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Integration of EBPR with mainstream anammox process to treat real municipal wastewater: Process performance and microbiology

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ABSTRACT

The mainstream application of anaerobic ammonium oxidation (anammox) for sustainable N removal remains a challenge. Similarly, with recent additional stringent regulations for P discharges, it is imperative to integrate N with P removal. This research studied integrated fixed film activated sludge (IFAS) technology to simultaneously remove N and P in real municipal wastewater by combining biofilm anammox with flocculent activated sludge for enhanced biological P removal (EBPR). This technology was assessed in a sequencing batch reactor (SBR) operated as a conventional A^2O (anaerobic-anoxic-oxic) process with a hydraulic retention time of 8.8 h. After a steady state operation was reached, robust reactor performance was obtained with average TIN and P removal efficiencies of 91.3 ± 4.1% and 98.4 ± 2.4%, respectively. The average TIN removal rate recorded over the last 100 d of reactor operation was 118 mg/L·d, which is a reasonable number for mainstream applications. The activity of denitrifying polyphosphate accumulating organisms (DPAOs) accounted for nearly 15.9% of P-uptake during the anoxic phase. DPAOs and canonical denitrifiers removed approximately 5.9 mg TIN/L in the anoxic phase. Batch activity assays, which showed that nearly 44.5% of TIN were removed by the biofilms during the aerobic phase. The functional gene expression data also confirmed anammox activities. The IFAS configuration of the SBR allowed operation at a low solid retention time (SRT) of 5-d without washing out biofilm ammonium-oxidizing and anammox bacteria. The low SRT, combined with low dissolved oxygen and intermittent aeration, provided a selective pressure to washout nitrite-oxidizing bacteria and glycogen-accumulating organisms, as relative abundances of

1. Introduction

Anaerobic ammonium oxidation (anammox) has been identified as a sustainable bioprocess for nitrogen removal from municipal wastewater (Agrawal et al., 2018; Arora et al., 2021; Fernández et al., 2016; Gonzalez-Martinez et al., 2018; Lackner et al., 2014; J. J. Li et al., 2018; Ma et al., 2016; Qiu et al., 2020; Ren et al., 2022). The main advantages of anammox include lower energy and no external organic carbon requirements, as well as lower sludge production and no greenhouse gas emissions compared to conventional nitrification-denitrification (Wen et al., 2020). However, despite the tremendous success of coupling partial nitritation (PN) and anammox collectively known as PNA systems to treat ammonium-rich waste streams in sidestream applications, mostly for high-strength anaerobic digester centrate (or filtrate), the full-scale implementation of mainstream PNA remains a challenge (Le et al., 2019). Low ammonium concentrations and fluctuating temperatures in mainstream conditions pose a challenge for suppressing the growth of nitrite-oxidizing bacteria (NOBs). Unsuppressed NOB growth leads to unstable PN and is the main obstacle to the adoption of mainstream PNA (Lotti et al., 2015). The presence of canonical heterotrophic denitrifying bacteria poses serious challenge to anammox bacteria for the available nitrate or nitrite. Moreover, effluents from PNA systems still contain considerable amounts of total inorganic nitrogen (TIN) in the form of residual NH_4^+-N (up to 5 mg/L), and nearly nitrate equivalent to11 to 16% of each mg NH_4^+-N removed is produced from anammox anabolism (Cao et al., 2017; Strous et al., 1998). As a result, the successful integration of anammox into the mainstream remains an unresolved challenge due to inherent operational challenges and the presence of unreacted nitrogen species (e.g., residual ammonium and in-situ produced nitrate) in the anammox effluents. The later becomes

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even more troublesome if the treatment plants are regulated TIN limits.

