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Enhanced selective enrichment of partial nitritation and anammox bacteria in a novel two-stage continuous flow system using flat-type poly (vinylalcohol) cryogel films

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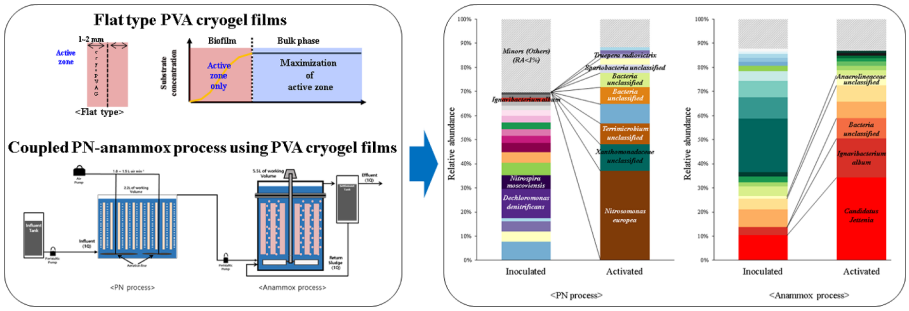
GRAPHICAL ABSTRACT



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ABSTRACT

To improve stability of nitrogen removal in partial nitritation (PN)-anammox process, flat-type cryogel films using poly (vinylalcohol) named as FT-CPVAF were applied in continuous reactors. Stable PN operation was maintained with short acclimation of 8 days and ammonium oxidation rate of 1.68 ± 0.12 kg N m^{-3} d^{-1} comparatively higher than previous studies. The nitrogen removal, initially inhibited by an oxygen shock, was immediately reactivated with short lag-period by immobilization of anammox bacteria in FT-CPVAF. A novel two-stage PN-anammox process was operated in a continuous flow using FT-CPVAF for treatment of ammonium-rich synthetic wastewater (influent 315 mg NH_4^+-N L^{-1}) showing 89.6 ± 0.76% of nitrogen removal at short hydraulic retention time (7.7 h). The use of FT-CPVAF enhanced selective enrichment of

Abbreviations: Anammox, Anaerobic ammonia oxidation; AOB, Ammonia oxidizing bacteria; AOR, Ammonia oxidation rate; AS, Activated sludge; BOD, Biological oxygen demand; BNR, Biological nitrogen removal; COD, Chemical oxygen demand; DO, Dissolved oxygen; FA, Free ammonia; FBBR, Fixed-bed bioreactor; FT-CPVAF, Flat-type PVA cryogel film; HRT, Hydraulic retention time; NCBI, National Centre for Biotechnology Information; NLR, Nitrogen loading rate; NOB, Nitrite oxidizing bacteria; NRE, Nitrogen removal efficiency; NRR, Nitrogen removal rate; OTU, Operational taxonomic unit; PAB, Pre-enriched anammox bacteria; PCR, Polymerase chain reaction; PN, Partial nitritation; PVA, Poly (vinylalcohol); SBR, Semi-batch reactor; VSS, Volatile suspended solid; WWTP, Wastewater treatment plant

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AOB and anammox bacter ia confirmed by high-throughput sequencing of i.e., relative abundances of *Nitrosomonas europaea* C-31 (37.14% in PN reactor) and '*Candidatus* Jettenia caeni' (34.36% in anammox reactor).

1. Introduction

The conversion of unreactive nitrogen (N_2) to reactive nitrogen species (such as ammonia and oxidized nitrogen species) is caused mainly by human activities through municipal, agricultural, and industrial undertakings. It has adversely affected natural ecosystems thus ensuing extreme changes in climate beyond a critical point, and further leading to human health risk in the world (Rockström et al., 2009). In particular, the anthropogenic nitrogen discharges on the land reach to aquatic environment via release of treated wastewater including human waste or sewage and food waste (18.1%), surface run-off to river (32.2%), volatilization to atmosphere (32.2%), and other losses (17.4%) (Matassa et al., 2015). The excessive reactive nitrogen released into aquatic environment causes severe ecological problems such as acidification of freshwater ecosystems, toxic algal bloom, and destroyed niches of marine lives (Camargo and Alonso, 2006). High nitrogen strength seems to be a key characteristic of piggery wastewater and human-generated wastewater including urine, and their anaerobically digested supernatants (260–7,000 mg NH_4^+-N L^{-1} for side-stream municipal wastewater) (Ahn, 2006) and industrial wastewater (approximately 250–400 mg NH_4^+-N L^{-1}) with low biological oxygen demand (BOD)(Tokutomi et al., 2010).

Biological nitrogen removal (BNR) processes mainly using bacteria are generally preferred to physicochemical processes because of their cost-effectiveness for the removal of nitrogenous pollutants from water environment (Ahn, 2006). In most of the current wastewater-treating BNR systems, a combination of bacterial nitrification and denitrification is used. However, the nitrification-denitrification coupled BNR system has limitations such as (i) high energy consumption due to aeration for complete nitrification (4.57 kg O_2 kg NH_4^+-N^{-1}), (ii) additional cost for external carbon source use (e.g., methanol) for stimulating the heterotrophic denitrification (0.349 kg COD kg NO_3^{-1}), (iii) production of wasted sludge due to the heterotrophic bacterial growth, and (iv) increased emission of greenhouse gases (N_2O and CO_2) in heterotrophic denitrification and respiration respectively (Okabe et al., 2011). These problems tend to be intensified when applied in treating high nitrogen strength wastewater. To circumvent the drawbacks of the currently available nitrification-denitrification coupled BNR system, the integration of partial nitritation (PN) and anaerobic ammonium oxidation (anammox) has been regarded as an effective alternative considering its following advantages: (i) significantly reduced aeration energy (57% reduction) due to use of PN, (ii) no requirement of external carbon source addition and reduced sludge production due to use of slow growing autotrophic bacteria, and (iii) reduced greenhouse gas emission due to little N_2O production in the PN and anammox pathways and less CO_2 production by the autotrophic carbon fixers (Maktabifard et al., 2018).

