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Mechanisms and characteristics of biofilm formation via novel DEAMOX system based on sequencing biofilm batch reactor

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A denitrifying ammonium oxidation (DEAMOX) process has been regarded as an innovative process to simultaneously treat ammonia and nitrate containing wastewaters, whereas very limited research has focused on its application in biofilm system. In this research, a novel DEAMOX process was established with fixed sponge carriers in a sequencing biofilm batch reactor (SBBR). To investigate biofilm formation process and characteristics can encourage further research on DEAMOX system optimization, deteriorated performance recovery strategies and application with actual wastewater. Total nitrogen removal efficiency was maintained at 93.0 % after 240 days of operation. With biofilm growth, the protein-like extracellular polymeric substances (EPS) and tightly-bound EPS (TB-EPS) of biofilms increased from 65.6 to 46.1, to 179.6 and 142.0 mg gVSS^{-1}, respectively, revealing that protein-like substances and TB-EPS promote biofilm formation. The mechanism of biofilm formation was discussed by analyzing the morphological development and functional bacterial activities of biofilms. Furthermore, high anammox activity was obtained in biofilms with specific NH_4^+–N removal rates over 4.29 mgN gVSS^{-1}h^{-1}, which were significantly higher than in suspended sludge (2.56 mgN gVSS^{-1}h^{-1}). Quantitative polymerase chain reaction results showed that the abundance of anammox bacteria in biofilms increased from 1.87 % to 11.48 % with biofilm growth. These results imply that mature biofilms formed on carriers and the anammox bacteria were sufficient enriched in DEAMOX-SBBR system.

[**Key words:** Anammox; Partial-denitrification; Sequencing batch biofilm reactor; Biofilm; Extracellular polymeric substance]

The anaerobic ammonium oxidation (anammox) process is an innovative biological nitrogen removal process that is cost-effective as it has a low demand for oxygen, does not require organic carbon source (1), has high nitrogen removal rates and a low sludge yield (2,3). These characteristics make the anammox process highly suitable for sewage treatment applications. Nitrite is a crucial substrate for anammox processes and is generally produced by partial nitrification or partial denitrification processes (Eq. 1) (4,5). A combination of the anammox process with partial nitrification in a single reactor [completely autotrophic nitrogen removal over nitrite (CANON) or simultaneously partial nitritation, anammox and denitrification (SNAD) processes] has been widely used for treating ammonia-containing wastewater (6–10). Recently, a novel partial denitrification process combined with the anammox process, named denitrifying ammonium oxidation (DEAMOX) was successfully used to treat ammonia and nitrate containing wastewater (Eq. 2) (11). However, the anammox bacteria have an extremely low growth rate and high sensitivity to environmental stress (e.g., pH, temperature, organic matter) (12–15), leading to lots of difficulties in their enrichment. Moreover, in DEAMOX process, heterotrophic denitrifying bacteria generally have a higher growth rate than autotrophic anammox bacteria (16). These microbiologic properties may increase the risk of complete denitrification in DEAMOX process and thus, deteriorating the nitrogen removal performance.

$$1.32\ NO_3^- + 0.55\ CH_3COO^- + 0.088NH_4^+ \rightarrow 1.32\ NO_2^- + 0.088\ C_5H_7NO_2 + 0.66\ HCO_3^- + 0.198\ H^+ + 0.264\ H_2O \quad (1)$$

$$NO_3^- + 0.519\ CH_3COO^- + 1.026\ NH_4^+ \rightarrow 0.926\ N_2 + 0.56\ HCO_3^- + 0.064\ H^+ + 0.083\ C_5H_7NO_{2\ (denitrifying)} + 0.062\ CH_2O_{0.5}N_{0.15\ (anammox)} + 2.16\ H_2O \quad (2)$$

Many studies (17,18) had reported that the formation of biofilms on carriers can reduce the wash-out of bacterial biomass and enhance the enrichment of functional bacteria and thus, they may be applied to anammox based processes. Different kinds of reactors have been used for cultivating anammox bacteria such as continuous stirred tank reactors (CSTR) (19), membrane aerated biofilm reactors (MABR) (20), and sequencing biofilm batch reactors (SBBR) (21). Generally, SBBR systems have higher biomass concentrations, greater resistance to shock loads and better volumetric loads, making SBBRs a highly suitable reactor for the growth of anammox bacteria (22,23). Additionally, as anammox bacteria prefer to grow in the form of biofilms on the surface of carriers, selective biomass wash-out is easier to realize in SBBR for DEAMOX process. Porous sponge carriers have been widely used for the enrichment of anammox bacteria because of the large-specific area and strong absorption capacity for the attachment of anammox bacteria (21,24,25). However, these carriers usually have been applied to

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cultivate anammox bacteria in ANAMMOX system, CANON system or SNAD system, their application in DEAMOX system where only heterotrophic denitrifying bacteria and autotrophic anammox bacteria were functional, is less common. Additionally, very few studies have investigated biofilm formation process and the characteristics of biofilm growth, especially in DEAMOX process.