In order to overcome these bottlenecks, different solutions have been proposed. One of these solutions is the integration of the partial denitrification (PDN) with anammox. PDN utilizes canonical heterotrophic denitrifiers to anoxically reduce nitrate partially to nitrite under controlled conditions. The in-situ generated nitrite through PDN could be used by anammox to further reduce it to nitrogen gas (Ahmad et al., 2021; Ahn et al., 2004; Cao et al., 2019; Cui et al., 2020; Du et al., 2020; Gao et al., 2021; Ji et al., 2020a; Ma et al., 2020; Xu et al., 2020; Zhang et al., 2021; Zhou et al., 2018; Zhuang et al., 2022). However, PDN still requires organic carbon which could be made available in the main stream waste treatment train. Theoretically, systems operating with PDN and anammox would reduce the cost of external carbon and aeration by 80% and 50% respectiviely. .

Another nutrient of concern is phosphorus (P). The biological removal pathway of P is accomplished through enhanced biological P removal (EBPR). With increasingly stringent regulations for P discharges into receiving waters, more and more utilities are focusing on EBPR. Hence, it becomes imperative to integrate any innovative N-removal process with EBPR for a holistic treatment of N and P in municipal wastewater.

A novel idea would be to pair EBPR-driven denitrification with anammox (Yin et al., 2021) to cut the organic carbon requirement further while concurrently removing P. Denitrifying polyphosphate accumulating organisms (DPAOs) are capable of endogenously reducing NO_3^- and NO_2^-, hence potentially serving as an effective denitrification pathway without the need for external organic carbon dosage. Dissolved oxygen (DO) demand is also reduced for the subsequence aerobic P-uptake, as PO_4^{3-} is partially taken up in the anoxic zone by DPAOs.

Another major challenge that hinders anammox processes in mainstream applications is the retention of the slow-growing anammox bacteria. However, this issue could be mitigated by having anammox bacteria grow on carriers as biofilms. Attached-growth biofilms have been found to be more effective in retaining anammox biomass than granular biofilms (Gao et al., 2022). Moreover, biofilms provide exclusive space for anammox bacteria to grow. In addition, anammox-based biofilms are more tolerant to high DO concentrations (Jiang et al., 2020), low temperatures (Gilbert et al., 2014; Lackner et al., 2015; Persson et al., 2014), and chemical inhibition (Zheng et al., 2017), thus making attached growth anammox an attractive candidate for mainstream applications. Attached-growth biofilms can also be combined with flocculent activated sludge in a technology known as integrated fixed film activated sludge (IFAS) for process intensification. IFAS technologies have been proven successful in increasing reactor stability and output for nitrification and anammox processes (Bai et al., 2016; Kim et al., 2011; Regmi et al., 2011; Veuillet et al., 2014; Waqas et al., 2020).

This study applied IFAS technology by combining attached growth biofilms with suspended growth flocs in a sequencing batch reactor (SBR) to remove P and N simultaneously. The reactor was fed with real primary effluent collected from a local treatment plant and operated in an A2O (anaerobic-anoxic-oxic) configuration. The biofilm biomass mainly consisted of ammonium-oxidizing (AOBs) and anammox bacteria for PNA, while the floc biomass mainly consisted of DPAOs/PAOs and other groups of bacteria for EBPR, denitrification, and aerobic C removal.

The main goal of this project was to assess the feasibility of combining EBPR, denitrification, nitritation, and anammox to simultaneously remove P, N, and C in real municipal wastewater without organic carbon dosage. The specific objectives of this study were (1) to determine the removal efficiency of P, N, and C; (2) to study the interactions between the different functional microorganisms at the substrate and molecular levels (16S rRNA gene-based microbial community analysis and functional gene expressions); and (3) to examine optimum conditions for the synergistic coexistence of these microorganisms for high and stable reactor performance. This study's engineering and gene expression data can inform the optimization of integrated EBPR-based denitrification and anammox processes to remove P, N, and C in wastewater treatment. Findings from this study will contribute to the ongoing efforts in advancing anammox technologies for mainstream energy- and carbon-efficient nutrient removal applications.