Currently PN-anammox coupled BNR systems are applied in full-scale treatment of high nitrogen strength wastewater (Lackner et al., 2014). They are operated generally in single-stage semi-batch reactor (SBR) modes with suspended granules. In a single-stage system, it is often difficult to provide an optimum operational condition to co-satisfy the different PN and anammox bacteria. If the ecological balance between the two vulnerable BNR autotrophs are subjected to any slight perturbations in operational condition(s), the start-up period for PN-anammox granule and recovery after perturbation can take long time because of their extremely slower growth rates (Ali and Okabe, 2015). Related to studies on full scale plants for the single-stage and two-stage PN-anammox process in case of side-stream wastewater treatment, NLR (nitrogen loading rate) has been found to be in the range 0.04–1.2 and 0.26–1.0 kg-N m^{-3} d^{-1} respectively (Fuchs et al., 2017; Lackner et al., 2014). In addition, SBR mode cannot be applied for continuous flow and hence disadvantageous for scale-up of treatment capacity (Mohan et al., 2016). To circumvent these limitations, two-stage continuous flow reactor systems have been applied with whole-cell immobilization techniques (Dosta et al., 2015; Li et al., 2014). However, the stabilities in BNR performance and selective enrichment of PN- and anammox-related microbial communities have yet to be examined in a long-term stable operation and maintenance under continuous flow conditions.

Among various materials available for whole-cell immobilization, poly (vinyl alcohol) (PVA) has been frequently adopted because of its non-toxicity to microorganisms, straightforward gelation process and relatively low cost (Choi et al., 2017). In the present study, the physical method employed for cryo-gelation technique using freezing and thawing is known to be advantageous in increasing gas permeability (Ingavle et al., 2015) and allow N_2 escape easily. However, the chemical method of production of PVA gel in other study (Choi et al., 2017) cause swelling of gel and obstruct N_2 coming out of the gel. The previous work (Choi et al., 2018) provided experimental evidence to support the applicability of the PVA cryogelation technique in anammox immobilization. In terms of cryo-gel configuration, cube-type or bead-type shape is generally used in most of whole cell immobilization techniques (Chen et al., 2015; Magrí et al., 2012). However, the accessibility of microorganisms to physiologically important substrates inside the cryogels is significantly limited with increase in the thickness (> 1.5 mm) of cube-type or bead-type cryogels (Choi et al., 2018). In the current PVA cryogel fabrication method, it is difficult to uniformly control the thickness of bead-type (or cube-type) gels at the level 1–3 mm. To solve this problem, flat-type PVA cryogel film (FT-CPVAF) formation was proposed in our previous work (Choi et al., 2018) wherein thin flat-type films of uniform thicknesses (1 mm, 2 mm, and 3 mm) could be consistently fabricated, and the optimal anammox enrichment was observed in the 2 mm-thick films. However, the applicability of FT-CPVAF in PN process has yet to be tested. If it works, it will let us develop a novel two-stage reactor system in which PN process can be coupled with anammox process using FT-CPVAFs in a continuous flow mode. In addition, the novel system development will let us experimentally examine if the two 'barely cultivatable' bacteria related to PN and anammox process can be selectively enriched using the FT-CPVAFs.

In this work, the objectives were (1) to develop a novel two-stage reactor system coupling PN process with anammox process using FT-CPVAFs, and (2) to experimentally examine the nitrogen removal performance and microbial community characteristics in the two-stage reactor system in a long-term stability under continuous flow conditions.

2. Materials and methods

2.1. Microbial seeding sources and PVA cryogel film fabrication

For immobilization of PN bacteria in cryogel films, an activate sludge (AS) was taken from an aerated bioreactor in a domestic wastewater treatment plant (WWTP) in Kyoung-gi province, Republic of Korea. For anammox immobilization, pre-enriched anammox bacteria (PAB) was taken from a bench-scale fixed bed continuous bioreactor fed with a synthetic wastewater containing 150 mg NH_4^+-N L^{-1} and 180 mg NO_2^-N L^{-1} (Choi et al., 2018). Prior to the following gelation, the biomass of the two seeding sources was concentrated by gravity settlement, and then pulverized by a homogenizer (IKA®, T 18 digital ULTRA-TURRAX, Germany) installed with a grinding element (IKA®, S18N-10G, Germany). After the biomass homogenization, the volatile suspended solid (VSS) concentrations were 8647.4 ± 121.3 mg

VSS L^{-1} for the PN biomass suspension, and 3413.3 ± 63.3 mg VSS L^{-1} for anammox biomass suspension.

For fabricating PVA gel, a 20% (v/w) PVA solution was autoclaved at 121 °C for 30 min, and then cooled to 37 °C. The 20% PVA solution was mixed with the same volume of the prepared biomass suspension (either AS or PAB), resulting in a mixture of 10% PVA and biomass. The prepared PVA-biomass mixture was poured into 2 mm-thick steel molds (60 mm width × 120 mm length). Since 2 mm thickness was optimal for anammox immobilization in our previous study (Choi et al., 2018), the same thickness was adopted for PN immobilization. The other procedures of PVA cryogel fabrication were the same as described by our previous study (Choi et al., 2018).

2.2. Activation of PN process

A 2.2 L fixed bed bioreactor (FBBR) installed with 10 FT-CPVAFs of 2 mm (packing ratio, 8.4% v/v) inoculated with AS was constructed at a laboratory scale, and then operated at 35 °C for 95 days under a continuous flow condition (Fig. 1(a)). A synthetic wastewater (Choi et al., 2018) was continuously fed into the reactor (flow rate = 11.41 L d^{-1}; hydraulic retention time, HRT = 4.63 h). The influent concentration of ammonium nitrogen was maintained at 499.50 ± 14.71 mg NH_4^+-N L^{-1}. Air was continuously supplied at a flow rate of 0.8 L min^{-1} for the former period of 47 days, and then changed to 0.5 L min^{-1} during the following stage (from the 48th to 79th day). The complete mixing in the reactors was ensured by measuring the level of DO and visually monitoring the uniform circulation of air bubbles in the beginning of the operation.

2.3. Activation of anammox process

The experiment to activate anammox process was conducted in two phases with different bioreactor configurations and mixing methods. In the initial phase, a 2.0 L air-tight sealed FBBR installed 10 FT-CPVAF of 2 mm (packing ratio of 6.8% v/v) inoculated with PAB was constructed and operated at 35 °C under a flowing condition (flow rate = 3.64 L d^{-1}; HRT = 13.18 h) for 78 days (Table 1). The bulk phase of FBBR was continuously mixed using magnetic stirrers. With exceptions of difference in ammonium-N and nitrite-N concentrations, the composition of synthetic wastewater feed to the reactor was the same as described by ~~our~~ the previous study (Choi et al., 2018). To facilitate step-wise acclimation of anammox bacteria, the influent ammonium-N and nitrite-N concentrations were maintained at low levels first (48.78 ± 3.35 mg NH_4^+-N L^{-1} and 48.12 ± 2.52 mg NO_2^--N L^{-1}, respectively, for the initial 55 days), and then increased to 96.33 ± 0.40 mg NH_4^+-N L^{-1} and 111.25 ± 0.50 mg NO_2^--N L^{-1} after 63th day. The behavior of acclimation of anammox immobilization was characterized by measuring lag-time required for activation of anammox in the films, and its corresponding nitrogen removal rate (NRR) in the reactor.