In this study, a lab scale SBBR reactor, equipped with porous sponge carriers at 30 ± 5 °C, was set up. The specific aims of this research were: (i) to establish a novel DEAMOX process in an SBBR reactor with adequate abundances of anammox bacteria; (ii) to analyze the EPS changes, functional bacteria activity and the enrichment of anammox bacteria between biofilms and suspended sludge, to help explain mechanisms and characteristics of biofilm formation; (iii) to propose a new solution of deteriorated nitrogen removal performance by realizing selective biomass was-out.

MATERIALS AND METHODS

SBBR setup and its operation As shown in Fig. 1, the DEAMOX process was operated in an SBBR reactor (working volume: 10.0 L). The reactor included a pH meter (WTW Company, WTW 3310, Munich, Germany) for monitoring the pH value and a heating rod for maintaining a stable operational temperature at 30 ± 5 °C. Two separated peristaltic pumps were utilized to supply influent substrates with 4.0 L synthetic water per cycle and carbon source to the reactor, respectively.

As shown in Fig. S1, the carriers were strung together by using fish wire and then fixed into a plastic rack with a 15 % of packing ratio. The carriers were cubic sponges made of polyurethane sponges with a specific surface area of 2395 m^2 m^{-3}. The volume of each cubic sponge was 3.375 cm^{-3} and the density was below 1g cm^{-3}. These sponge carriers have a porous structure and large-specific area, which provided them a strong absorption capacity for the attachment of biomass (Table 2).

As shown in Table 1, the operation stage of the SBBR reactor included four stages: 5 min feeding, 4.0 h anaerobic reaction in phase I, and it was decreased to 3.0 h in phase II and phase III, then to 2.5 h in phase IV, finally to 2.0 h in phase V; followed by 50 min settling in phase I, and it was shorten to 40 min in phase II and phase III, then to 15 min in phase IV and 10 min in phase V, finally 8 min discharging of 4.0 L effluent. The SBBR reactor was operated in five different phases with a stepwise decreased hydraulic retention time (HRT) from 10.0 h to 5.0 h, resulting in an increased nitrogen loading rate (NLR) from 0.78 ± 0.04 to 1.70 ± 0.07 kgN $m^{-3}d^{-1}$ (Table 1). A sodium acetate solution acted as the organic carbon source in DEAMOX system.

Synthetic wastewater and seeding sludge The influent synthetic wastewater adopted in this research was made up of NH_4Cl, $NaNO_3$, mineral salts and trace element solution. The composition of the mineral medium contained KH_2PO_4 (30 mg L^{-1}), $MgSO_4\cdot 7H_2O$ (140 mg L^{-1}), $CaCl_2\cdot 2H_2O$ (140 mg L^{-1}) and 1 ml L^{-1} trace element solution A and B. Trace element solution A contained (L^{-1}): EDTA·2Na, 6.37 g; $FeSO_4\cdot 7H_2O$, 9.15 g; and trace element solution B contained (L^{-1}): EDTA·2Na, 19.1 g; $CoCl_2\cdot 6H_2O$, 0.24 g; $CuSO_4\cdot 5H_2O$, 0.25 g; $NaMoO_4\cdot 2H_2O$, 0.22 g; $NiCl_2\cdot 6H_2O$, 0.19 g; H_3BO_4, 0.014 g; $ZnSO_4\cdot 7H_2O$, 0.43 g; $MnCl_2\cdot 4H_2O$, 0.99 g.

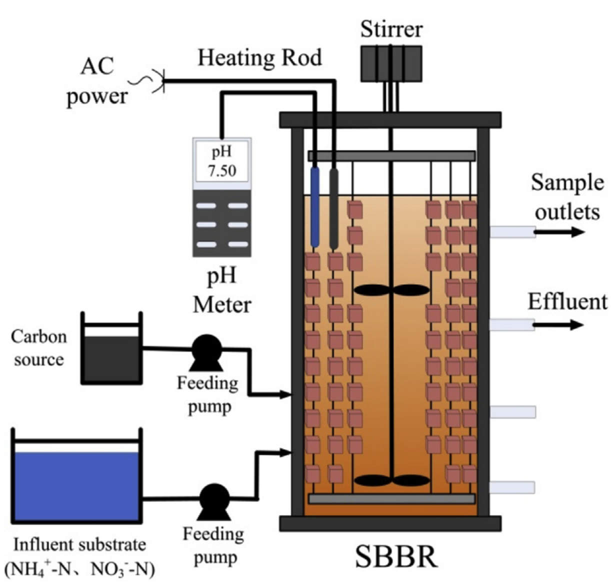

FIG. 1. Schematic diagram of SBBR process.

TABLE 1. The operational conditions of the SBBR reactor.