2. Materials and methods

2.1. Reactor operation

A 5-L SBR with an IFAS process was investigated in this study. The SBR was operated in a sequence of anaerobic-anoxic-aerobic phases as found in traditional A^2O processes with a cycle length of 6 h (4 cycles/d). Each cycle consisted of a 50-min anaerobic phase, which included 10-min of feeding, a 40-min anoxic phase, a 4-h aerobic phase, and a 30-min settling/decanting phase. At the beginning of the anoxic phase, exogenous sodium nitrate ($NaNO_3$) was fed to the reactor at a total loading of 19 mg N for each cycle (equivalent to 10 mg N/L in the feed of 1.9 L) to simulate the internal nitrified recycling of the aerobic effluent to the anoxic zone in the conventional continuous-flow A2O process. The reactor was seeded with suspended biomass from an ongoing lab-scale EBPR reactor (~20%) and activated sludge from Central Valley Water Reclamation Facility (CVWRF; Salt Lake City, Utah) to reach an initial total suspended solids (TSS) concentration of 3 g/L. Mature anammox/AOB (ammonium oxidizing bacteria) biofilms grown on carriers (AnoxK™5) from a PNA reactor (Supplementary Information, SI, Text S1) were added to the reactor stepwise to make-up to 5% of the mixed liquor volume on day 10, 10% on day 50, and finally, 15% on day 75. During the startup from day 1 to 10, the working volume was 3 L with an exchange volume of 1 L, resulting in a hydraulic retention time (HRT) of 18 h. From day 11 to 75, the working volume was decreased to 2.63 – 2.75 L (increased as the biofilms were added while the mixed liquor volume remained 2.5 L) with an exchange volume of 1.5 L (overall HRT of 10 h). From day 75 onward, the working volume of the reactor was increased to 3.2 L (2.78 L mixed liquor volume), and the exchange volume was increased to 1.9 L (HRT of 8.8 h). Total solids retention time (SRT) for the flocs was maintained at 5 d TSS and volatile suspended solids (VSS) concentrations of the flocs after steady-state were approximately 1.3 and 1.0 g/L, respectively. The reactor was fed with real primary effluent collected weekly and stored at 4 °C from CVWRF. The characteristics of the primary effluent are listed in Table 1. pH was not controlled but regularly monitored and fluctuated between 7.3 – 8.3 within a cycle. N_2 was continuously purged at 0.25 – 0.35 L/min during the anaerobic and anoxic phases. Intermittent aeration (7 min on/off) was adopted during the aerobic phase. Air was purged at 0.2 – 0.4 L/min to achieve a DO setpoint of 0.4 ± 0.1 mg/L during each aeration cycle. The reactor was operated at room temperature (22 ±3 °C).

Table 1
Characteristics of the primary effluents used in this study.

Parameter	Range, mg/L
PO_4^{3-}-P	0.8 – 4.41
NH_4^+-N	27.28 – 44.71
NO_3^--N*	10
TN*	43.35 – 61.82
tCOD	190 – 285
sCOD	83 – 180
C/N	6.3 – 7
Acetate	25.55 – 64.96
Propionate	5.95 – 18.62

* After adding $NaNO_3$ in the anoxic phase. TN, total nitrogen. C/N, tCOD/NH_4^+-N.