Thereafter HRT was decreased to 6.61 h. However, in the initial phase bioreactor experiment, unexpectedly an operational failure (after 63 days) was noted due to entry of air and by-passing flows. To resolve the operational problems, the bioreactor configuration and operational conditions were modified in the second phase experiment (Fig. 1(b)).

After recovery of anammox activity (106 days) of the initial phase experiment, the same PVA cryogel films immobilizing anammox-enriched microbes which were already acclimated in the first bioreactor were moved to a newly constructed FBBR. To avoid clogging and by-pass flow, the same number (10) of the acclimated PVA cryogel films were installed in a larger volume (5.5 L) reactor, resulting in reduction of its packing ratio to 2.12% (v/v). For uniform mixing and even distribution of biomass, the shape of reactor bottom was changed from rectangular to square. Mixing was enhanced by installing a motor driven stirring bar at 100 rpm avoiding biomass settlement without damaging flocs. To minimize washout, a settlement tank was added to collect suspended biomass, and the settled biomass (sludge) was returned to the new FBBR with the flow rate equivalent to the influent flow rate (1Q). The new FBBR was fed with the same synthetic wastewater (Choi et al., 2018) differing in ammonium-N and nitrite-N concentrations of 126.39 ± 1.93 mg NH_4^+-N L^{-1} and 144.22 ± 2.41 mg NO_2^--N L^{-1} respectively. The reactor was operated at 35 °C under continuous flowing conditions with two different HRTs (8.6 h between 107th day and 108th day and then 5.89 h between 109th day and 139th day).

2.4. Two-stage system coupling PN and anammox

A two-stage system was set up by coupling the PN-FBBR with the anammox-FBBR (Fig. 1(c)). After the nitrogen removal in the PN-FBBR (Fig. 1(a)) became stabilized with the air flow rate of 0.5 L min^{-1}, the effluent of the PN reactor was linked into the influent port of the anammox-FBBR (Fig. 1(b)) through a flow control reservoir. The two-

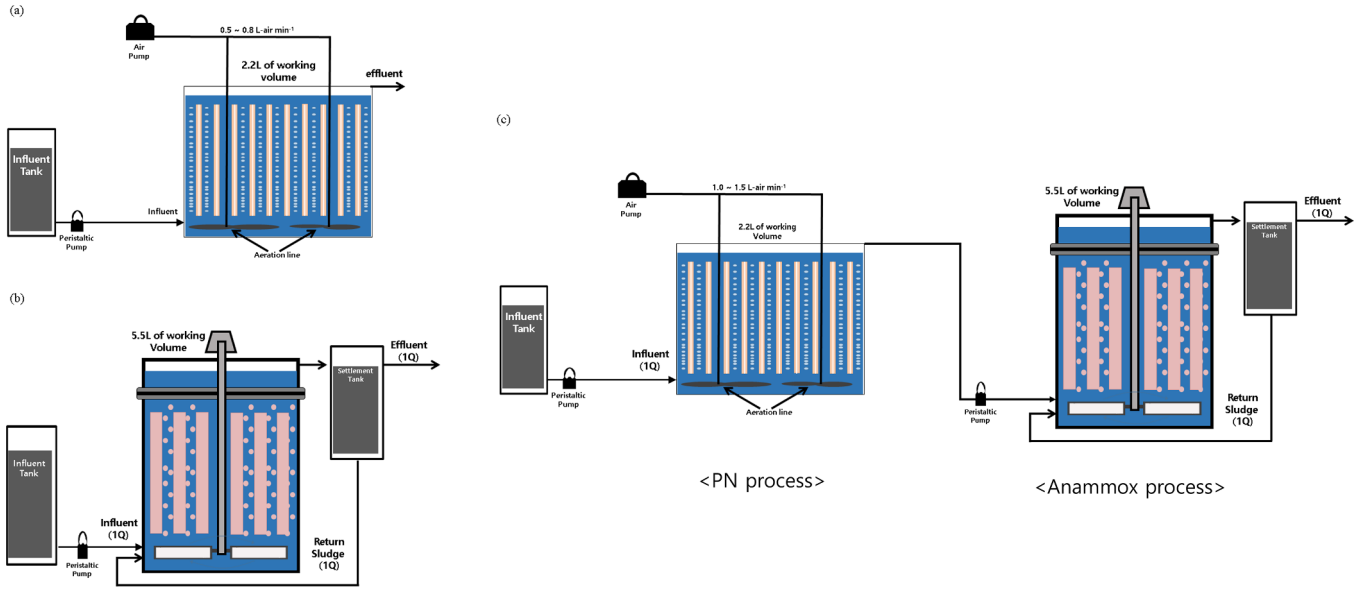

Fig. 1. Experimental setups for continuous flow fixed-bed bioreactors (FBBRs) with flat-type PVA cryogel films: (a) FBBR for only PN process (PN-FBBR), (b) FBBR for only anammox process (anammox-FBBR), and (c) two-stage system coupling PN with anammox.

Table 1
Operational conditions in anammox-FBBRs.

Phase	Days	Feeding conditions			
		Average ammonium-N concentration. (mg NH_4^+-N L^{-1})	Average nitrite-N concentration (mg NO_2^--N L^{-1})	HRT (h)	Maximum NLR (kg-N m d^{-1})
Initial	0–55	48.78 ± 3.35	48.12 ± 2.52	13.18	0.18
	56–62	75.50 ± 0.11	75.50 ± 0.58	13.18	0.28
	63–78	96.33 ± 0.40	111.25 ± 0.50	13.18	0.38
	79–95*	98.47 ± 1.23	106.00 ± 7.42	6.61	0.83
	96–106**	94.98 ± 2.69	99.25 ± 2.50	6.61	0.77
Modified	107–108	126.92 ± 1.19	144.19 ± 2.58	8.61	0.74
	109–139			5.88	1.12

* Occurrence of operation failure.
** Recovery period of anammox activity.

stage system was run at 35 °C with a continuous flow rate of 23.8 L d^{-1} and combined HRT of 7.7 h (2.2 h for PN process and 5.5 h for anammox process). The same synthetic wastewater was fed to the PN stage bioreactor of the system, but the level of feeding ammonium concentration was lowered into 300 and 315 NH_4^+-N L^{-1} (compared to those used in the individual PN bioreactor), which can provide the similar influent nitrogen condition (approximately 130 NH_4^+-N L^{-1} with 1:13 nitrite-N to ammonium-N ratio) in which the individual anammox bioreactor was previously optimized.