Phase	Time (day)	COD/NO_3^-−N	HRT (h)	Influent nitrogen concentration (mg L^{-1})		NLR (kgN$m^{-3}d^{-1}$)
				NH_4^+−N	NO_3^-−N	
I	1–25	2.5	10	156.8 ± 2.4	165.9 ± 7.6	0.78 ± 0.04
II	26–89	2.5	7.5	161.1 ± 6.6	170.9 ± 9.3	1.05 ± 0.15
III	90–112	2.5	7.5	150.9 ± 9.9	189.6 ± 9.8	1.13 ± 0.10
IV	113–163	2.0	6.3	161.8 ± 16	197.1 ± 13	1.38 ± 0.14
V	164–243	2.0	5.0	151.5 ± 4.2	200.7 ± 8.3	1.70 ± 0.07

The inoculated sludge treating synthetic wastewater was collected from a DEAMOX process of suspended sludge system in our laboratory, which simultaneously removed NH_4^+−N and NO_3^-−N with TN removal efficiency over 90% by feeding with synthetic wastewater containing NH_4^+−N (150 mg L^{-1}) and NO_3^-−N (150 mg L^{-1}) (11). The volatile suspended solid (VSS) of the SBBR was 2600 mgVSS L^{-1} after inoculation.

Analytical methods The effluent liquor samples were collected every operational day and stored in a refrigerator at 4 °C. The NH_4^+−N, NO_2^-−N and NO_3^-−N of samples were measured on the basis of the standard methods (26). The COD was measured by a COD quick-analysis apparatus (model 5B-3C, Lian Hua Tech. Co., Ltd., Lanzhou, China). The VSS were measured using the standard methods (26). Temperature and pH value were monitored by a pH/Oxi 3310 analyzer (WTW 3310, WTW Company, Munich, Germany).

Specific activity of anammox and partial-denitrifying bacteria batch tests To investigate biofilm formation, batch tests were carried out to assess the specific activity of anammox and partial-denitrifying bacteria in biofilms as compared with suspended sludge. Two 0.5 L glass bottles (S_1 and S_0) containing NH_4Cl and $NaNO_3$ were used to test the activity of functional bacteria. S_1 contained carriers taken from the SBBR reactor at a packing ratio consistent with the SBBR reactor, while S_0 was filled with suspended sludge and its SS was consistent with the SBBR reactor. Both test systems were operated for 5.0 h in anoxic conditions, with the temperature controlled at 30 °C in an incubator (LRH-250F, Blue Pard Instruments Co., Ltd., Shanghai, China) and continual agitation with a magnetic stirrer at 250 rpm. The influent substrate concentrations for NH_4^+−N and NO_3^-−N, were 60 mg L^{-1} for both, and the COD/NO_3^-−N ratio was fixed at 2.5. A sample was taken from the test system at the times of 0, 5, 10, 20, 40, 60, 90, 120, 180, 240, and 300 min, and the NH_4^+−N, NO_2^-−N and NO_3^-−N concentrations were measured. Batch tests were carried out at each phase of the SBBR reactor and the rate of ANAMMOX and partial-denitrification per VSS (mgN $gVSS^{-1}h^{-1}$) were calculated, allowing assessment of the specific activity of functional bacteria.

Calculations The specific activity of anammox was measured based on the specific NH_4^+−N removal rate (SARR), while the specific activity of partial-denitrification was measured based on the specific NO_3^-−N reduction rate (SNRR) and specific NO_2^-−N accumulation rate (SNAR). The SARR, SNRR and SNAR were calculated according to Du et al. (11).

EPS extraction and analysis The attached biofilms and suspended sludge were taken from the SBBR reactor for EPS analysis during the steady state of each phase. The EPS extraction method described by Li and Yang (27) and Zhao et al. (28) was applied, on the basis of the modified heat extraction method. All EPS samples were filtered through polyvinylidene fluoride membranes with a 0.45 μm pore size, prior to measurement. The protein (PN) and polysaccharides (PS) content of EPS fractions were measured according to the Lowry–Folin methods (29) and the phenol–sulfuric acid methods (30). The 3D-EEM spectra of EPS samples were recorded according to the method of Miao et al. (21).

DNA isolation and qPCR analysis The attached biofilms and suspended sludge samples from the SBBR were taken out at phase II (day 48) and phase IV (day 161). The DNA of sludge samples were extracted according to the method of Du et al. (11). The abundance of total bacteria and anammox bacteria were measured by qPCR technique according to the method of Miao et al. (21). Additionally, the primers of anammox bacteria and total bacteria for qPCR included Amx368f-Amx820r (31) and 341f-543r (32), respectively.

TABLE 2. Abundance of anammox bacteria in biofilms and suspended sludge in different operational periods.

Parameter	Phase II (day 48)		Phase IV (day 161)	
	Biofilm	Suspended sludge	Biofilm	Suspended sludge
Anammox bacteria (copies (g dry sludge)$^{-1}$)	6.08×10^9	6.14×10^9	1.00×10^{11}	2.76×10^{10}
Total bacteria (copies (g dry sludge)$^{-1}$)	3.26×10^{11}	6.44×10^{11}	8.74×10^{11}	2.88×10^{12}
Anammox bacteria/total bacteria ratio (%)	1.87	0.95	11.48	0.96

