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Optimizing start-up strategies for the two-inflow nitritation/anammox process: Influence on biofilm microbial community composition

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ABSTRACT

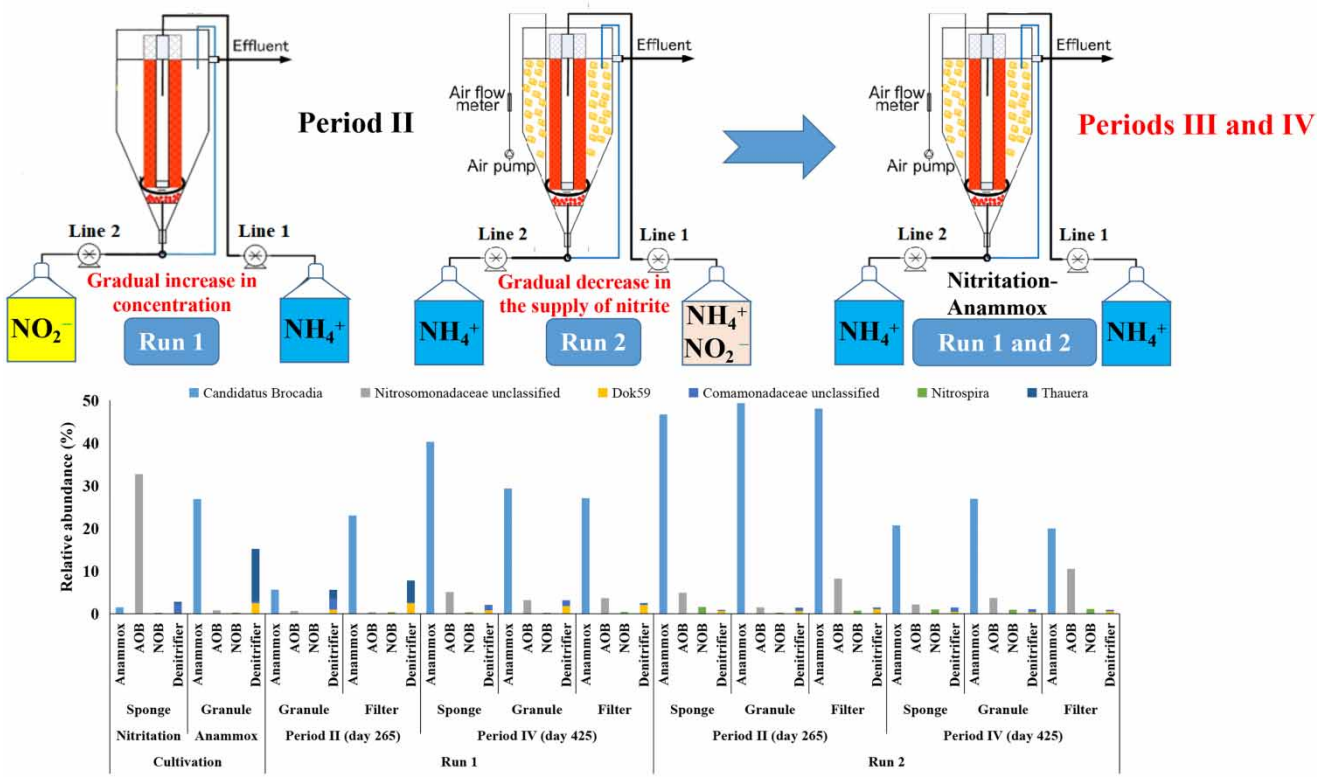
Low-energy nitrogen removal from ammonium-rich wastewater is crucial in preserving the water environment. A one-stage nitritation/anammox process with two inflows treating ammonium-containing wastewater, supplied from inside and outside the wound filter, is expected to stably remove nitrogen. Laboratory-scale reactors were operated using different start-up strategies; the first involved adding nitritation inoculum after anammox biomass formation in the filter, which presented a relatively low nitrogen removal rate (0.171 kg $N/m^3 \cdot d$), at a nitrogen loading rate of 1.0 kg $N/m^3 \cdot d$. Conversely, the second involved the gradual cultivation of anammox and nitritation microorganisms, which increased the nitrogen removal rate (0.276 kg $N/m^3 \cdot d$). Furthermore, anammox (*Candidatus Brocadia*) and nitritation bacteria (Nitrosomonadaceae) coexisted in the biofilm formed on the filter surface. The abundance of nitritation bacteria (10.5%) in the reactor biofilm using the second start-up strategy was higher than that using the first (3.7%). Thus, the two-inflow nitritation/anammox process effectively induced habitat segregation using a suitable start-up strategy.

Key words: biofilm, *Candidatus Brocadia*, habitat segregation, Nitrosomonadaceae, one-stage nitritation/anammox process

HIGHLIGHTS

- The addition of nitritation inoculum after the anammox biofilm formation in the two-inflow partial nitritation/anammox (PNA) process does not increase nitrogen removal.
- Gradual cultivation of anammox and nitritation microorganisms leads to better nitrogen removal.
- The two-inflow PNA process effectively induced habitat segregation and *Candidatus Brocadia* and Nitrosomonadaceae formed a thick biofilm on the filter.