2.5. Bioreactor microbiome analysis

Firstly, FT-CPVAFs were sampled for initial microbiome before the start of the reactor operation (sample name: “Inoculated”) having immobilized AS (in case of PN) or PAB (in case of anammox), and after the end of the PN-anammox process (sample name: “Activated”). Then, genomic DNA from PVA cryogel films were extracted using a DNA extraction kit (Mobio Inc., Carlsbad, CA, USA). Bacterial 16S rRNA genes in the V3 to V4 region were amplified by PCRs (polymer chain reactions) with amplication target specific universal primers (341F and 785R) (Klindworth et al., 2013). Illumina MiSeq (Illumina Inc., San Diego, CA, USA) was applied for high-throughput sequencing of the PCR amplicons from the microbiomes of the samples. Generated raw sequences were processed and analyzed using Mothur v1.42.3 following the MiSeq SOP (Schloss et al., 2009). The sequences were trimmed using filtering command if the sequences were shorter than 550 bps or/and the homopolymers were longer than 8 nucleotides. The remaining sequences were aligned in Mothur using Silva v132 reference database. Chimeric sequences were removed using the UCHIME algorithm v4.2. Operational taxonomic units (OTUs) based on the sequences were generated with the cluster database with at least 97% sequence similarity. The OTUs were finally classified to the genus level using the naïve Bayesian classifier (Wang et al., 2007) in Mothur with the RDP 16S rRNA training set v16 reference database. Using blast database of the National Center for Biotechnology Information (NCBI), the bacterial OTUs were used for matching the closest species. Richness, evenness, and diversity were also conducted in Mothur.

2.6. Chemical analysis

The concentration of ammonium was determined using a Kjeltec auto-analyzer (Kjeltec 2300, Foss, Denmark). The concentrations of nitrite and VSS were measured by standard methods (APHA, 1998). The concentration of nitrate was measured by a commercial kit using cadmium reduction mechanism (program 355, HACH®, USA). pH and DO were analyzed using a pH meter (Orion 5–Star BENCHTOP MULTI, Thermo Fisher Scientific Inc., USA) and a portable DO meter (HQ30d, HACH, USA) with probe (LDO101 sensor Hach, USA), respectively. Free ammonia (FA) concentration was calculated by Equation (1) (Sun et al., 2010)

$$FA = \frac{17}{14} \times \frac{[Ammonium - N] \times 10^{pH}}{\left(\frac{K_b}{K_w}\right) + 10^{pH}} \quad (1)$$

where *Ammonium-N* denotes the concentration of ammonium–N in FBBR, *pH* denotes pH in the FBBR, K_b and *Kw* denote the ionization constants for the ammonia equilibrium equation and pure water, respectively, and $K_b/K_w = e^{(6344/273 + \text{temperature } (^\circ C))}$.

3. Results and discussion

3.1. PN activation with PVA cryogel film

After the cryogel films inoculated with AS were incubated in the bioreactor, it took only 8 days for PN process to be activated i.e., acclimation period (Table 2) shorter than previous reports in literature (Isaka et al., 2011; Kunapongkiti et al., 2019; Rostron et al., 2001; Tsuneda et al., 2003), and the activated PN was maintained stably in the reactor. These observations were made on the basis of ammonium and nitrite concentrations in the effluent (Fig. 2(a)) as well as the rates of ammonium removal and nitrite accumulation (Fig. 2(b)).

Ammonium was the sole nitrogen source in the synthetic wastewater feed and its removal may have been attributed to PN process including very little nitrate accumulation, and ~6.54% ammonia loss by air stripping at pH 8.00 ± 0.35 was calculated based on nitrogen mass balance of the influent and the effluent. In the former phase of air supply at 0.8 L min^{-1}, the average ammonium oxidation rate (AOR) was 1.68 ± 0.12 kg N m^{-3} d^{-1} after the PN activation (Fig. 2(b)). The AOR values measured in the experiment are higher than those reported in literature (Table 2), with one exception of the report by Isaka et al. (2011) where highly enriched nitrifying bacteria had been used as inoculum. The high AOR result suggests that PN was effectively activated in the FT-CPVAF, at least compared to the cube-type PVA cryogel.

The average ratio of nitrite-N to ammonium-N (1.64 ± 0.31) was higher than the optimal ratio for anammox process, i.e., 1.32 (Strous et al., 1998). To adjust the nitrite-to-ammonium ratio, the air flow rate was slightly lowered to 0.5 L min^{-1}. Within a period of 4 days, nitrite-to-ammonium ratio was optimized at 1.03 ± 0.11 (Fig. 2(a)).

Effluent nitrate concentration was maintained below 4.49 ± 1.88% of influent ammonium concentration during the entire operation period (Fig. 2(b)). In the former phase (0.8 L min^{-1} air supply), the nitrate production rates were 0.034 ± 0.0067 kg N m^{-3} d^{-1}, which is nearly 50 times smaller than the nitrite production rates (1.67 ± 0.12 kg-N m^{-3} d^{-1}), indicating that nitrite-to-nitrate oxidation activity was negligible compared to ammonium-to-nitrite oxidation. A similar trend was also observed after lowering the air flow rate to 0.5 L min^{-1} (in the latter phase). These suggest the possibility of suppressed nitratation process by nitrite oxidizing bacteria (NOB). The possibility may be supported by the observed DO (< 0.2 mg-O_2 L^{-1}) and FA concentrations (data not shown) (Anthonisen et al., 1976; Garrido et al., 1997). As DO concentration

Table 2
Comparison of ammonium oxidation rates (AORs) and acclimation times between the present PN-FBBR and previous works for continuous flow.