RESULTS AND DISCUSSION

Nitrogen removal performance of the DEAMOX-SBBR process The DEAMOX-SBBR process was successfully operated for more than 240 days for biofilm formation. As shown in Fig. 2b, the reactor seed sludge quickly adapted to the high strength wastewater, with a maintained TN removal efficiency of 97.4 % in phase I. The effluent NH_4^+–N, NO_3^-–N and NO_2^-–N concentrations remained in the range of 0.12–12.63 mg L^{-1}, 0–11.5 mg L^{-1} and 0.05–7.93 mg L^{-1}, respectively. During phase I, carriers were added into the SBBR to form biofilms and promote anammox bacteria cultivation. From phase II to phase IV, the average TN removal efficiency remained high at 93 %, with the NLR increasing to 1.38 ± 0.14 kgN $m^{-3}d^{-1}$ and HRT decreasing to 6.3 h. Although, the influent nitrogen load showed some fluctuation, the reactor still exhibited high nitrogen removal performance when treating synthetic high concentration wastewater. The gradual formation of biofilms on carriers allowed bacterial biomass (especially anammox bacteria) to be effectively retained in the reactor, contributing to its stable performance over a long-term operational period. In phase V, the NLR continually increased to 1.70 ± 0.07 kgN $m^{-3}d^{-1}$, with the HRT set at 5.0 h. The TN removal efficiency reduced to 85.7 % in the first 30 days of operation, and then gradually increased to 94.4 %. The effluent NH_4^+–N, NO_2^-–N and NO_3^-–N concentrations were in the range of 0.3–29.2 mg L^{-1}, 11.0–48.0 mg L^{-1} and 2.5–37.8 mg L^{-1}, respectively. Due to the short settling time, an obviously sludge wash-out occurred in DEAMOX–SBBR, resulting in the reduction of nitrogen removal performance. Also, a continually increasing NLR probably have a shock effect on the activity of anammox bacteria. Under the circumstances, we collected the wash-out sludge and returned back to the DEAMOX–SBBR until the nitrogen removal performance was recovered. After 30 days of operation, the SBBR performance recovered and then remained stable. The average effluent NH_4^+–N, NO_3^-–N and NO_2^-–N concentrations were below 0.68, 1.92 and 9.90 mg L^{-1}, respectively.

Studies by Wei et al. (33) and Hosseini and Borghei (34) established that both SBBR and MBBR systems had a good resistance to varying loading rates, with reactor returning to a steady state within a short period. These results confirmed that the biofilm system of DEAMOX process had a strong adaptability to the varying loading rate. Du et al. (11) reported that the anammox process was the main nitrogen removal route in the novel DEAMOX system, further suggesting that the stability and high activity of the anammox bacterial community are the key factors for achieving

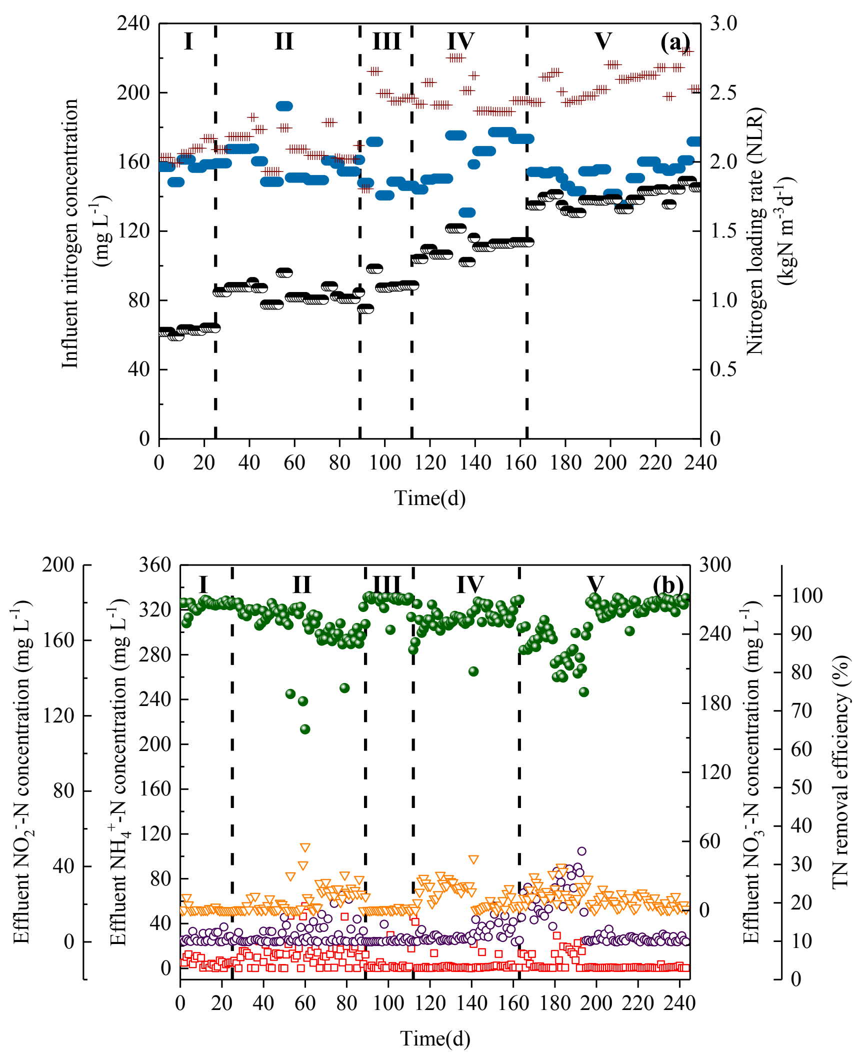

FIG. 2. (a) Variations of influent nitrogen compounds and NLR. Blue circles, inf NH_4^+–N; cross sign, inf NO_3^-–N; black and white circles, NLR. (b) Variations of effluent nitrogen compounds and TN removal efficiency. Red square, eff NH_4^+–N; purple circle, eff NO_2^-–N; yellow inverted triangle, eff NO_3^-–N; green circle, TN removal efficiency. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

