

Performance and microbial community of the CANON process in a sequencing batch membrane bioreactor with elevated COD/N ratios

Xiaoling Zhang, Xincong Liu and Meng Zhang

ABSTRACT

In this study, the effects of elevated chemical oxygen demand/nitrogen (COD/N) ratios on nitrogen removal, production and composition of the extracellular polymer substances (EPS) and microbial community of a completely autotrophic nitrogen removal via nitrite (CANON) process were studied in a sequencing batch membrane bioreactor (SBMBR). The whole experiment was divided into two stages: the CANON stage (without organic matter in influent) and the simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) stage (with organic matter in influent). When the inflow ammonia nitrogen was 420 mg/L and the COD/N ratio was no higher than 0.8, the addition of COD was helpful to the CANON process; the total nitrogen removal efficiency (TNE) was improved from approximately 65% to more than 75%, and the nitrogen removal rate (NRR) was improved from approximately 0.255 kgN/(m³·d) to approximately 0.278 kgN/(m³·d), while the TNE decreased to 60%, and the NRR decreased to 0.236 kgN/(m³·d) when the COD/N ratio was elevated to 1.0. For the EPS, the amounts of soluble EPS (SEPS) and loosely bound EPS (LB-EPS) were both higher in the CANON stage than in the SNAD stage, while the amount of tightly bound EPS (TB-EPS) in the SNAD stage was significantly higher due to the proliferation of heterotrophic bacteria. The metagenome sequencing technique was used to analyse the microbial community in the SBMBR. The results showed that the addition of COD altered the structure of the bacterial community in the SBMBR. The amounts of *Candidatus* 'Anammoxoglobus' of anaerobic ammonia oxidation bacteria (AAOB) and *Nitrosomonas* of ammonia oxidizing bacteria (AOB) both decreased significantly, and *Nitrospira* of nitrite oxidizing bacteria (NOB) was always in the reactor, although the amount changed slightly. A proliferation of denitrifiers related to the genera of *Thauera*, *Dokdonella* and *Azospira* was found in the SBMBR.

Key words | CANON, COD/N ratio, extracellular polymeric substance, SBMBR, SNAD

INTRODUCTION

The completely autotrophic nitrogen removal over nitrite (CANON) process, in which ammonia oxidizing bacteria (AOB) and anaerobic ammonia oxidation bacteria (AAOB) are simultaneously grown in one reactor, has been extensively studied because this process reduces oxygen demand, does not need chemical oxygen demand (COD), and produces less sludge (Slijkers *et al.* 2002). For the successful start-up and operation of the CANON process, the key points are the enrichment and balanced relationship of AOB and AAOB, and the total suppression or out-selection of nitrite oxidizing bacteria (NOB), which are related to factors, such as pH, dissolved oxygen (DO), temperature and reactor configuration (Liu *et al.*

2016; Yue *et al.* 2018). Reactors with an efficient retention of biomass prefer the CANON process due to the low growth rate of AAOB (doubling time of 10–12 days) (Kartal *et al.* 2010), and the long start-up period is one of the limitations of the CANON process. Recently, the application of the CANON process in a membrane bioreactor (MBR) was proposed because of the total retention of biomass in this type of bioreactor (Wang *et al.* 2013; Zhang *et al.* 2013; Dai *et al.* 2015; Ma *et al.* 2019). Based on studies about the MBR-CANON process, the MBR has been concluded to be ideal for the CANON process due to its operational simplicity, high effluent quality, high performance stability and short start-up period. These studies all

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adopt continuous inflow, while MBR can also be operated in sequencing batch mode, and sequencing batch membrane bioreactors (SBMBRs) for wastewater treatment, especially for biological nutrient removal, have attracted widespread attention in recent years (Bae *et al.* 2003; You & Chen 2008; Dong & Jiang 2009). This reactor combines the advantages of MBRs and SBRs to make MBRs more flexible for CANON start-up and performance by supplying a suitable environment for the growth of both AOB and AAOB. However, there is limited research on the start-up and performance of the CANON process in SBMBRs.

Although AOB and AAOB are both autotrophic bacteria, the CANON process is accomplished without organic matter (OM), while in general, nitrogen and OM coexist in most wastewaters. When COD is introduced into the CANON process, another new process emerges. In this process, partial nitrification, anammox and denitrification work simultaneously in a single reactor (SNAD). Chen *et al.* (2009) reported a chemical oxygen demand/nitrogen (COD/N) ratio of 0.5 in the SNAD process, and the ammonium removal efficiency reached 79%, while it decreased to 52% with a COD/N ratio of 0.75. Lackner & Horn (2013) observed that the overall TN removal in SBRs and MBRs was approximately 80–85%, respectively, with a COD/N ratio of 1.0. Zhang *et al.* (2015) demonstrated that the suppressing threshold value of the COD/N ratio for the CANON process was 1.7. Notably, researchers unanimously agree with respect to the COD/N threshold value for the CANON process. The variation of the nitrogen removal performance at different COD/N ratios is only a surface phenomenon, and the focus should be the shift of the microbial community due to the influent COD/N ratio. Zhang *et al.* (2015) reported that an excessively high COD concentration suppressed both AOB and AAOB, and AAOB and AOB biodiversity both decreased with increasing COD. Some studies observed that the addition of organic matter changed the bacterial community structure and increased the proliferation of heterotrophic bacteria (Sabumon 2007; Liang *et al.* 2015; García-Ruiz *et al.* 2018).