Type of Immobilization	Initial inoculum	Initially stabilized AOR [1] (kg-N m^{-3} d^{-1})	Acclimation time [2] (d)	Packing ratio (v/v %)	VSS of inoculum (kg-VSS m^{-3})	Maximal AOR [3] (kg-N m^{-3} d^{-1})	Reference
Suspended biomass (UASB)	Activated sludge	~ 0.9	100	NA[4]	NA	1.5	Tsuneda et al. (2003)
PVA-bead type	Activated sludge	0.27	20	NA	2.0	0.27	Kunapongkiti et al. (2019)
PEG – cube type	Activated sludge	0.82	29	20	40	1.7	Isaka et al. (2011)
PVA cryogel – cube type	Nitrifying sludge	~0.50	83	20	2.5	0.7	Rostron et al. (2001)
PVA cryogel – flat-type	Activated sludge	1.37–1.68	8	8.4	8.6	1.68	This study

The AOR values were measured at the initially stabilized steady state conditions in the experiments.
The values are corresponding to the lengths of acclimation periods required to reach to the initially stabilized steady AOR values.
The values are the maximal AORs reported in the studies.
NA stands for 'not available', meaning the values are not available in the literature or cannot be calculated due to lack of information.

becomes lower, AOB may exhibit higher affinity to DO than NOB, i.e., the Monod half-saturation constants for AOB (0.03–1.18) versus those for NOB (0.16–3.00) (Arnaldos et al., 2015). The level of DO concentrations in the FBBR experiment (0.1–0.2 mg-O_2 L^{-1}) may have been too low for NOB but appropriate for AOB. In the case of FA concentration, the FA concentration (32.0 ± 16 mg-NH_3 L^{-1}) in the PN-FBBR were within the range 10 mg-NH_3 L^{-1} < FA < 150 mg-NH_3 L^{-1} where the growth of NOB is inhibited unlike AOB (Anthonisen et al., 1976). The NOB suppression was indeed confirmed by the following microbiome analysis results (see Section 3.4).

In the PN process using PVA cryogel films, washout loss of microorganisms was minimized. A low level of effluent SS concentrations (< 10 mg L^{-1} d^{-1}) was observed in the FBBR experiment. This suggests that the cryogel film may have acted as an effective Supporting material for the attached-growth of PN-exhibiting microorganisms (probably AOB). This possibility was also confirmed by the following microbiome analysis (see Section 3.4).

The findings from the PN process using FT-CPVAF suggest that the FT-CPVAF may have beneficial effects in decreasing the time to reach AOR (initiation of microbial activity) and maximum ammonium oxidation rate. It was observed that flat-type film enhanced AOB activity in the comparison to cube or bead type carrier in previous studies. This is because flat-type film can control substrate diffusion owing to its thickness (Choi et al., 2018). Additionally, flat-type films seem to selectively enrich active AOB and effectively suppress NOB, compared to cube-type PVA cryogel. The minimized biomass washout loss is operationally advantageous because of no need to put an additional settlement tank after the PN bioreactor.

3.2. Anammox activation with PVA cryogel film

In the first phase of anammox bioreactor with the PVA cryogel film inoculated with the anammox-active microbial consortium, nitrate-N was produced (Fig. 2(c) and (d)). The nitrate production may have been partially attributed to oxygen intrusion into the bioreactor. Despite the occurrence of oxic nitrification, anammox may have been activated in the FT-CPVAF equipped bioreactor. The influent ammonium and nitrite concentrations were further increased stepwise to 96.33 ± 0.40 mg NH_4^+-N L^{-1} and 111.25 ± 0.50 mg NO_2^--N L^{-1}, respectively between the 63rd day to the 78th day to enhance the activity of anammox bacteria (Fig. 2(c)). The molar ratio ammonium-N removed: nitrite-N removed: nitrate-N produced was experimentally determined as 1.00: 1.20 ± 0.03: 0.35 ± 0.07. This showed a minor deviation from the theoretical stoichiometric value for anammox reaction (1.00: 1.32: 0.26) (Strous et al., 1998) possibly due to the co-occurrence of nitrification in the bioreactor and the presence of different bacterial consortia and predominance of different bacterial species in anammox reactor (Dosta et al., 2015). However, the ratio was within the range of other reported value (Li et al., 2014). pH of the initial anammox reactor was maintained at 7.90 ± 0.27.

The entry of air caused operational failure with extended lag time of 63 days to achieve NRR of 0.32 kg N m^{-3} d^{-1} (Fig. 2(d)) in the initial anammox-FBBR (Table 3) due to nitrification of ammonium by oxygen. With the oxygen intrusion, anammox activity was inactivated showing no ammonia removal. However, the inactivation did not kill the anammox microbes immobilized in the gel, rather it might have caused the reversible suppression of enzyme activity of anammox bacteria (Dalsgaard et al., 2014). This was evident from a very short lag-time for reactivation of anammox bacteria after the oxygen intrusion was stopped by reactor modification (Fig. 2(c)). To circumvent the anammox inactivation due to oxygen shock (after 69 days), initial anammox-FBBR was tightly sealed with silicone rubber to inhibit air entry (Table 3). This measure improved in decreasing the lag period to 18 days and increasing NRR to 0.61 kg N m^{-3} d^{-1} (after 92 days, Table 3). The accidental failure in anammox activity was mainly due to a certain air intrusion with evidence of a sudden decline of anammox