GRAPHICAL ABSTRACT



INTRODUCTION

Anammox bacteria have gained increasing usage in the removal of nitrogen from ammonium-rich wastewater, such as industrial wastewater (Daverey *et al.* 2013), landfill leachate (Wang *et al.* 2016), and digester supernatant (Gut *et al.* 2006; Pereira *et al.* 2019), because of its low-energy consumption and low-sludge generation (Strous *et al.* 1999; Tsushima *et al.* 2007). Furthermore, stable operation can be achieved in the two-stage process, wherein nitritation (partial nitrification) and anammox reactions are conducted in separate reactors (Sharon-Anammox) (Hellinga *et al.* 1998) and a large number of plants are constructed (Van der Star *et al.* 2007). In contrast, a one-stage nitritation/anammox process, wherein both reactions occur in a single reactor, was developed for treating ammonia-containing wastewater due to reduced investment costs (Sliekers *et al.* 2003; Lackner *et al.* 2014). However, providing and maintaining an appropriate environment for both slow-growing ruler microorganisms, the ammonium-oxidizing bacteria (AOB), and anammox bacteria pose a challenge in the one-stage nitritation/anammox process (Strous *et al.* 1998). Low dissolved oxygen (DO) and intermittent aeration promote simultaneous microbial reactions and enhance total nitrogen removal (Jetten *et al.* 2001; Yang *et al.* 2015). Several types of reactors, such as the sequential batch reactor (Joss *et al.* 2009; Daverey *et al.* 2013), up-flow anaerobic sludge blanket (UASB) (Li & Sung 2015), and integrated fixed-film activated sludge (Zhang *et al.* 2015) have been used for one-stage anammox processes. Different types of biofilm carriers, including granular sludge (Pérez *et al.* 2014), sponge (Zhang *et al.* 2015), immobilized gel (Isaka *et al.* 2011), and membranes (Third *et al.* 2001) have been used to create necessary conditions for coexistence (Pérez *et al.* 2014). Cho *et al.* (2011) observed AOB growth on the outer portion of the carrier with anammox bacteria growing internally, whereas Li & Sung (2015) reported that the growth of both bacteria overlapped in the UASB reactor. AOB oxidizes ammonium to nitrite, creating an anoxic niche, which preserves the growth of anammox bacteria; however, each granule must maintain a suitable microbial consortium, which can increase the sensitivity of the long-term operation.

Zulkarnaini *et al.* (2018) proposed a novel approach, the one-stage partial nitritation/anammox (PNA) process involving two inflows to induce habitat segregation within each microorganism. In this process, wastewater was supplied to a reactor from two lines: Line 1, through the string wound filter placed inside the reactor and Line 2, directly from the bottom of the

reactor. The ammonium supplied from the bottom of the reactor was oxidized to nitrite, which subsequently reacted with the ammonium supplied from the filter within. This design facilitated the growth of specific microorganisms at each respective site. A laboratory-scale experiment using a two-inflow PNA process could successfully treat the artificial wastewater; however, the microbial community in the reactor biomass remains unverified. Furthermore, start-up processes are crucial in improving reactor performance. In this study, two reactors were operated with different start-up strategies to determine the optimum start-up method of the two-inflow PNA process and the microbial community in the reactor was analyzed to verify habitat segregation.

MATERIAL AND METHODS

Experimental reactor and operational conditions

Figure 1 shows a schematic diagram of the experimental reactor, which consists of an acrylic vessel with an active volume of 4 L. A polyester wound cartridge filter (length: 25 cm, 3.0 cm bore × 3.0 mm wall thickness, pore size: 10 μm) (Toyo Advantec type TCW-10-EPS, Japan) was placed at the center of the reactor. Substrate was added through Line 1 (inside the filter) and Line 2 (bottom of the reactor) using a peristaltic pump (EYELA, Japan). The bulk solution was mixed via circulating effluent water into the reactor using a metering pump (IWAKI, Japan) at a flow rate 10 times the inflow rate. All reactors were installed in a room with temperature controlled at 35 °C. The operational conditions are listed in Table 1. Different reactor-start-up strategies were used in Runs 1 and 2. In Run 1, anammox granules that were cultivated in a UASB reactor at 35 °C were inoculated, and anammox bacteria were enriched in the reactor via feeding ammonium and nitrite media through Line 1 during Period I. These media were then fed from Lines 1 and 2, respectively, by increasing the nitrogen loading rate (NLR) of 0.20–0.50 kg $N/m^3 \cdot d$ (Period II). An N_2-filled gas bag was connected to the substrate tank during these periods. AOB were cultivated separately under semi-aerobic conditions, using a vessel packed with a sponge carrier fed with ammonium medium (Zulkarnaini *et al.* 2018). At the initial stage of Period III, sponges from the nitritation reactor were placed in the two-inflow reactor and aeration was initiated. Ammonium medium was added from both lines at NLR of 0.5 kg $N/m^3 \cdot d$. During Period IV, the NLR was increased to 1.0 kg $N/m^3 \cdot d$ via increasing flow rate, which was then decreased to 0.8 kg $N/m^3 \cdot d$ during Period V via decreasing the influent ammonium concentration. During Periods III to V, aeration was regulated to maintain the DO concentration at 0.5–1.5 mg/L.