Extracellular polymer substances (EPS), which are released by microbes, are a kind of high molecular polymer that contains polysaccharides (PS), DNA, proteins (PN), lipids and humic acids (Zhang *et al.* 2014b; Lotti *et al.* 2019). EPS are often divided into two major fractions: soluble EPS (SEPS) and bound EPS; SEPS are often dissolved in solution due to the weak adhesion of SEPS to cells, and bound EPS can be divided into an inner layer of tightly bound EPS (TB-EPS) and an outer layer of loosely bound EPS (LB-EPS) (Chen *et al.* 2013).

Previous studies have reported that the production and composition of EPS were influenced by operating conditions, environmental conditions, carbon sources and COD/N ratios (Avella *et al.* 2010; Miqueleto *et al.* 2010; Dvořák *et al.* 2011; Feng *et al.* 2012; Hao *et al.* 2016). Some studies have found that a higher COD/N ratio promoted the production of more EPS and a higher carbohydrate proportion in EPS (Miqueleto *et al.* 2010; Feng *et al.* 2012; Hao *et al.* 2016). Most studies on MBRs have focused on the effect of EPS on membrane fouling under different COD/N ratios. However, little research has been conducted on the characteristics of EPS in CANON systems characterized by low COD/N ratios.

In this study, a membrane bioreactor in sequencing batch mode (SBMBR) was adopted for the CANON and SNAD stage to investigate the relationship among nitrogen removal, the characteristics of the EPS and microbial community and the elevated influent COD/N ratios in the CANON and SNAD stage. Therefore, the nitrogen compounds in the influent and effluent and the production and composition of EPS were examined for increasing COD/N ratios from 0 to 1.0. Additionally, the microbial communities of influents with and without organic material were analyzed by the metagenome sequencing technique.

MATERIAL AND METHODS

Experimental setup and operating conditions

The laboratory-scale submerged SBMBR system, as shown in Figure 1, had a 10 L working volume and contained a hollow fibre membrane module (Tianjin Mo Timo Membrane Technology Co. Ltd, Tianjin, China). The hollow fibre membrane was made of polyvinylidene fluoride (PVDF) and had a pore size of 0.1 mm and an effective area of 0.2 m². The SBMBR was operated in 8 h cycles distributed in three phases: the filling phase (10 min), the reacting phase (460 min) and the decanting phase (10 min). Three cycles were performed each day, and the hydraulic retention time (HRT) was 24 h. An immersed fine air bubble diffuser was installed under the membrane module for aeration to maintain satisfactory DO levels during the aerobic period, and the exchange volume was fixed at 30%. Mechanical mixing was achieved by using a mechanical stirrer. The temperature of the bioreactor was maintained at 28 °C–30 °C by means of a water jacket.

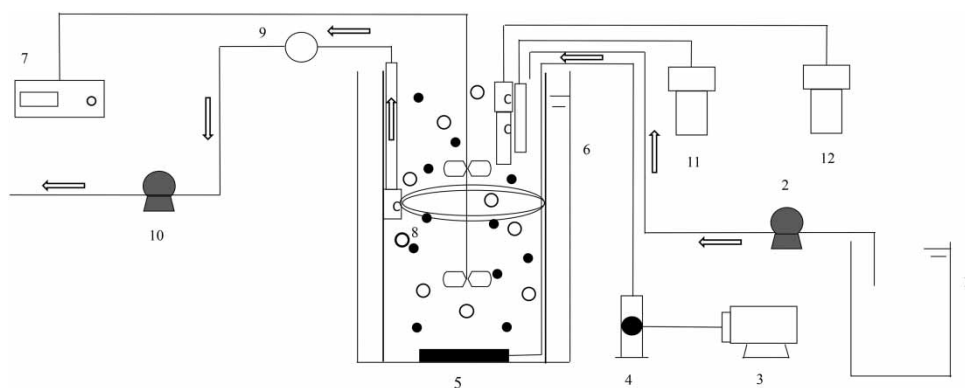


Figure 1 | Setup of sequencing batch membrane bioreactor. 1. Influent tank, 2. influent pump, 3. blower, 4. gas flow meter, 5. diffuser, 6. water jacket, 7. mixer, 8. membrane module, 9. manometer, 10. effluent pump, 11. pH meter, 12. DO analyzer.