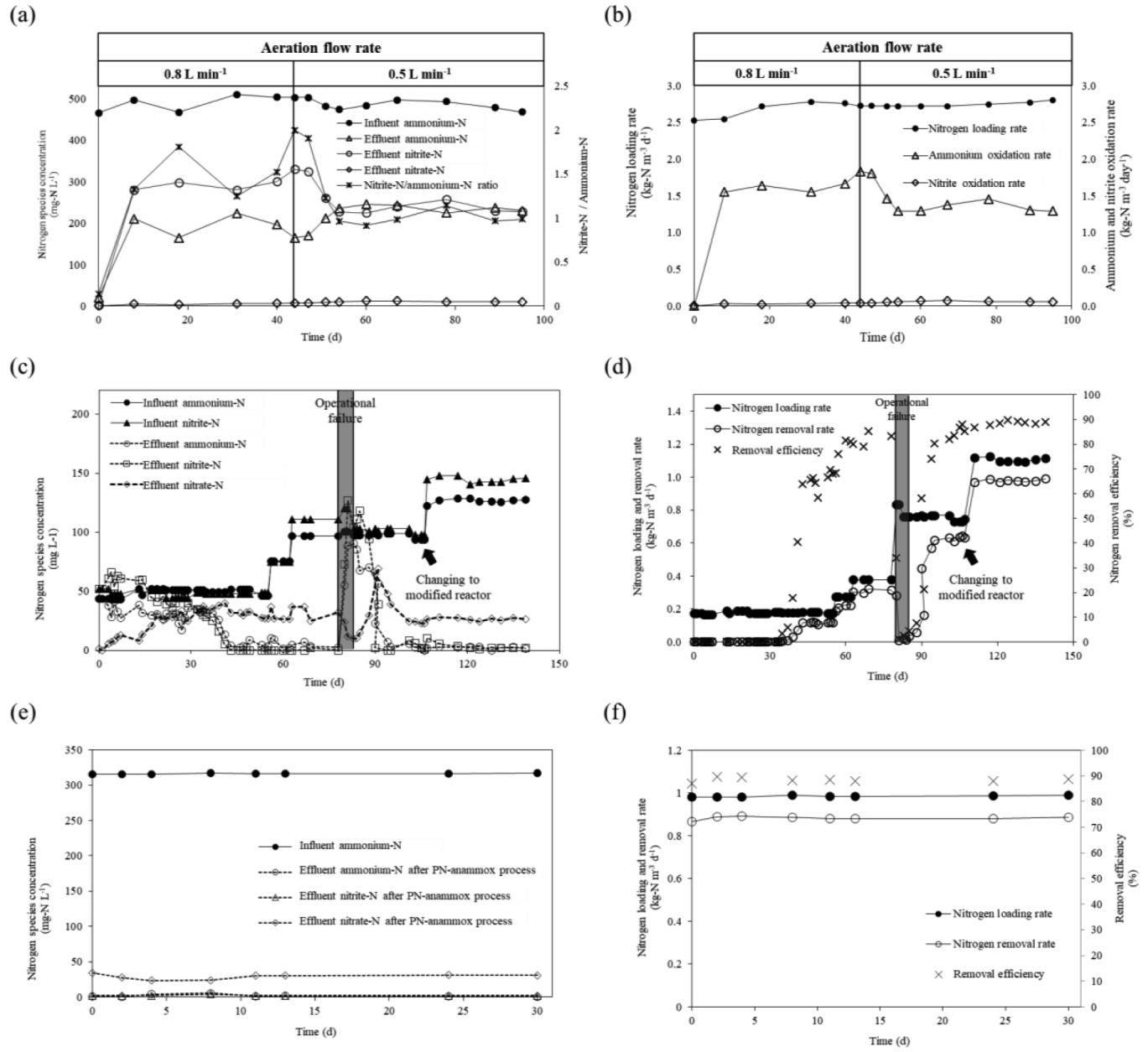

Fig. 2. (a) Influent and effluent concentrations of nitrogen species (ammonium-N, nitrite-N and nitrate-N) and nitrite-N/ammonium-N ratios in PN-FBBR; (b) the rates of nitrogen loading, ammonium oxidation, and nitrite oxidation in PN-FBBR; (c) influent and effluent concentrations of nitrogen species (ammonium-N, nitrite-N and nitrate-N) in anammox-FBBR; (d) the rates of nitrogen loading and of nitrogen removal, and nitrogen removal efficiencies in anammox-FBBR; (e) Influent and effluent concentrations of nitrogen species (ammonium-N, nitrite-N, and nitrate-N) in the two-stage system coupling PN with anammox; (f) the rates of nitrogen loading and of nitrogen removal, and nitrogen removal efficiencies in the two-stage system coupling PN with anammox.

activity (NRR) and unexpected increase of nitrite-N and nitrate-N.

Then, a further improvement in anammox-FBBR was done by changing the agitation type to motor-driven impeller, use of sludge recycle, and increasing the space between cryogel films (Fig. 1(b)). After the anammox-active PVA cryogel films (previously acclimated in the first experiment) were transferred into the modified bioreactor (Table 3), stable activation of anammox process was observed with no lag-time (Fig. 2(c) and (d)) possibly due to maturing of PVA cryogel films in the optimized. The matured gels in the modified reactor showed enhancement in terms of NRR value over former anammox-FBBR (NRR as 0.61 kg N m^{-3} d^{-1}). Through sludge return system, the suspended biomass concentration in modified anammox-FBBR was maintained at 1254.18 ± 172.40 mg-VSS L^{-1}, and the average effluent SS concentration was measured to be < 50 mg L^{-1}. pH of the modified anammox reactor was observed at 8.34 ± 0.34. Considering the currently operated full scale treatment plants for PN-anammox process (Fuchs et al., 2017; Lackner et al., 2014), the previous reported NRR values are in the range up to 1.5 kg N m^{-3} d^{-1}. After the acclimation period, maximal NRR value of 0.99 kg N m^{-3} d^{-1} observed in the modified anammox-FBBR was comparable with the previously reported NRR values (Chen et al., 2015; Cho et al., 2017; Cho et al., 2018; Choi et al., 2018; Kowalski et al., 2018; Magrí et al., 2012; Xie et al., 2016) for anammox process (Table 3). A strong linear correlation ($y = 0.9225x - 0.0465$, $R^2 = 0.9982$) was observed between NRR and NLR (range 0.18–1.11 kg-N m^{-3} d^{-1}, data not shown). In addition, after the acclimation period, almost complete removals of ammonium and nitrite were observed (98.33 ± 0.85% and 98.80 ± 0.53%, respectively). However, it took relatively longer time to reach the initially

Table 3
Nitrogen removal rates (NRRs) and their corresponding times to reach NRR of the present anammox-FBBR and previous studies for continuous flow.