Basal medium composition: $KHCO_3$, 500 mg; KH_2PO_4, 27.2 mg; $MgSO_4.7H_2O$, 300 mg; $CaCl_2.2H_2O$, 180 mg; $CaCl_2$, 136 mg; and 1 mL trace element solutions I and II (Van De Graaf *et al.* 1996), supplemented with each nitrogen concentration of $(NH)_2SO_4$ and $NaNO_2$ (per L ELIX water).

In Run 2, the anammox granules were inoculated and anammox bacteria were cultivated under anaerobic conditions at a low NLR (Period I), which was identical to that of Period I in Run 1. In the initial stage of Period II, the sponges from the nitritation reactor were transferred to the experimental two-inflow reactor and aeration was initiated. The

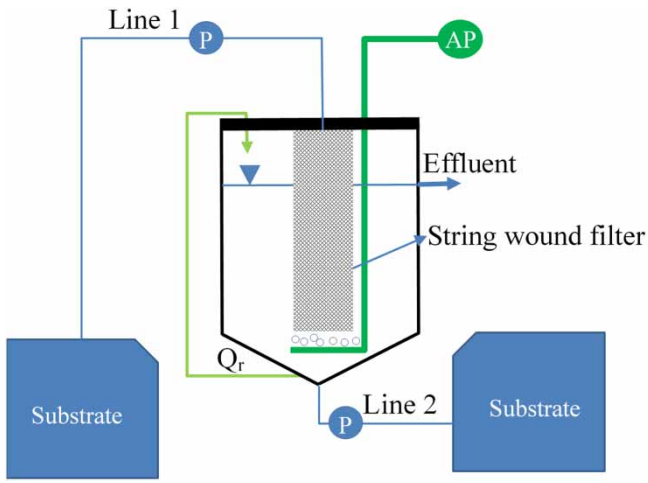


Figure 1 | Experimental reactor. Operated at 35 °C. Q_r: recirculation.

Table 1 | Operational conditions in the reactors

Run	Period no.	Time (d)	HRT (h)	Airflow rate (L/min)	Nitrogen concentrations in each medium (mg N/L)						NLR (kg N/m^3 · d)
					Line 1		Line 2		Average		
					NH_4^+	NO_2^-	NH_4^+	NO_2^-	NH_4^+	NO_2^-	
1	I	0–53	24	–	100	100	–	–	100	100	0.20
	II	54–270	24	–	200–500			200–500	100–250	100–250	0.20–0.50
	III	271–390	24	0.15–0.20	500	0	500	0	500	0	0.50
	IV	391–430	12	0.20	500	0	500	0	500	0	1.00
	V	431–497	12	0.10	400	0	400	0	400	0	0.80
2	I	0–53	24	–	100	100	–	–	100	100	0.20
	II	54–240	24	0.18	100–300	100–0	500	0	300–400	50–0	0.35–0.40
	III	241–390	24	0.20	500	0	500	0	500	0	0.50
	IV	391–430	12	0.20	500	0	500	0	500	0	1.00
	V	431–497	12	0.2	400	0	400	0	400	0	0.80

anammox medium, which consisted of ammonium and nitrite, was supplied from Line 1. The concentration of nitrite subsequently reduced from 200 to 100 mg/L and the ammonium concentrations increased periodically from 100 to 300 mg/L. The ammonium medium was also added from Line 2. The DO concentration was maintained at 0.5–1.5 mg/L. During Periods III to V, the ammonium medium was supplied from both lines at the same NLR similar to that of Run 1.

The influent and effluent waters were sampled once weekly and filtered using 0.2 μm pore size membranes (Merck Millipore Ltd, Germany). The concentrations of ammonium, nitrite, and nitrate were analyzed using an ion chromatograph (Shimadzu HIC-SP, Japan). The pH was measured using a pH meter (HORIBA F-71, Japan) and DO was measured using a DO meter (HACH HQ30d, Germany).

The reactor performance was evaluated based on nitrogen balance. The nitrogen removal rates (NRRs, kg N/m^3 · d) were calculated according to the following equation:

$$\mathrm{NRR} = \frac{[\mathrm{NH_4^+ - N}]_{\mathrm{in}} + [\mathrm{NO_2^- - N}]_{\mathrm{in}} - [\mathrm{NH_4^+ - N}]_{\mathrm{out}} - [\mathrm{NO_2^- - N}]_{\mathrm{out}} - [\mathrm{NO_3^- - N}]_{\mathrm{out}}}{\mathrm{HRT}} \quad (1)$$

where: HRT = hydraulic retention time.

Microbial community analysis

Biofilms attached to the filter surface and on the sponge, and granules settled at the bottom were collected on days 265 (Period II) and 425 (Period IV) from both runs. Additionally, inoculated anammox granules and biofilms on the sponge in the nitritation reactor were collected.

