilm during operational period; c2: the bottom layer of CANON biofilm during operational period).

microorganism that was found. When the CANON process with SABF was successfully started up, the genus *Candidatus* Brocadia was obviously enriched and its relative abundance increased to 14.3% in the upper layer and 16.6% in the bottom layer of the biofilm, respectively. Whereas, the genera *Planctomycetes* and the unnamed microorganism behaved differently. When the CANON process began the operational stage, the genus *Candidatus* Brocadia was continuously accumulated and its relative abundance increased to 20.3% in the upper layer and 30.6% in the bottom layer of the biofilm, respectively. From the above phenomenon, it can be seen that the genus *Candidatus* Brocadia was the major contributor to nitrogen removal in this system, which was the same as in some reported researches (Huang *et al.* 2013; Gilbert *et al.* 2014; Lotti *et al.* 2014; Laureni *et al.* 2016; Persson *et al.* 2017). Moreover, other recent researches found that the *Candidatus* Jettenia and Kuenenia were also the detected genera that performed the anammox process (Wang *et al.* 2016; Sun *et al.* 2017), which were not be detected in this research. This might be related to different species of seed sludge and different reactor types.

In summary, the originality of this research is that the SABF has been firstly employed to start up and perform the CANON process. It plays an important role in promoting further development of the CANON process.

CONCLUSIONS

In this experiment, the CANON process with a SABF was investigated to treat synthetic wastewater with an appropriate concentration of ammonium nitrogen over 290 days. The investigation was divided into two different phases: a start-up period (phase I, days 1–55) and long-term performance period (phase II, days 56–290). The experimental conditions were a temperature of $30 \pm 1\ ^{\circ}C$ and controlled DO of 0.2–0.8 $mg \cdot L^{-1}$ with the average ANRE and TNRE achieved at 97.4% and 75.7%, respectively. In addition, the phyla Proteobacteria and Planctomycetes were confirmed as the AOB and AnAOB, respectively, of this CANON process with a SABF according to 16S rRNA macro genome sequencing analysis. Finally, the major contributor of nitrogen removal was the genus *Candidatus* Brocadia in Brocadiae. In summary, this research could promote the development of the CANON process in the future.

ACKNOWLEDGEMENTS

This work is supported by the Science and Technology Program of Guangzhou, China (201707010428). This work is also supported by the Science and Technology Program of Guangdong, China (2016B090918036).

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First received 28 March 2018; accepted in revised form 2 July 2018. Available online 13 July 2018