high nitrogen removal efficiency. In this study, relatively stable and highly efficient nitrogen removal (>90 %) was achieved for almost 240 days, suggesting that the use of porous sponge carriers may provide a significant advantage for the immobilization of biomass and enhance the retention of anammox bacteria within the reactor. Moreover, Tatara et al. (35) have found that anammox microorganisms could be efficiently acclimated and enriched under relatively high NLR with short HRT. Therefore, batch tests were carried out to assess whether the anammox bacteria had been successfully cultivated on carriers, by analyzing the microbial activity.

Specific activities of functional bacteria in biofilms and suspended sludge The anammox activity was characterized by the SARR in both biofilms and suspended sludge. Fig. 3a shows the variations of SARR for biofilms and suspended sludge in DEAMOX−SBBR during different operational phases. The biofilm SARR increased from 1.6 mgN $gVSS^{-1}h^{-1}$ in phase I (day 13) to 4.3 mgN $gVSS^{-1}h^{-1}$ in phase III (day 92), with the formation of biofilm. The biofilm then maintained stable anammox activity with the SARR ranging from 3.6 to 4.0 mgN $gVSS^{-1}h^{-1}$, which may indicate that mature biofilms had formed on the carriers. The SARR of suspended sludge ranged from 1.49 to 2.56 mgN $gVSS^{-1}h^{-1}$ during the whole operational period, indicating that the fixed porous sponge carriers have a greater capacity to immobilize and retain anammox bacteria than suspended sludge. As a result, the abundance of anammox bacteria was enhanced in DEAMOX−SBBR. Meanwhile, these carriers provide anammox bacteria with a protective barrier against environment stress (36), the anammox bacteria activities of suspended sludge are more likely to be inhibited. Liu et al. (19) also reported that biofilms had a higher activity and abundance of anammox bacteria than suspended sludge. Conversely, biofilms have been found to have