DNA was extracted using a PowerSoil DNA Isolation Kit (QIAGEN, Germany) as per manufacturer's instructions. The 16S rRNA genes were amplified via polymerase chain reaction (PCR) using universal forward primer 515F/universal reverse primer 806R (Caporaso *et al.* 2012). The PCR program consisted of 25 cycles of 10 s at 98 °C, 15 s at 55 °C, and 45 s at 68 °C, and was performed using an Applied Biosystems 2720 thermal cycler (Thermo Fisher Scientific, Japan). The PCR products were sequenced using the Illumina MiSeq method (Illumina, USA). The sequences were processed using the UPARSE line (Edgar 2013) and QIIME software with SILVA_128 as the reference database (Caporaso *et al.* 2010).

RESULTS AND DISCUSSION

Reactor performances

Figures 2 and 3 show the reactor performances for Runs 1 and 2, respectively. NRRs are summarized in Table 2. In Period I, the substrate consisted of 100 mg N/L ammonium and 100 mg N/L nitrite was supplied to the reactor via Line 1 to accelerate anammox biofilm formation on the filter surface. The anammox reaction occurred immediately due to the inoculation of anammox granules. After 50 days, the anammox bacterial growth was observed on the filter surface. Furthermore, NRR reached

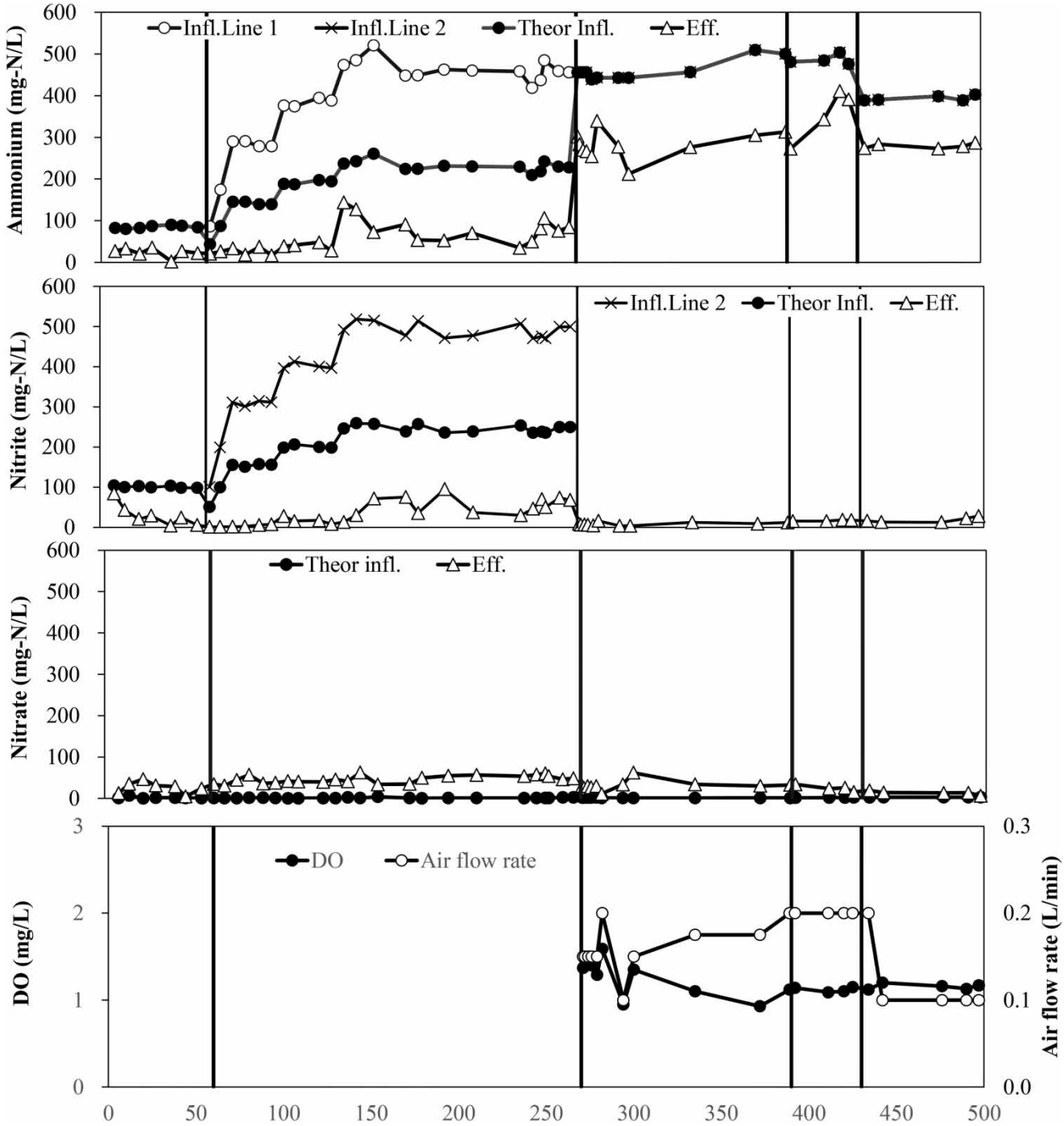


Figure 2 | Reactor performance of Run 1.