Biomass and synthetic wastewater

In this study, the experiment was divided into two stages: the CANON stage and the SNAD stage. In the CANON stage, conventional nitrification sludge from the oxidation ditch of a municipal wastewater plant was inoculated and developed to partial nitrification in the SBMBR. Then, a small amount of CANON biofilm biomass was inoculated in the SBMBR to strengthen the anaerobic ammonia oxidation and achieve the CANON process in the SBMBR. The initial mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) of the SBMBR were 3,000 and 1,620 mg/L, respectively. In the SNAD stage, the influent COD/N ratio was increased in a step-wise manner by adding the required volume of a sodium acetate solution (Figure 3) to maintain a COD/N ratio of 0.5 for 10 days, 0.8 for 10 days and 1.0 for 7 days. The synthetic wastewater sample contained the following components: 1.13–2.82 g/L NH_4HCO_3 , 1.20–2.40 g/L NaHCO_3 , 0.02 g/L KH_2PO_4 , 0.08 g/L MgSO_4 , 0.08 g/L CaCl_2 , 0–0.538 g/L CH_3COONa and 0.5 mL trace element

solution (Van de Graaf *et al.* 1996). The detailed operational conditions of the SBMBR are shown in Table 1.

Analytical methods

Wastewater quality measurement

The concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$, MLSS and MLVSS were measured according to standard methods. The total nitrogen (TN) was defined as the sum of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$. The dissolved oxygen (DO) concentrations and pH values in the reactor were determined with a portable DO analyser (HQ30D, HACH, USA) and a pH meter (pHS-10, Fangzhou, China), respectively.

EPS extraction and measurement

The SEPS, LB-EPS and TB-EPS of the sludge samples were extracted using the method developed by Liang *et al.* (2010). 20 mL of mixed liquid was taken from the reactor. The

Table 1 | Detailed operational conditions of the SBMBR

Stage	Phase	Period (d)	DO (mg/L)	HRT (h)	Aeration mode	Aeration duration (h)	Aeration: no aeration	$\text{NH}_4\text{-N}$ in influent (mg/L)	COD in influent (mg/L)
CANON	Phase I	1–40	0.2–1.2	24	Continuous	7	–	285–500	0
	Phase II	41–60	0.1–0.15	24	Intermittent	4	2 h:2 h	200–300	0
	Phase III	61–77	0.15–0.2	24	Intermittent	4	2 h:2 h	300–370	0
SNAD	Phase I	115–119	0.15–0.2	24	Intermittent	4	2 h:2 h	420	0(as compared period)
	Phase II	120–129	0.15–0.2	24	Intermittent	4	2 h:2 h	420	210
	Phase III	130–139	0.15–0.2	24	Intermittent	4	2 h:2 h	420	336
	Phase IV	140–146	0.15–0.2	24	Intermittent	4	2 h:2 h	420	420

mixed liquid was pre-treated ultrasonically at 20 kHz and 30 W for 60 s and centrifuged at 4 °C and 2,000 g for 15 min. The supernatant liquor was collected as the soluble EPS. The collected bottom sediments were resuspended to their original volume using 20 mL of deionised water. Then, this solution was centrifuged (5,000 g, 15 min, 4 °C), and the supernatant was collected to analyse the chemical composition of the LB-EPS. The sediments were resuspended to their initial volume with phosphate buffered saline to extract the TB-EPS by heating (suspensions were heated to 80 °C for 45 mins). All supernatant fluids were filtered through a 0.45 µm filter and stored at -18 °C before chemical analysis. The extraction tests were performed twice. The polysaccharide content was determined by anthrone colorimetry (Raunkjaer *et al.* 1994). The humic acid content and the protein content were determined using a modified Folin-Lowry method (Frolund *et al.* 1995). The DNA content was determined with the diphenylamine colorimetric method (Sun *et al.* 1999).

DNA analysis

Two sludge samples were obtained separately from the SBMBR, one was at the end of the CANON stage and the other was at the end of the SNAD stage, for the metagenome sequencing to analyse the shift in the microbial community. DNA was extracted from the sludge samples using a DNA extraction kit and was then visualized using AGE agarose gel electrophoresis. The qualified DNA was sequenced using a MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) by the Sangon Company, and the obtained sequences were compared with similar sequences of the reference organisms using a BLAST search.