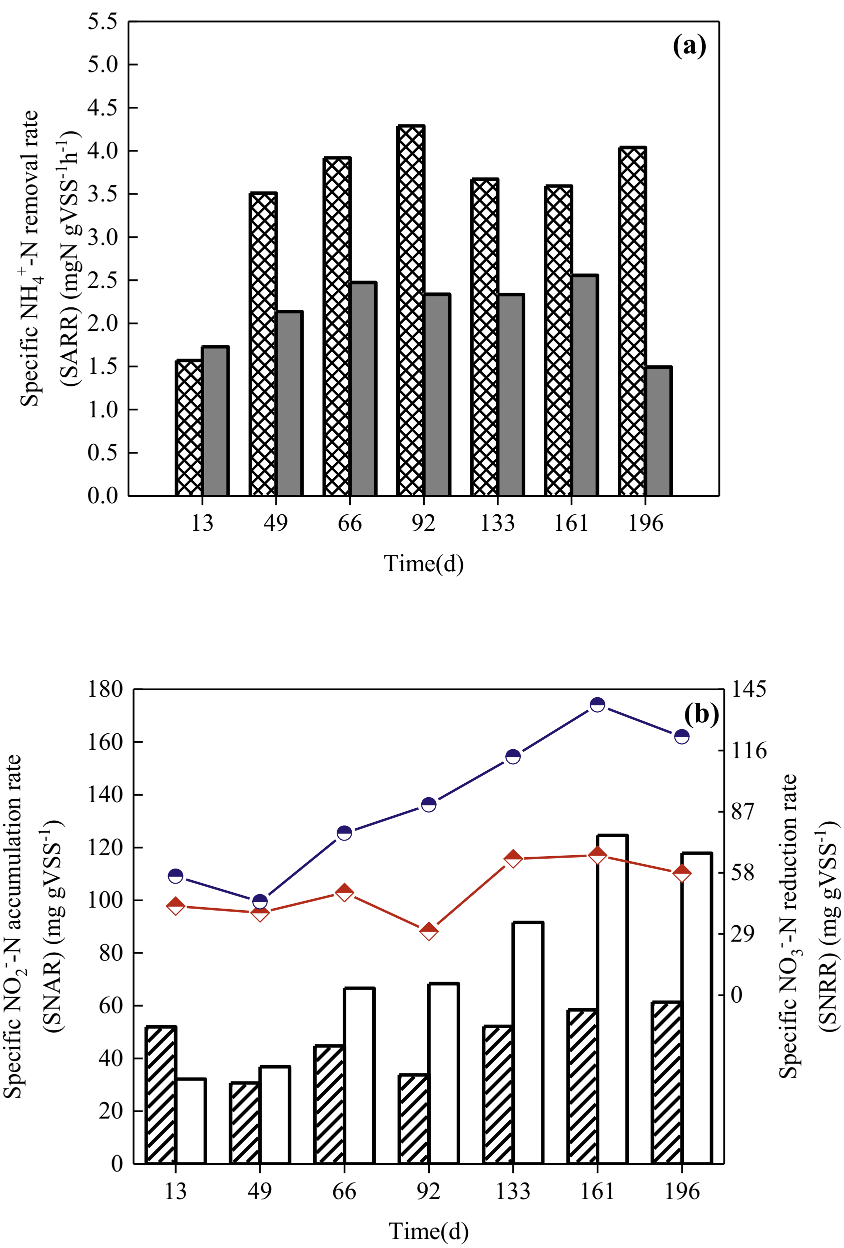

FIG. 3. Comparisons of nitrogen removal activity between biofilm and suspended sludge (S-sludge). (a) Specific NH_4^+−N removal rate (SARR) in anammox period. Grid columns, SARR of biofilm; shaded columns, SARR of S-sludge. (b) Specific NO_2^-−N accumulation rate (SNAR) and specific NO_3^-−N reduction rate (SNRR) in partial-denitrification period. Hatched columns, SNAR of biofilm; open columns, SNAR of S-sludge; red and white diamonds, SNRR of biofilm; blue and white circles, SNRR of S-sludge. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

more EPS than suspended sludge, which supplies a higher abundance of extracellular enzymes and nutrients for microbes (37), enhancing the activity of anammox bacteria.

The partial-denitrification activity was characterized by SNAR and SNRR in both biofilms and suspended sludge. As shown in the Fig. 3b, the SNAR of biofilms increased from 30.69 mgN $gVSS^{-1}h^{-1}$ in phase II (day 49) to 61.33 mgN $gVSS^{-1}h^{-1}$ in phase V (day 196) and SNRR of biofilms increased from 42.3 mgN $gVSS^{-1}h^{-1}$ in phase II (day 49) to 57.7 mgN $gVSS^{-1}h^{-1}$ in phase V (day 196). These values were much lower than in suspended sludge, where SNAR increased from 36.87 mgN $gVSS^{-1}h^{-1}$ in phase II (day 49) to 124.6 mgN $gVSS^{-1}h^{-1}$ in phase IV (day 161) and SNRR increased from 44.29 mgN $gVSS^{-1}h^{-1}$ in phase II (day 49) to 137.6 mgN $gVSS^{-1}h^{-1}$ in phase IV (day 161). This finding indicates that heterotrophic denitrifying bacteria tend to preferably grow in suspended sludge, rather than in biofilm environments, due to the richer COD and substrates. Because of this, when the nitrogen removal performance was deteriorated, heterotrophic denitrifying bacteria could be selectively washed out by discharging the suspended sludge without the loss of anammox bacteria. Due to the low activity of denitrifying bacteria in biofilms, the anammox bacteria had sufficient nitrite to maintain a relatively high anammox microbial activity with a low risk of complete denitrification. These results indicate that a stable and mature biofilm formed on carriers which helped to maintain a balance between functional bacteria and the nitrogen removal performance. Therefore, an investigation into biofilm development on carriers was performed to confirm whether the biofilm reached a stable maturity.

Biofilm development on carriers The variations of observed biofilm morphology were investigated at different operational phases. As shown in Fig. S2a, b, the blank sponge carrier presented a red color after attachment of the inoculated sludge in phase I (day 9) with an NLR of 0.78 ± 0.04 kgN $m^{-3}d^{-1}$. As the NLR increased from 1.05 ± 0.15 (phase II, day 52, 67) to 1.13 ± 0.10 kgN $m^{-3}d^{-1}$ (phase III, day 91), the biofilm gradually grew thicker and turned red which is the typical biofilm color observed with anammox bacterial enrichment (Fig. S2c, d), indicating that anammox bacteria grew well in the biomass attached on the carriers (38). It is of note, that as the NLR increased further to 1.38 ± 0.14 and 1.70 ± 0.07 $kgNm^{-3}d^{-1}$ in phase IV and phase V (day 138 and day 196, respectively), the biofilm surface began to be covered by a layer of yellow-gray bacteria which were considered to be heterotrophic denitrification bacteria, while the inner layer remained red (Fig. S2e, f). Because of the high organic matter contents in SBBR reactor, heterotrophic denitrifying bacteria may tend to grow on the surface of biofilms, protecting anammox bacteria from organic matter suppression (39–41). As the thickness of biofilms increased, an anoxic zone formed inside the biofilm, providing favorable conditions for the growth of anammox bacteria.

As the experimental results suggest that porous sponger carriers could effectively attach to anammox bacteria and enhance biomass retention. The biomass concentrations in biofilms were measured during the biofilm formation period, to assess whether the biomass was attached to carriers. As shown in Fig. 4, the biomass concentrations in biofilms increased to 3819 mgVSS L^{-1} over the entire period of operation, while the suspended sludge biomass concentrations remained almost unchanged with an average biomass maintaining 4734 mgVSS L^{-1}. All the above results indicate that a stable and mature biofilm had formed on carriers, increasing the biomass concentrations in SBBR reactor. It has often been reported that the formation and generation of biofilms are closely related to EPS, so analysis of the variations in EPS during biofilm growth are essential.