0.141 kg N/m^3 · d at NLR of 0.20 kg N/m^3 · d. During Period II, the ammonium and the nitrite media were supplied from Lines 1 and 2, respectively. Subsequently, both ammonium and nitrite concentrations in the media increased periodically to enhance the NRR via the anammox reaction. Initially, NRR decreased to 0.038 kg N/m^3 · d at the start of Period II; however, upon transitioning to the two-line system, NRR increased in relation to NLR and reached 0.279 kg N/m^3 · d at an NLR of 0.5 kg N/m^3 · d. A red-colored biofilm was formed on the filter surface. At the beginning of Period III, the sponge from the nitritation reactor, which supplied ammonium and aerated the media, was introduced to the two-inflow reactor and aerated.

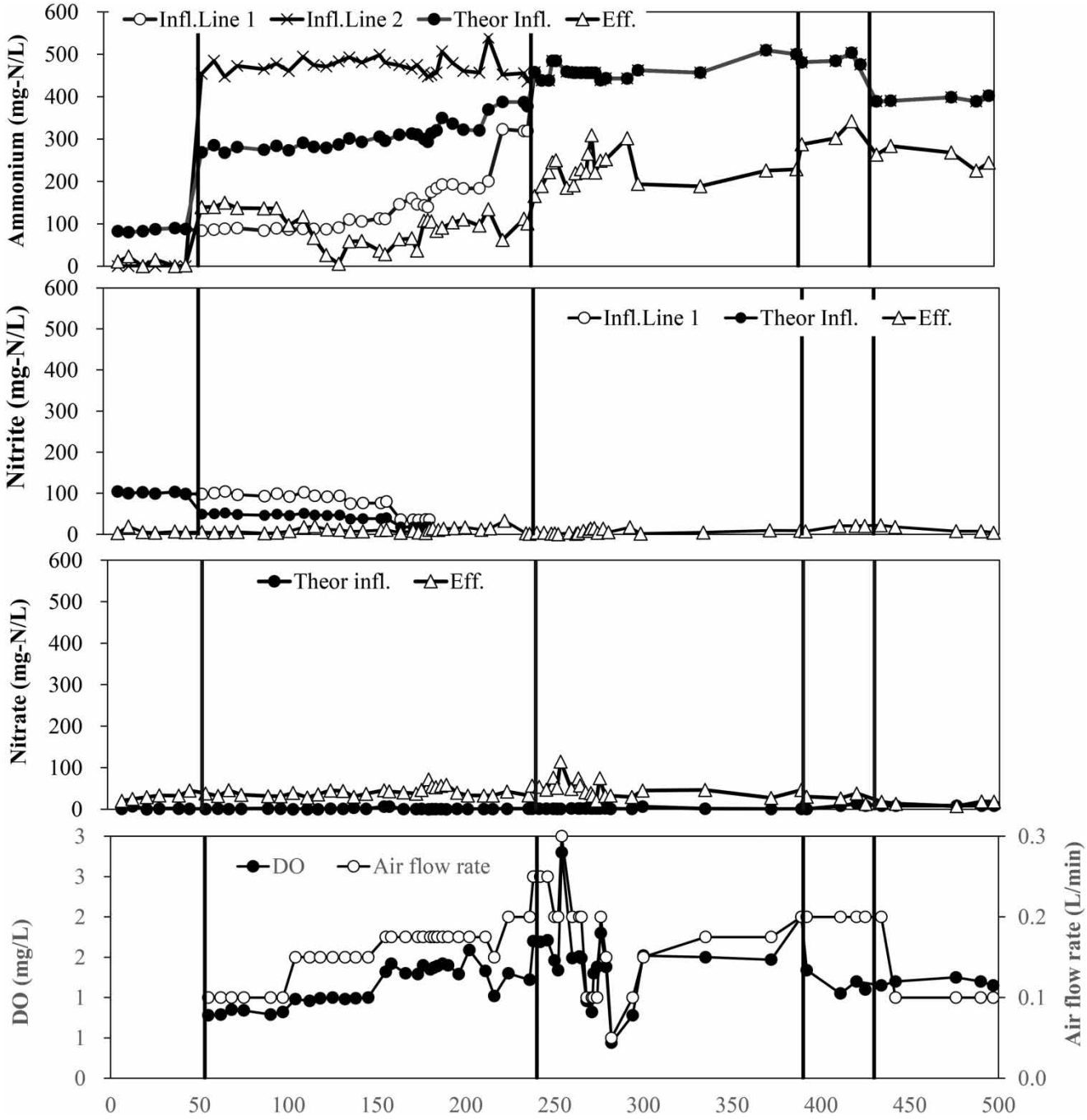


Figure 3 | Reactor performance of Run 2.

Table 2 | NRR* in each period

Period	I	II	III	IV	V
Run 1	0.141	0.279	0.153	0.105	0.171
Run 2	0.144	0.237	0.227	0.278	0.276

*Unit: kg N/m^3 · d.