RESULTS AND DISCUSSION

Start-up of the CANON process in SBMBR

The completely autotrophic nitrogen removal via nitrite process includes two reactions: first, part of the ammonia in the wastewater is oxidized to nitrite by AOB under low DO, and then anammox bacteria convert the remaining ammonia and nitrite to nitrogen gas (Nielsen *et al.* 2005). In general, two strategies were used to start the CANON process. (1) The reactor was started under anoxic conditions after inoculation with biomass from a reactor performing anammox or common activated sludge. Subsequently, a limited amount of oxygen was supplied to the reactor, and ammonia was

oxidized to nitrite. In this process, AOB and AAOB became the dominant bacteria, while the growth of NOB was prevented due to their lower affinity for oxygen compared to AOB and for nitrite compared to AAOB (Slikers *et al.* 2002, 2003). (2) The reactor was started by nitrification, in which nitrite accumulated in the reactor by decreasing the aeration amount and then further lowering the DO to cultivate AAOB in the reactor or inoculating AAOB biomass from another anammox (or CANON) reactor (Vázquez-Padín *et al.* 2009; Zhang *et al.* 2014a). The second start-up strategy was more suitable because the activity of AAOB in the first start-up strategy decreased sharply due to oxygen, and a large amount of nitrite was consumed at the beginning of the start-up.

In this stage, the operation of an SBMBR can be divided into three phases: partial nitrification (phase I, 1–40 days), complete autotroph nitrogen removal (phase II, 41–60 days), and increased nitrogen loading (phase III, 61–77 days). The detailed operational conditions of the SBMBR in each phase are summarized in Table 1.

Nitrogen removal performance of the partial nitrification phase

Large amounts of free ammonia (FA) and a small concentration of dissolved oxygen (DO) were used in this study to achieve NOB inhibition, and thus high nitrite accumulation. Large FA values were attained by maintaining a high pH (7.8–8.1) and a high influent ammonia nitrogen concentration (285–500 mg/L). The DO concentration in the reactor was lowered gradually from 1.32 mg/L to 0.2 mg/L by reducing the aeration rate. The performance of the SBMBR during the partial nitrification phase (phase I) is shown in Figure 2.

Figure 2 shows that on the first day, 400 mg/L of $\text{NH}_4^+\text{-N}$ was converted to 270.8 mg/L of $\text{NO}_3^-\text{-N}$ and 11.8 mg/L of $\text{NO}_2^-\text{-N}$. Further, the effluent $\text{NH}_4^+\text{-N}$ concentration was 31.2 mg/L, indicating that the inoculated biomass performed conventional nitrification. In the following days, the $\text{NO}_3^-\text{-N}$ of the effluent decreased, and the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations in the effluent both increased as the $\text{NH}_4^+\text{-N}$ concentration increased and the DO concentration decreased. Until the 9th day, the nitrite accumulation rate (NAR), which is equal to the effluent nitrite/(effluent nitrite + effluent nitrate), reached 94%, and remained at more than 80% during the following days in this phase. The $\text{NH}_4^+\text{-N}$ concentration of the effluent reached 400 mg/L and the ammonia removal efficiency (ARE) was only 13.6% on the 24th day, indicating that the

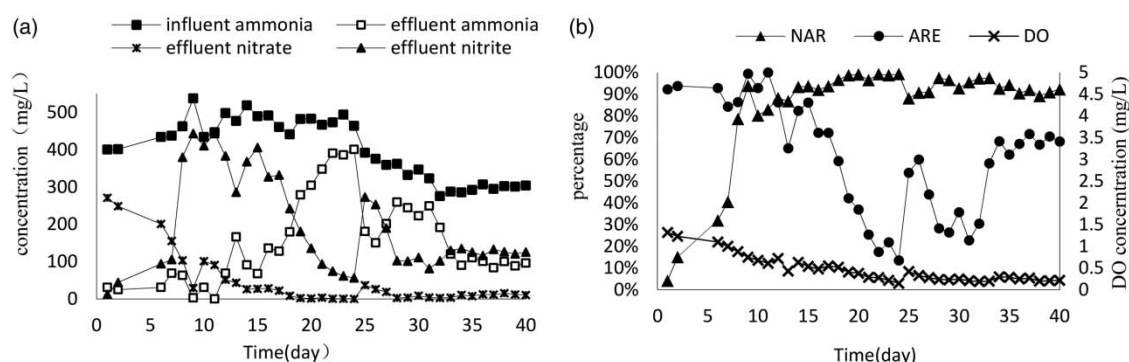


Figure 2 | Performance of the SBMBR during the partial nitrification phase (phase I). (a) Concentrations of nitrogen compounds in the influent and effluent; (b) the nitrite accumulation rate (NAR), the ammonia removal efficiency (ARE) and the DO concentration in the SBMBR.