Type of Immobilization	Initial inoculum	Initially stabilized NRR [1] (kg-N m^{-3} d^{-1})	Acclimation Time [2] (d)	VSS of inoculum (kg-VSS m^{-3})	Packing ratio (v/v %)	Maximal NRR [3] (kg-N m^{-3} d^{-1})	Reference
Attached biofilm	Anammox-active microbial consortium (different inocula in the different works)	~0.12	130	3	NA	1.03	Xie et al. (2016)
Attached biofilm		0.14	50	1.25	NA	0.53	Kowalski et al. (2018)
Polyurethane – Cube type		0.32	35	NA	10.0	1.50	Chen et al. (2015)
PVA-SA – bead type		0.20	9	0.85	18.5	1.34	Cho et al. (2017)
PVA cryogel – cube type		0.52	30	11.3	50.0	0.60	Magrí et al. (2012)
PVA cryogel – cube type		0.78	94	3.8	80.0	0.78	Cho et al. (2018)
PVA cryogel – flat-type		0.93	69	5.3	1.7	1.00	Choi et al. (2018)
PVA cryogel – flat-type		0.32*	63*	3.4	6.8	0.99	This study
		0.61**	18**	3.4	2.2	0.99	
		0.98***	0***	3.4	2.2	0.99	

The values are the initially stabilized steady NRRs observed in the studies.
The values are corresponding to the lengths of acclimation periods required to reach to the initially stabilized steady NRR values.
The values are the maximal NRRs reported in the studies.
*Initially stabilized NRR and its corresponding acclimation time for initial anammox-FBBR (until operational failure).
**Initially stabilized NRR and its corresponding acclimation time after the oxygen perturbation (air intrusion) in the initial anammox-FBBR.
***Initially stabilized NRR and its corresponding acclimation time after shifting into the modified anammox-FBBR.

stabilized steady NRR in this study than few reported studies (Table 3). It could be possibly due to inactivation of anammox bacteria by freezing and thawing during the cryogel fabrication (Magrí et al., 2012) and oxygen intrusion in the reactor. Further research is needed to mitigate this issue by using cryoprotective agents (e.g., glycerol) (Ali and Okabe, 2015) during the cryogel fabrication.

Continuous feeding system has the advantage over the intermittent feed SBR system with regard to its easy scale-up for large treatment facilities (Val del Río et al., 2016). Rapid growth of the microorganisms and the high dilution rate are required to make such systems robust (Cho et al., 2017). However, anammox process generally requires a long sludge retention time due to a very slow growth rate and difficulty in applying a short HRT (< 1 day) (Cho et al., 2017). On the other hand, the findings in this study highlight the novel characteristic developed on anammox process with FT-CPVAF having a shorter HRT (5.88 h) for NRR of 0.99 kg N m^{-3} d^{-1}.

3.3. BNR performance in two-stage FBBR system coupling PN with anammox

After the activation of the PN- and anammox-FBBRs separately, the PN-FBBR was coupled with the anammox-FBBR (Fig. 1(c)) to examine if the two-stage system is applicable in treating moderately high ammonium-N wastewater (315 mg-N L^{-1}) in a continuous flow mode. During the PN-FBBR operation (see E-supplementary data), PN was well established with negligible loss of ammonia by air stripping (0.34% of total nitrogen removal) as well as effective NOB suppression (3.73 ± 0.41% of nitrate accumulation), and the molar ratio of nitrite-to-ammonium in effluent was 1.13 ± 0.03 which is suitable for the following anammox process. DO concentrations for PN process always remained below 0.2 mg L^{-1}. Building on that observation, it was assumed that oxygen level was well below the inhibitory effect for anammox process. After the effluent was treated by the following anammox- FBBR (see E-supplementary data), the final effluent nitrogen concentrations of ammonium and nitrite from the two-stage system were lower than 2 mg N L^{-1} (Fig. 2 (e)), showing high efficiency of nitrogen removal (> 99%). It was found that 13.4 ± 2.57% of influent ammonium-N was converted to nitrate-N in the final effluent.

Using a PVA cryogel film containing a variety of microorganisms, the two-stage PN-anammox process in our present study showed an NLR of 0.99 ± 0.0028 kg N m^{-3} d ^{1} with an NRR of 0.88 ± 0.0075 kg N m^{-3} d^{-1} (average nitrogen removal efficiency, NRE = 89.6 ± 0.76%) at HRT of 7.7 h (Fig. 2(f)). The observed pH values for PN and anammox were 8.21 ± 0.07 and 8.49 ± 0.12 respectively. The continuous flow system with two-stage linking PN and anammox together, in our present study, is also feasible for scale-up. As per the performance data of full scale plants for the single-stage and two-stage PN-anammox process, the NLR was in the range 0.04–1.2 and 0.26–1.0 kg-N m^{-3} d^{-1} respectively in case of side-stream wastewater treatment (Fuchs et al., 2017; Lackner et al., 2014).

In this study, the nitrogen removal process in PN and anammox reactors using FT-CPVAFs were performed independently, linked together as one unit. The ammonia influent concentration was about 315 mg NH_4^+-N L^{-1} and it is considered that high-rate treatment is possible for moderate strength side-stream levels (300–400 mg NH_4^+-N L^{-1}). Comparing with previous similar studies for one-stage and two-stage reactors of PN-anammox process (Bae et al., 2017; Cho et al., 2011; Dosta et al., 2015; Li et al., 2014; Qiao et al., 2013), the present study showed comparable NRR and HRT (see e-Supplementary data). The results suggest that the two-stage PN-anammox process using the newly fabricated FT-CPVAFs with microorganisms can be used as alternative in BNR process for side-stream wastewater treatment with a continuous flow mode. Future studies are needed dealing with treatment of a wide range of side-streams having 500–2000 mg NH_4^+-N L^{-1}.