Changes in EPS during biofilm formation EPS is an organic macromolecule secreted by microorganisms, which adhere to cell surfaces and enhance the aggregation of bacteria, supporting the formation of stable microbial community structures (42). In SBBR system, EPS is directly related to microbial aggregation, adhesion and biofilm formation. Therefore, to investigate EPS variations can help explain biofilm formation. EPS can be classified into three fractions: soluble EPS (S-EPS), loosely-bound EPS (LB-EPS), and tightly-bound EPS (TB-EPS). As shown in Fig. 5, EPS from biofilms and suspended sludge were analyzed at phase I (day 20), phase II (day 48, 67), phase III (day 95, 110), phase IV (day 128, 140), and phase V (day 161, 196). Compared to suspended sludge, the total EPS (PN+PS) concentrations of biofilms increased from

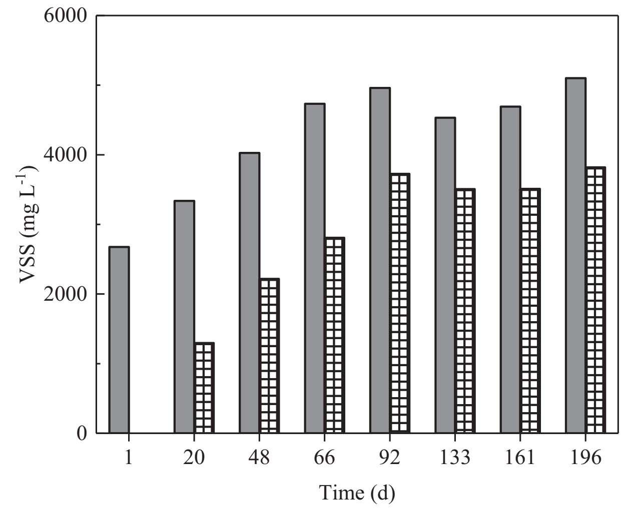

FIG. 4. Variations of VSS during long-term operational period of biofilms and suspended sludge. Grid columns, biofilm; shaded columns, S-sludge.

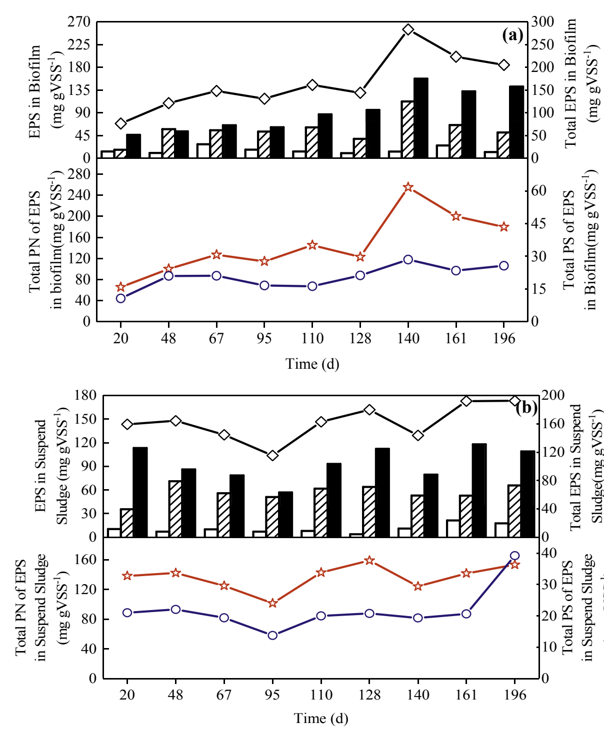

FIG. 5. Total EPS concentrations and variations of EPS contents (PN and PS) (a) in biofilms; and (b) in suspended sludge throughout the operational period. Open columns, S-EPS; hatched columns, LB-EPS; closed columns, TB-EPS; diamonds, total EPS; red five-pointed stars, total PN; blue circles, total PS. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

76.3 mg $gVSS^{-1}$ to 205.3 mg $gVSS^{-1}$ throughout the operational period, while the EPS variations in suspended sludge were not obvious. This finding is mainly because the EPS secreted from microorganisms helps the biomass adhere to carriers first, with further microbial growth on the carriers occurring as the nitrogen load increased. At the same time, the large amount of EPS secreted by attached microorganisms promotes the biofilm formation. Choi et al. (43) also reported that the extractable concentrations of EPS from biofilms increased during biofilm growth. As the biomass concentrations of suspended sludge did not show any obvious change, it can be assumed that the EPS from suspended sludge was maintained at a stable level.

It is of note, that the TB-EPS from biofilms increased from 46.1 mg $gVSS^{-1}$ to 142.0 mg $gVSS^{-1}$ during biofilm formation, while the TB-EPS from suspended sludge were always below 120 mg $gVSS^{-1}$ throughout the entire operational period. It has been reported that TB-EPS is beneficial to microbial adhesion and aggregation by bridging cells together in clusters (44). The increasing TB-EPS concentrations in biofilms observed in this study, suggests that TB-EPS plays an important role in microbial attachment in biofilm systems, supporting microbial cell adhesion to carriers. Miao et al. (45) and Gu et al. (46) also found that TB-EPS played a significant role in the formation of biofilms.