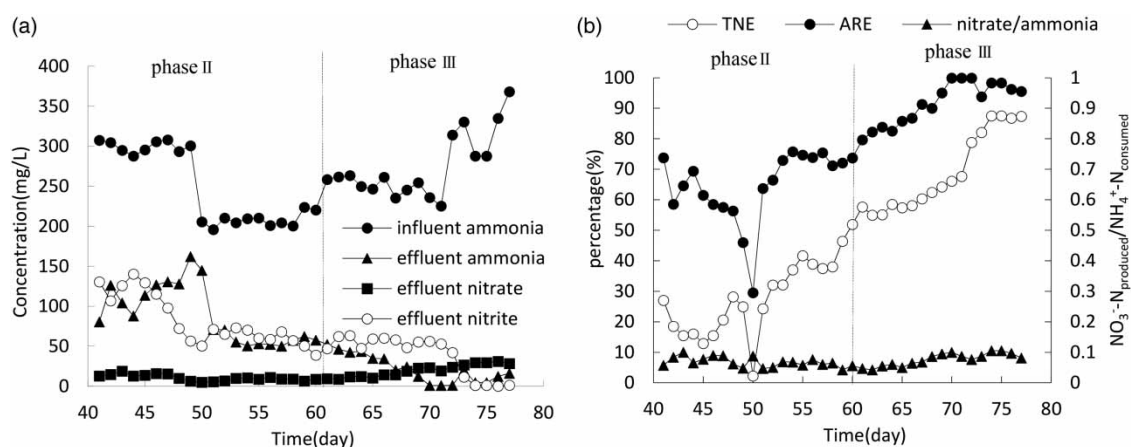


Figure 3 | Performance of the SBMBR during the start-up and nitrogen loading increase CANON phases. (a) Concentrations of nitrogen compounds in the influent and effluent; (b) the total nitrogen removal efficiency (TRE), the ammonia removal efficiency (ARE) and the $\text{NO}_3\text{-N}_{\text{production}}/\text{NH}_4\text{-N}_{\text{consumption}}$ ratio in the SBMBR.

activity of the AOB also decreased with the decrease in DO. To recover the activity of the AOB and avoid influencing the nitrite accumulation, we decreased the $\text{NH}_4^+\text{-N}$ concentration of the influent on the 25th day. The $\text{NH}_4^+\text{-N}$ concentration of the effluent began to decrease, although there was some fluctuation. The ratio of $\text{NO}_2\text{-N}$ to $\text{NH}_4^+\text{-N}$ in the effluent was approximately 1.32:1 (the theoretical ratio of nitrite to ammonia in the anammox reaction) (Strous *et al.* 1999), and the NAR was approximately 90%, which showed that partial nitrification succeeded in the SBMBR and the contents of the reactor were well prepared for the anammox process.

Nitrogen removal performance during the establishment of the CANON process

The CANON process was initiated by adding 0.3 g of the CANON biofilm biomass into the SBMBR. The DO concentration in the reactor was lowered further to

0.1–0.15 mg/L, and the aeration mode was changed to intermittent aeration. In addition, the influent $\text{NH}_4^+\text{-N}$ concentration was maintained at approximately 300 mg/L. The performance of the SBMBR during the start-up and nitrogen loading increasing CANON phases is shown in Figure 3. In the first 9 days of phase II, the effluent $\text{NO}_2\text{-N}$ concentration basically showed a downward trend, and the total nitrogen removal efficiency (TNE) was in the range of 12–27%. However, the effluent $\text{NH}_4^+\text{-N}$ concentration rose from 80.6 mg/L (day 41) to 162.3 mg/L (day 49) because the activity of the AOB was inhibited by the limited oxygen. To resolve this problem, the influent $\text{NH}_4^+\text{-N}$ concentration was decreased to approximately 200 mg/L. During the next 11 days (day 50 to day 60), the effluent $\text{NH}_4^+\text{-N}$ concentration decreased regularly from 158.9 mg/L to 57.9 mg/L, and the ammonia removal efficiency (ARE) increased from 22.7% to 73.8%. On day 60, the TN removal efficiency and the nitrogen removal rate reached 51.9% and

0.1 kgN/(m³·d), respectively. It was apparent that complete autotrophic nitrogen removal via nitrite (CANON) was achieved by the close cooperation between the AOB and the AAOB in the SBMBR.

After the start-up of the CANON process, to increase the TN loading rate, the influent NH₄⁺-N concentration was increased gradually and the DO concentration was increased slightly and maintained at 0.15–0.2 mg/L. It was found that the TN removal efficiency reached 87.4%, which is close to the theoretical nitrogen removal of the CANON process. Furthermore, the NO₃⁻-N_{production}/NH₄⁺-N_{consumption} ratio was approximately 0.11 (the theoretical value in the anammox reaction) (Strous et al. 1999).