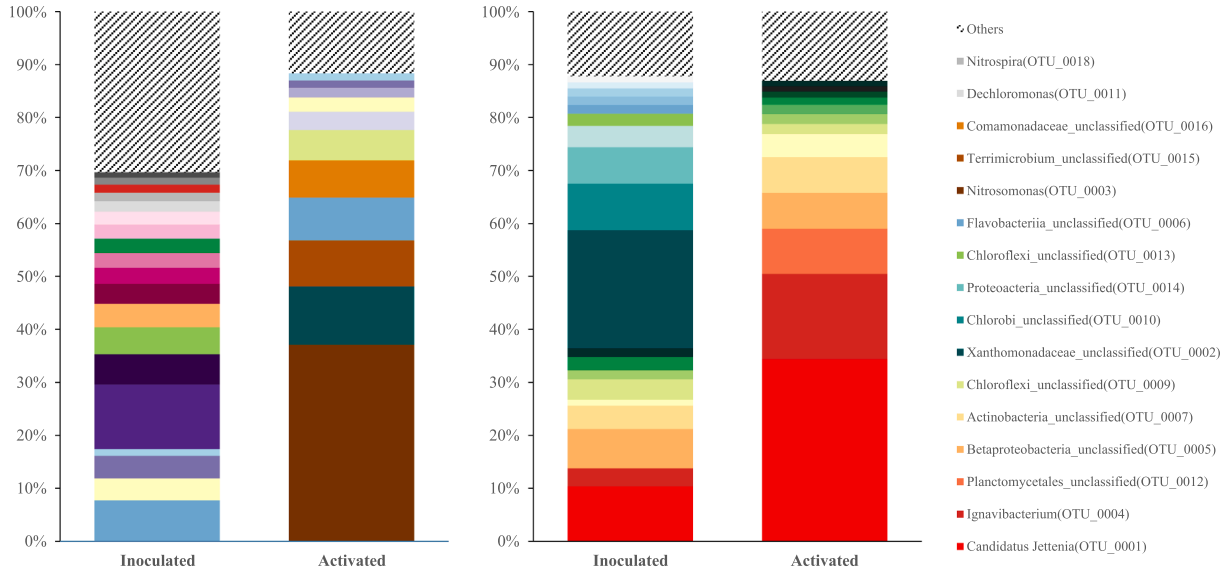

Fig. 3. Relative abundances (%) of OTUs (genus level) for (a) PN process and (b) anammox process ("Inoculated" means the PVA cryogel film sample before the start of the reactor operation having immobilized AS (in case of PN) or PAB (in case of anammox), and "Activated" means the PVA cryogel film sample after the end of the two-stage system coupling PN with anammox).

3.4. Bioreactor microbial community characteristics

To investigate the possibility of selective enrichment of AOB and anammox bacteria in the FT-CPVAF in the PN- and anammox-FBBRs, the immobilized microbial communities at the end of the individual PN- and anammox-FBBR experiments were analyzed and compared with the initial inocula (from AS and PAB). It was found that alpha-diversity values (OTU richness and Shannon index) were lowered for PN process microbial community compared to the initial inoculum (AS) (see E-supplementary data), and the bacterial community structures (at species level) differed significantly (paired *t*-test p-value < 0.05) between the inoculum and the final immobilized microbial community. A similar trend was observed in the anammox-FBBR experiment. These findings reveal that the activation of PN and anammox by using the FT-CPVAF significantly shifted the bacterial community structure to a reduced population diversity, indicating the occurrence of selective enrichment at species level.

To confirm the selective enrichment of PN and anammox bacteria in the FT-CPVAFs, the dominant bacterial populations (relative abundance cut-off > 1%) detected by the 16S rRNA gene sequencing were identified. Most of bacterial populations were identified as 'unclassified' (Wu et al., 2019) matching with known bacterial genus or species (Fig. 43, see E-supplementary data). *Nitrosomonas europaea* C-31, a well-known AOB (Schramm et al., 1999), was selectively enriched (relative abundance of 37.14%) in the FT-CPVAF in the PN-FBBR experiment (Fig. 43(a)) and comparatively higher than relative abundances of similar AOB reported in literature (see E-supplementary data) (Kunapongkiti et al., 2019; Wang et al., 2018). *Nitrospira moscoviensis*, one of NOB found in activated sludge (Daims et al., 2001), and *Dechloromonas denitrificans*, one of representative denitrifiers in activated sludge (Horn et al., 2005), present in the AS (inoculum) were found to disappear in the final FT-CPVAF thereby confirming the effective suppression of NOB and denitrifying bacteria with the use of the FT-CPVAF. In the case of anammox FBBR experiment, a very well-known anammox bacteria '*Candidatus* Jettenia caeni' (Cho et al., 2017) was selectively enriched (relative abundance of 34.36%) in the FT-CPVAF (Fig. 3 (b)) and comparatively higher than relative abundances of similar anammox bacteria reported in literature (see E-supplementary data) (Cho et al., 2018; Choi et al., 2018; Xie et al., 2016). The *Ignavibacterium* JCM 16,511 (relative abundance of 10.84%), frequently found in anammox reactors (Cho et al., 2018), was also detected in the anammox-activated FT-CPVAF sample (Choi et al., 2018). These confirm that the use of FT-CPVAFs could improve the selective enrichment of AOB and anammox bacteria.

AOB can be divided into two groups with different substrate utilization strategies: r-strategists which use relatively higher ammonium concentration, and K-strategists which grow rapidly at lower ammonium concentration (Schramm et al., 1999). *Nitrosomonas europaea* C-31 belongs to r-strategists (Schramm et al., 1999). Thus, *Nitrosomonas europaea* C-31 may be suitable for high concentration nitrogen removal.

The predominant anammox bacteria in our present study was '*Candidatus* Jettenia' and different from a previous study (Choi et al., 2018) which reported '*Candidatus* Brocadia' as the dominant genus. Despite the similarity in the seeding inoculum and NLR, there were differences in operational conditions e.g., ammonium-N and nitrite-N concentrations, and HRT. '*Candidatus* Jettenia' is generally found in the wastewater treatment system and is known to grow in high strength nitrogen treatment systems (Cho et al., 2017). Nevertheless, '*Candidatus* Brocadia' might outnumber '*Candidatus* Jettenia' in the continuous flow type of anammox process with high NRL owing to its physiological traits such as higher maximum specific growth rate and improved resistance to nitrite-N (Cho et al., 2017). On the other hand, '*Candidatus* Jettenia' was dominant instead of '*Candidatus* Brocadia' in anammox system using bead type of PVA-SA under high NLR conditions (1.45 kg-N m^{-3} d^{-1}) with high NRR (1.34 kg-N^{-3} d^{-1}) (Cho et al., 2017). These results indicate that '*Candidatus* Jettenia' and '*Candidatus* Brocadia' might be crucial anammox bacteria in continuous flow anammox systems, and consequently it is expected that FT-CPVAF with '*Candidatus* Jettenia' would be suitable for treatment of nitrogen-containing wastewater with high NRR and NRE.

4. Conclusion

This study validated FT-CPVAF for stable operation of PN and anammox processes together with two-stage PN-anammox coupling for nitrogenous wastewater treatment. PN was maintained with shorter acclimation (8 days) achieving high AOR (~1.68 kg-N m^{-3} d^{-1}), compared to most previous studies. Immobilized anammox bacteria remarkably reactivated after oxygen shock, with negligible lag-time, facilitated by matured PVA cryogel in modified FBBR. Two-stage system demonstrated ~90% NRE at short HRT (7.7 h). Microbial community data confirmed selective enrichment of PN- and anammox

bacteria. Additional studies on PN-anammox process are required to improve NRE and optimize operational factors for influent NH_4^+-N > 500 mg L^{-1}.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122546.

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