In the present study, PN and PS were found to be the major components in EPS, as well as small amounts of nucleic acids, lipids, and humic substances, among others. An analysis of EPS composition showed that proteins made up a significant proportion in both biofilms and suspended sludge (87.1 % and 87.6 %, respectively), followed by polysaccharides, which is consistent with the findings of Qian et al. (47). The protein contents in biofilms increased continuously from 65.6 mg $gVSS^{-1}$ to 179.6 mg $gVSS^{-1}$; however, the protein contents in suspended sludge increased only slightly from 138.2 mg $gVSS^{-1}$ to 153.2 mg $gVSS^{-1}$. With further biofilm growth, the bacteria continued to secrete high levels of protein. These proteins played a critical role in the aggregation of slow growing bacteria by increasing cell density and enhancing cell to cell interactions (48,49). As a result, high protein levels increase the information transfer and corporate action of anammox bacteria in biofilms, which may partly explain why the anammox activity in biofilms was higher than in suspended sludge. Ni et al. (41) reported that abundant polysaccharides could strengthen cell adhesion into the carriers. As the shown in Fig. 5b, the polysaccharide levels in biofilms increased from 10.68 mg $gVSS^{-1}$ to 25.7 mg $gVSS^{-1}$, promoting biofilm formation.

3D-EEM fluorescence spectra of different fractions of biofilm and suspended sludge Fig. S3 shows the 3D-EEM fluorescence spectra of LB-EPS and TB-EPS from biofilms and suspended sludge at phase II and phase V. Table S1 summarizes the fluorescence peak location and intensity in 3D-EEM fluorescence spectra for each fraction. As shown in Fig. S3, two main fluorescence peaks (peak A and peak B) could be observed. Peak A was observed at the excitation/emission wavelengths (Ex/Em) of 210–220 nm/340–355 nm, indicating aromatic protein-like substances. Peak B was identified at the Ex/Em of 280 nm/330–350 nm, indicating tryptophan protein-like substances. Peak A and peakB both appeared in the spectra LB-EPS and TB-EPS from biofilms and suspended sludge (Fig. S3a–h), while the peak C and peak D were only detected in the LB-EPS from suspended sludge (Fig. S3d, c). Peak C (280/420–445 nm) and peak D (340/410–415 nm) were assigned to humic acid-like substances generated from the decomposition of dead cells or macromolecular organics substances, such as proteins and polysaccharides (50,51). These results revealed that protein-like substances played a significant role in the formation of biofilms. Additionally, the biofilm was found to have a stronger resistance to environmental stress than suspended sludge, leading to the differences in EPS compositions observed between biofilms and suspended sludge. The fluorescence intensities of peak A and peak B greatly increased in both LB-EPS and TB-EPS of biofilms from phase II to phase V (Fig. S3a, b, e, f). This finding may suggest that the concentrations of protein-like substances increased with the biofilm growth and were the dominant components of EPS, contributing to biofilm formation and stability.

Quantitative microbial analysis of biofilms and suspended sludge The variations in abundance of functional anammox microorganisms in biofilms and suspended sludge, were assessed by quantitative PCR microbial analysis (q-PCR) on phase II (day 48) and phase IV (day 161). The results show that the abundance of anammox bacteria in biofilms increased by 2 orders of magnitudes from 6.08×10^9 to 1.00×10^{11} copies g^{-1} dry sludge, between phase II and phase IV, while the abundance of anammox bacteria in suspended sludge increased by 1 order of magnitude from 6.14×10^9 to 2.76×10^{10} copies g^{-1} dry sludge. This indicates that anammox bacteria adhered more effectively to carriers, with the biofilm gradually reaching maturity. Additionally, the proportion of anammox bacteria in biofilms significantly increased from 1.87 % to 11.48 % during biofilm formation, while the proportion of anammox bacteria in suspended sludge remained constant at 0.95 % and 0.96 %.

The proportion of anammox bacteria in biofilms was found to be much higher than in suspended sludge at the end of day 161 (phase IV), further confirming that anammox bacteria were successfully enriched in biofilms of DEAMOX–SBBR system. Compared to the former DEAMOX process of suspended sludge system, the DEAMOX–SBBR had some advantages in cultivating functional bacteria. The anammox bacteria and denitrifying bacteria have a different niche in this system. As the biofilm grew thick, biofilm provided a favorable environment for the growth of anammox bacteria, where the mass transfer of COD was limited. The denitrifying bacteria mostly existed in surface of biofilm and suspended sludge, owing to the richer COD and substrates. Consequently, the biofilm provided a protective barrier for anammox bacteria against environmental stress and a high proportion of anammox bacteria were achieved in biofilms.

Generally, the functional fixed carriers are beneficial to the biofilm formation and the growth of anammox bacteria, which providing a significant advantage for the immobilization of biomass and enhancing the enrichment of anammox bacteria within the reactor. It can be inferred that the biofilm formed on functional fixed carriers played a crucial role in the achievement of high nitrogen removal potential in DEAMOX–SBBR system.

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