Performance and EPS characteristics in the SBMBR with elevated COD/N ratios

To study the effect of COD on the CANON process, sodium acetate was introduced into the synthetic wastewater influent on the 120th day of operation of the SBMBR. Figure 4 shows the nitrogen and COD removal performance with elevated COD/N ratios ranging from 0 to 1.0. The average COD concentrations were 0 mg/L (115–119 day), 210 mg/L (120–129 day), 336 mg/L (130–139 day) and 420 mg/L (140–146 day), while average influent NH₄⁺-N

concentration was maintained at 420 mg/L, resulting in COD/N ratios of 0, 0.5, 0.8 and 1.0. From day 115 to 139, the nitrogen compounds in the effluent were basically stable. From days 120 to 139, the COD removal efficiency increased from 75% (day 120) to 93% (day 139). These results suggested that partial nitrification, anammox and denitrification (SNAD) took place simultaneously in the SBMBR.

The nitrogen components in the influent and effluent of the four phases with different C/N ratios are summarized in Table 2, including ammonia concentration, nitrite concentration, nitrate concentration and COD concentration (average data are used in Table 2). Denitrification ratio was estimated according to theoretical stoichiometric relationships between COD and nitrate nitrogen (1 g of NO₃⁻-N consumed 2.86 g of COD), ignoring the assimilation of heterotrophic bacteria and the amount of COD consumed under aerobic conditions. The average nitrogen removal rate (NRR) increased from 0.255 kgN/(m³·d) to 0.287 kgN/(m³·d) as the COD/N ratio increased from 0 to 0.8. However, the effluent NO₃⁻-N concentration increased significantly and the average NRR decreased to 0.236 kgN/(m³·d) when the COD/N ratio increased to 1.0. As shown in Table 2, both CANON and denitrification processes contributed to TN removal in the four phases with

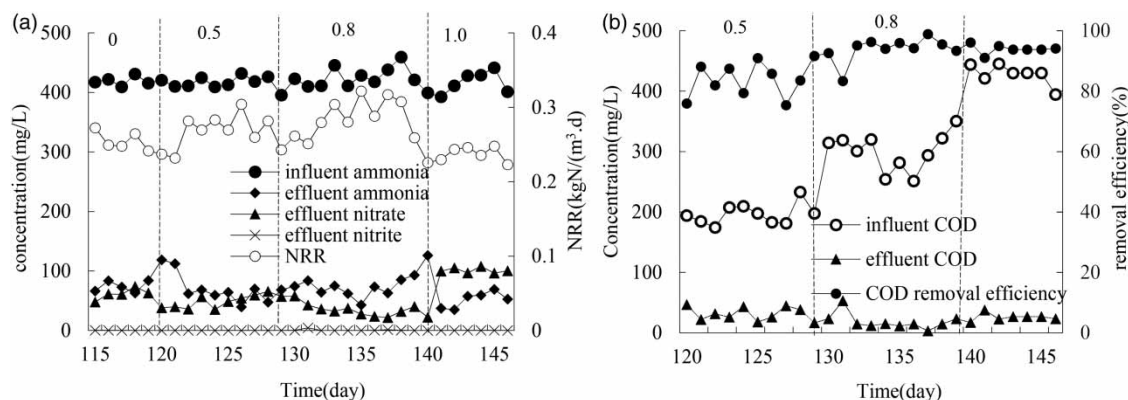


Figure 4 | Performance of SBMBR with different COD/N in influent. (a) Nitrogen removal performance; (b) COD removal performance.

Table 2 | Nitrogen removal and denitrification ratio in the four phases with different COD/N

Phases	COD/N	Inf.ammonia	Eff.ammonia	Eff.nitrite	Eff.nitrate	Inf.COD	Eff.COD	NRR (KgN/(m ³ ·d))	Denitrification ratio
I	0:1	418.9	74.0	0.2	61.3	—	—	0.255	—
II	0.5:1	416.0	71.0	0.2	49.1	196.2	31.3	0.266	20%
III	0.8:1	426.5	71.7	0.8	35.5	300.7	18.5	0.287	31%
IV	1:1	414.6	62.5	0.2	89.8	427.8	25.8	0.236	54%

COD feeding. Although 1.0 of COD/N ratio resulted in the lowest TN removal, the denitrification ratio was the highest. The increase of NO_3N concentration in effluent was due to nitrification, since the anammox process was inhibited.

Figure 5 shows that the contents and constituents of the EPS extracted from the activated sludge samples are related to the conditions of the influent without organic carbon and COD/N ratios of 0.5, 0.8 and 1.0. The amount of SEPS (total content of PS, PN, HA and DNA) in the EPS decreased gradually when the influent COD/N ratio increased from 0 to 0.8 and increased slightly when the COD/N ratio was 1.0. The DNA decreased the most compared to the other SEPS contents, which suggested a reduction in cell lysis as the amount of organic carbon increased. The amount of LB-EPS also decreased because SEPS and LB-EPS can be transformed into one another. However, the increase in the amount of TB-EPS with the increases in the COD/N ratio should be related to the increase of the abundance of total bacteria 16 s RNA and heterotrophic bacteria (seen in 3.3). It is believed that heterotrophic bacteria secrete more EPS than autotrophic

bacteria (Shao *et al.* 2019). Although the results in our study were in accordance with many previous studies, the reason for the increase of TB-EPS with the increasing COD/N ratio was not the same as these previous studies, which demonstrated that increases in the amount of EPS with higher COD/N ratios are due bacterial growth under the limitation of nitrogen (Miqueleto *et al.* 2010; Feng *et al.* 2012; Hao *et al.* 2016).