An ammonium medium (500 mg N/L) was supplied from both lines. The ammonium concentration decreased and a low concentration of nitrate was detected in the effluent. Furthermore, nitrite accumulation was observed, whereas nitration was not, indicating the occurrence of both nitritation and anammox reactions in the reactor. Initially, the DO concentration occasionally increased during this period, resulting in unstable effluent concentrations of ammonium and nitrite; however, after one month, the effluent quality stabilized via the DO control at approximately 1.0 mg/L, and an NRR of 0.153 kg $N/m^3 \cdot d$ was achieved. In Period IV, the NLR was increased to 1.0 kg $N/m^3 \cdot d$ via increasing the flow rate, which increased the effluent ammonium concentration and decreased NRR to 0.105 kg $N/m^3 \cdot d$. In Period V, NRR increased to 0.171 kg $N/m^3 \cdot d$ at an NLR 0.8 kg $N/m^3 \cdot d$.

In Run 2 (Figure 3), the operational process in period I was identical to that of Run 1, yielding similar results with an NRR of 0.144 kg $N/m^3 \cdot d$ at NLR 0.20 kg $N/m^3 \cdot d$. In Period II, nitritation sponges were added and aeration was initiated. The ammonium medium was supplied from Line 1, whereas the nitrite medium was supplied from Line 2. The nitrite concentration from Line 2 was gradually decreased from 100 mg N/L to zero, whereas the ammonium concentration was increased from 300 to 400 mg N/L. The DO concentration was maintained below 1.5 mg/L via air flow regulation. The anammox reaction efficiently eliminated most of the nitrite and approximately 80% of the ammonium. Both ammonium oxidation and anammox reaction occurred, resulting in an NRR of 0.237 kg $N/m^3 \cdot d$ at an NLR of 0.260 kg $N/m^3 \cdot d$. In Period III, nitrite supplementation was discontinued and the ammonium concentration in both lines was maintained at 500 mg N/L. The nitritation/anammox reaction occurred stably. Ammonium conversion efficiency (ACE) was approximately 50% and NRR was 0.277 kg $N/m^3 \cdot d$. Moreover, NLR increased in Period IV and decreased in Period V via varying the flow rate; however, this did not alter the reactor performance. Although the trend of the reactor performance after Period III was similar to that in Run 1, the maximum NRR and nitrogen removal rate (NRE) in Run 2 were higher than those in Run 1. These results suggest that the start-up strategy affects reactor performance and gradual acclimation of bacterial facilitation of nitritation is crucial for enhancing the nitritation/anammox process.

Microbial community

The phylogenetic classification of bacterial sequences at the phylum level is presented in Table 3. Significant differences between the inocula and biomass in the reactors were observed. Dominant phyla in the inoculated anammox granules were Chloroflexi (40.7%) and Planctomycetes (31.9%). Proteobacteria (18.1%), Bacteroidetes (3.6%), and Acidobacteria (2.5%) were also detected. In contrast, Proteobacteria was detected in high abundance (42.0%) in the sponge inoculum. In the two-inflow reactors, Planctomycetes (20.6–52.9%), Chloroflexi (2.4–49.1%), Chlorobi (2.5–38.7%), and Proteobacteria (4.2–12.3%) were detected in all samples.

Figure 4 shows the presence of anammox bacteria, AOB, nitrite-oxidizing bacteria (NOB), and denitrification bacteria in each sample. Table S4 provides an overview of the bacterial sequence distribution at the taxonomic levels.

In the inoculated granules, the most abundant bacterial genus was *Candidatus Brocadia* (26.9%), which is a common anammox bacterium. Uncultured bacteria envOPS12 (7.3%) and SBR1031 (A4b: 19.5%; SJA101: 11.2%), belonging to Anaerolineae of the Chloroflexi phylum, were also detected. The Chloroflexi phylum plays a beneficial role in providing a filamentous scaffold for floc formation, fermenting carbohydrates, and degrading complex organic compounds to support the growth of other bacterial populations in activated sludge (Speirs *et al.* 2019). SBR1031 is a heterotrophic bacterium commonly found in anammox reactors without organic carbon compounds. It can utilize extracellular polymeric substances as a carbon source and contribute to the removal of cellular debris and extracellular proteins, which are essential for cell aggregation and anammox sludge granulation (Li *et al.* 2023; Yu *et al.* 2023). Denitrifying bacteria *Thauera* and *Dok59*, belonging to the family Rhodocyclaceae, also grew (12.3 and 2.5%, respectively) using endogenous organic substances. Additionally, low abundance of Phycisphaerales belonging to the phylum Planctomycetes was detected. In the inoculated sponge in the nitritation reactor fed with ammonium under semi-aerobic conditions, the most abundant bacteria were unclassified Nitrosomonadaceae, an ammonium-oxidizing bacterium. NOB–*Nitrospira*, were not detected, implying that partial nitrification occurred in the sponge reactor.