Shift of the microbial community in response to the elevated COD/N ratio in the SBMBR

Figure 6 shows the community of the dominant bacteria in the CANON stage and SNAD stage at a genus level. 30946 and 33486 sequences were obtained from the two sludge samples on the 119th day (the end of the CANON stage) and the 146th day (the end of the SNAD stage), respectively, and the OTU numbers of the two samples were 1820 and 2088, respectively. The OTU number increased after COD feeding, which could be attributed to the culturing of some heterotrophic species due to the introduction of

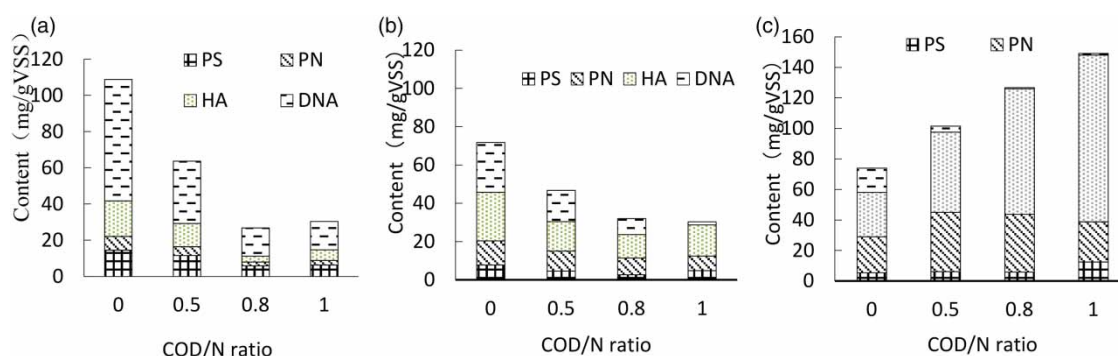


Figure 5 | The contents and constituents of the EPS under different COD ratios. (a) SEPS, (b) LB-EPS, (c) TB-EPS.

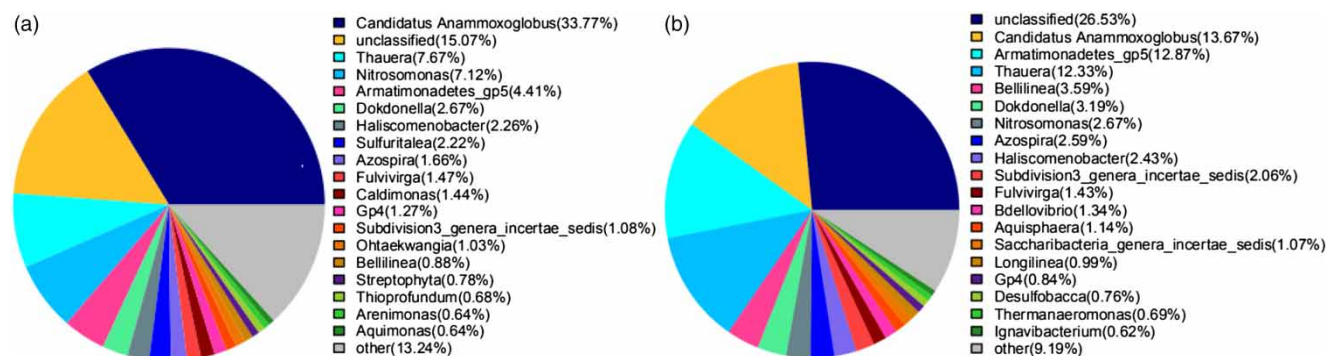


Figure 6 | Microbial community composition of the SBMBR sludge based on metagenome sequencing technique at genus level. (a) Sample taken from CANON stage. (b) Sample taken from SNAD stage.

organic carbon. The top ten genera in the CANON stage were *Candidatus* 'Anammoxoglobus' (33.77%), unclassified (15.07%), *Thauera* (7.67%), *Nitrosomonas* (7.12%), *Armatimonadetes_gp5* (4.41%), *Dokdonella* (2.67%), *Haliscomenobacter* (2.26%), *Azospira* (2.22%), *Fulvivirga* (1.47%) and *Caldimonas* (1.44%). While in the SNAD stage, the top ten genera were unclassified (26.53%), *Candidatus* 'Anammoxoglobus' (13.67%), *Armatimonadetes_gp5* (12.87%), *Thauera* (12.33%), *Bellilinea* (3.59%), *Dokdonella* (3.19%), *Nitrosomonas* (2.67%), *Azospira* (2.59%), *Haliscomenobacter* (2.43%), *Subdivision3_genera_incertae_sedis* (2.06%). *Nitrosomonas* is a type of AerAOB bacteria, while *Candidatus* 'Anammoxoglobus' is a type of AnAOB. However, previous studies have suggested that *Nitrosomonas* and *Candidatus* 'Kuenenia' were the predominant functional bacteria in the CANON system, especially in the MBR-based CANON system (Zhang *et al.* 2013). It was first reported that *Candidatus* 'Anammoxoglobus' was the predominant AAOB genera in an MBR in our study.