In Run 1, the reactor received ammonium via Line 1 and a sufficient amount of nitrite via Line 2 without aeration after seeding anammox granules during Period II. In the granules collected from the bottom of the reactor collected on day 265 (final of Period II), the abundance of *Candidatus Brocadia* decreased to 5.6% and Phycisphaerales belonging to phyla Planctomycetes increased, because nitrite alone was added from the bottom of the reactor. Furthermore, the abundance of denitrifiers (*Thauera* and *Dok59*) decreased. Conversely, the abundance of *Candidatus Brocadia* was 33.1% in the biofilm

Table 3 | Relative abundance of sequences at the phylum level (%)

	Inoculum		Run 1					Run 2					
			Day 265		Day 425			Day 265			Day 425		
	Sponge	Granule	Granule	Filter biofilm	Sponge	Granule	Filter biofilm	Sponge	Granule	Filter biofilm	Sponge	Granule	Filter biofilm
Planctomycetes	2.0	31.9	31.5	35.6	41.1	34.1	30.7	47.8	52.9	49.1	21.6	28.3	20.6
Chloroflexi	8.0	40.7	49.1	34.0	2.4	22.9	29.4	15.7	23.6	18.1	18.0	24.9	30.6
Chlorobi	10.2	0.8	2.5	9.0	27.2	16.5	18.1	10.9	8.0	9.3	38.7	26.2	15.5
Proteobacteria	42.0	18.1	11.3	12.3	10.4	10.7	9.0	7.0	4.2	10.4	6.0	7.0	13.8
Bacteroidetes	14.6	3.6	0.1	1.6	7.0	6.5	6.4	10.0	4.1	7.9	9.2	8.1	14.2
BRC1	4.2	0.8	2.8	2.5	1.8	2.3	1.6	2.0	3.2	2.0	0.5	1.0	1.1
Acidobacteria	10.4	2.5	0.6	2.4	5.7	3.0	2.0	3.3	1.4	1.1	3.0	2.1	1.4
OD1	0.0	0.3	0.2	0.6	0.0	0.0	0.0	0.4	1.0	0.5	0.2	0.1	0.0
Armatimonadetes	5.3	0.3	1.0	0.7	2.3	2.1	1.5	0.5	0.7	0.4	1.2	0.5	0.3
Nitrospirae	0.0	0.0	0.1	0.0	0.1	0.2	0.2	1.6	0.3	0.7	1.0	0.9	1.1
Others	3.2	1.0	0.6	1.5	1.9	1.6	1.0	0.9	0.6	0.5	0.7	0.8	1.2

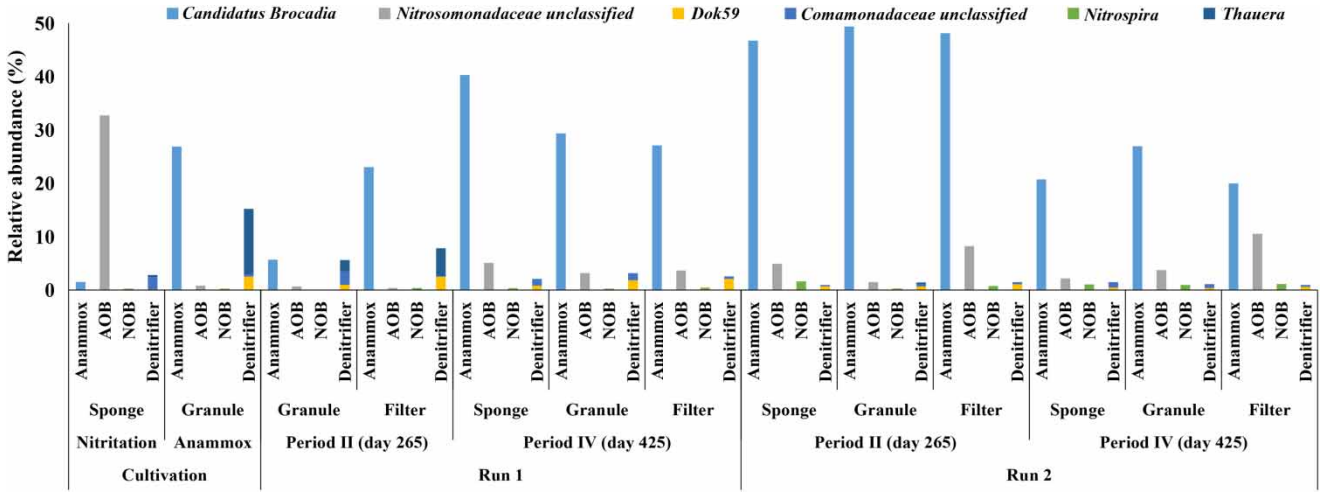


Figure 4 | Abundance of anammox, ammonia oxidation, nitrite oxidation, and denitrification bacteria in each sample.

attached to the filter surface. Anaerolineae envOPS12 was also detected with high abundance (27.1%); anammox bacteria and Anaerolineae may have detached from the granules and formed a biofilm on the filter surface. On day 425 of Period V in Run 1, in which ammonium was supplied from both lines and aerated after inoculation of the nitritation sponge in Period III, similar communities were observed in all biomasses. *Candidatus Brocadia* was detected in the range of 26.9–27.1%, and envOPS12 and Ignavibacteriaceae (phyla Chlorobi) were detected in high abundance. Ignavibacteriaceae a is non-phototrophic chemoheterotrophic facultative-anaerobic bacterium family that uses organic matter from other cells for biofilm formation (Connan *et al.* 2017). Biofilm forming bacteria shifted from anaerobic to facultative-anaerobic via aeration. Although Nitrosomonadaceae in the sponge decreased, they were detected with an abundance of 3.2–3.3% in all biomasses, implying that both nitritation and anammox occurred in the entire reactor.