Figure 6 shows that AerAOB and AnAOB are both suppressed after COD is introduced into the SBMBR. The *Nitrosomonas* abundance decreased from 7.12% (in the CANON stage) to 2.67% (in the SNAD stage), and the abundance of *Candidatus* 'Anammoxoglobus' decreased from 33.77% (in the CANON stage) to 13.67% (in the SNAD stage). This result was consistent with the NRR decrease shown in Figure 4. However, there was a significant increase in the abundance of denitrifying bacteria, such as *Thauera*, *Dokdonella* and *Azospira*, in the top ten genera. *Thauera* has been confirmed to be a typical denitrifier and can be enhanced by acetate (Han *et al.* 2018), while *Dokadonella* is a completely aerobic denitrifier (Pishgar *et al.* 2019). The abundance of NOB decreased slightly (from 0.24% to 0.18%), and it was confirmed that the bioactivity and biodiversity of NOB both decreased with the increase of the C/N ratio when the influent C/N ratio was lower than 1.0 (Zhang *et al.* 2017). Moreover, the abundance of total bacterial 16S rRNA genes increased when the condition changed from CANON to SNAD, which might also explain the higher TB-EPS production in the SNAD stage compared to the CANON stage. The decrease in the amounts of SEPS and LB-EPS with the increase of the COD/N ratio could be due to the utilization of some contents by the proliferating heterotrophic bacteria. Zhang & Bishop (2003) discovered that biofilm EPS were biodegradable by their own producers and by other microorganisms when they were starved. Miao *et al.* (2018) stated that the high levels of protein and carbohydrate in the biofilm might effectively enrich the anammox bacteria and supply the nutrients and enzymes. Li *et al.*

(2006) reported that the heterotrophic bacteria coexisted with AOB and NOB even in an MBR fed with inorganic ammonia-bearing wastewater, living mainly on EPS and dead AOB and NOB. Kim & Nakhla (2010) reported that DPAO denitrification increased the relative hydrophobicity (RH) of bEPS but decreased the carbohydrate/protein ratio, possibly due to carbohydrate utilization in EPS by ordinary heterotrophic organisms (OHO) under substrate-limiting conditions for denitrification. These studies demonstrated that EPS could be utilized by microorganisms.

Previous studies have demonstrated that the EPS composition in a bioreactor was related to the microbial community structure (Shao *et al.* 2019). For instance, it has been generally recognized that autotrophic nitrifying bacteria have much slower biofilm formation rates than other heterotrophic bacteria due to their lower growth rate and lack of EPS production ability (Tsuneda *et al.* 2001), and Stehr *et al.* (1995) reported there was a *Nitrosomonas* strain isolated from the Elbe estuary that lacked EPS production capacity. And Tsuneda *et al.* (2001) demonstrated that heterotrophic bacteria can produce higher amounts of EPS than autotrophic bacteria. Ni *et al.* (2018) reported that the EPS content of the anammox granules increased to 87.7 mg/g MLVSS after 200 mg COD/L NaAc treatment for 2 d; as a result, increased EPS secretion and the slightly proliferated heterotrophic bacteria in the outer layer of the granule could intercept Zn(II), contributing to the improved adaptability of the anammox granules to Zn(II) shock. These studies may help to explain the observed EPS difference between the CANON stage and the SNAD stage in our study.

CONCLUSION

The influent COD/N ratio affected the nitrogen performance, the characteristics of the EPS and the microbial communities of a CANON bioreactor configured as an SBMBR. The nitrogen removal increased by denitrification when the COD/N ratio increased from 0 to 0.8; however, increasing the COD/N ratio to 1.0 led to the suppression of AOB and AAOB, which resulted in a decrease in nitrogen removal. The amounts of SEPS and LB-EPS decreased as the COD/N ratio increased, while the amount of TB-EPS increased in accordance with the proliferation of bacteria in the SBMBR. The addition of organic matter also changed the bacterial community structure and increased the proliferation of denitrifying bacteria associated with the genera of *Thauera*, *Dokdonella* and *Azospira*. Thus, for a

stable CANON process in an SBMBR, the influent COD/N ratio should be maintained below 1.0.

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