In Run 2, the sponge was added during the initial of Period 2 (day 54) and nitrite was supplied from Line 2 at a low concentration until day 240. Only ammonium was supplied via both lines and the reactor was aerated on day 264; the abundance of *Candidatus Brocadia* was >40% in all biomasses. The abundance of Nitrosomonadaceae in the sponge and filter biofilm was relatively high (4.9 and 8.2%, respectively). NOB was not detected in the granules and biofilm; however, only 1.59% of *Nitrospira* was detected in the sponge. As the sponges remained afloat in the reactor, sufficient oxygen was supplied to the sponge surface. In the 425-day sample of Run 2, a consortium similar to that of Run 1 was observed in the sponge and biofilm, which presented a high abundance of *Candidatus Brocadia*, envOPS12, and Ignavibacteriaceae. In the granule, the abundance of envOPS12 significantly decreased; however, the decrease was observed as they loosened and decreased in quantity. Conversely, the abundance of Nitrosomonadaceae was considerably higher than that in Run 1. Although the total biomass concentration in each site was unclear, the biofilm thickness increased to approximately 5 mm. The nitritation/anammox reaction is considered to mainly occur in the biofilm. Figure 5 shows the biofilm consortium in the reactor. On the biofilm surface, AOB produces nitrite under semi-aerobic conditions. In the deep biofilm, anammox bacteria produce nitrogen gas using nitrite supplied from the biofilm surface and ammonium supplied from inside the filter.

As mentioned in the section 'Reactor performances', the NRR in Run 2 was considerably higher than in Run 1. These results indicate that the growth of nitritation bacteria on the biofilm surface is an important factor in improving NRR. In this study, the sponge helped accelerate the nitritation reaction. The two-inflow reactor facilitated ecological channel maintenance between both bacteria. A sudden change in the environment, such as the start-up in Run 1, was unsuitable for the formation of the biofilm consortium; however, the NRR was relatively low, despite being within the range of previous reports. Beyond its conventional role in nitrogen removal, DO plays an important role in maintaining the balance between AOB and anammox and inhibiting the growth of NOB. Notably, in this study, the DO level was higher than those of previous studies, which typically reported a DO of 0.5 mg/L (Antwi *et al.* 2019; Li *et al.* 2019). Despite the elevated DO, nitrate was undetectable and the abundance AOB remained low, indicating effective control over nitrite oxidation. Furthermore, the significantly increased anammox abundance, in comparison to a single inflow reactor, suggests that the two-inflow system created optimal conditions for anammox bacteria.

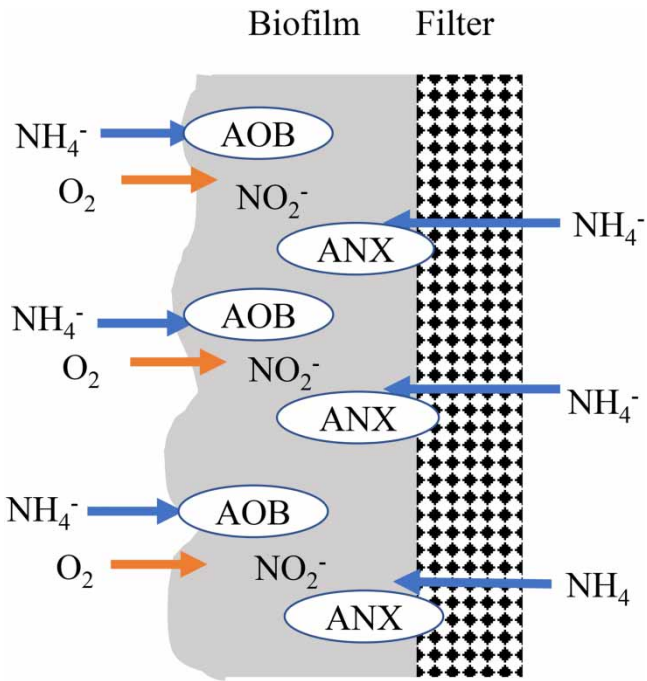


Figure 5 | Model of the biofilm. AOB: ammonia oxidation bacteria, NOB: nitrite oxidation bacteria, and ANX: anammox bacteria.

The filter surface area versus water volume was low due to the presence of a single filter; hence, the biomass amount was insufficient for NRR improvement. The multi-filter process could improve reactor performance. In addition, the sponges attacked the filter surface biomass and prevented its growth on the filter. Sponge removal after AOB acclimation is considered optimal.

CONCLUSIONS

The two-inflow PNA process demonstrated a stable performance. The start-up strategy of Run 2, which involved a gradual decrease in nitrite concentration in the influent, resulted in better performance than that of Run 1. The results of microbial community analysis revealed that *Candidatus Brocadia* and Nitrosomonadaceae were detached from the inoculated granule and sponge biomass, respectively, and formed a thick biofilm on the filter. The two-inflow PNA reactor could induce habitat segregation; however, the NRR did not increase as in previous reports. Further research is required to improve reactor performance.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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