2.2. P-uptake activity batch test

Batch experiments were conducted to assess the activities of PAOs and DPAOs for PO_4^{3-} uptake with different electron acceptors (O_2 (air), NO_2^-, and NO_3^-). Effluent from the reactor was filter sterilized by passing through 0.22 μm membrane filters. The filtrate was adjusted to a pH of 7.5 by adding 1 N HCl. Suspended biomass was collected from the reactor at the end of the anaerobic phase and washed twice with the pH-adjusted filtrate. 10 mL of the washed settled flocs were transferred to 125 mL serum bottles, followed by 40 mL of the pH-adjusted filtrate to obtain the final volume of 50 mL. A well-mixed solution (5-mL) was then collected for TSS and VSS analyses. For aerobic experiments, the bottles were left open to the atmosphere on a shaker at 200 RPM to maintain well mixed conditions. For the NO_2^- and NO_3^- assays, the bottles were sealed and purged with N_2 for 10 min to establish anoxic and well mixed conditions. PO_4^{3-} was then directly injected into the bottles of each of the respective assays, targeting an initial concentration of 20 mg PO_4^{3-}-P/L. In the aerobic assays, air was purged through needles vigorously and continuously in the bottles from an aquarium pump. The experiments were carried out for 120 min. For chemical analysis, a 5-mL aliquot was withdrawn from each bottle at times 0, 15, 30, 60, 90, and 120 min. All tests were done in duplicate.

2.3. Anammox activity assays

Towards the end of the study, two batch assays were performed on days 219 and 226 to confirm the activity of anammox bacteria and to quantify their contributions to the overall TIN removal during the micro-aerobic phase. As feed (real primary effluent) was collected weekly, different batches of feed may have had different effects on anammox activities. For this reason, the assays were conducted a week apart for two different batches of feed. Approximately 250 mL of the mixed liquor containing only flocs were withdrawn from the reactor into a flask at the end of the anoxic phase (just before the aerobic phase started) and ran in parallel to the reactor containing both flocs and AOB/anammox biofilms till the end of the aerobic phase. Air purging was provided in the flask (flocs only) from the same air pump used for the reactor for intermittent aeration synchronization. DO in the flask was manually controlled. Samples were withdrawn from both vessels for chemical analysis at the beginning and the end of the tests.

2.4. Complete cycle analysis and gene expression

On day 230 of reactor operation, a gene expression study was conducted. Samples for chemical analyses were taken at 0 (feed), 10 (after feeding), 25 (mid-anaerobic), 50 (end of anaerobic/start of anoxic), 70 (mid-anoxic), 90 (end of anoxic/start of aerobic), 110, 160, 220, 280, 330 (end of aerobic) and 360 min (effluent, end of cycle). Sludge samples were also collected at the same time intervals (except at 0 and 360 min) for the gene expression analysis. At each sampling point, two aliquots of approximately 2 mL of mixed liquor containing flocs were collected and centrifuged immediately at 7000 g for 30 s. The supernatants were collected for chemical analysis, while the pellet from one aliquot was transferred to a Lysis Matrix E tube containing glass beads from MP Medicals for RNA extraction, while the other was processed for PHA analysis. For the biofilm biomass, about 1/8 of a carrier was cut and collected in a Lysis Matrix E tube. Then 800 μL of the RNA lysis buffer (section 2.7) was added to the lysis tube. The mixture was mechanically homogenized using a bead mill (BEAD RUPTOR 12, Omni-International) at 6 m/s for 3 cycles of 1 min running and 5 min resting. The lysates were stored at 4 °C and RNA was then extracted from the lysate on the next day. N_2O concentrations in the off-gas were measured at the beginning of the anoxic phase until the end of the aerobic phase.

2.5. DNA and RNA extraction and two-step RT-qPCR

DNA from collected sludge samples was extracted using Qiagen PowerSoil Pro (Qiagen, Germany) kits per the manufacturer's protocols. Total RNA was extracted using Quick-RNA Kits (Zymo Research, USA). Genomic DNA (gDNA) was removed by an on-column DNase kit included in the kits. The isolated RNA was treated with TURBO™ DNase (ThermoFisher, USA) to remove trace DNA contamination further. After the treatment, RNA was purified using the same kits. The quantity and quality of RNA were checked using NanoDrop 2000c (ThermoFisher, USA). Reverse transcription for cDNA synthesis was conducted using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, USA) by adding 1000 ng RNA for each sample. Quantitative real-time PCR (qPCR) was done to quantify anammox 16S rRNA, Accumulibacter (DPAOs/PAOs) 16S rRNA, *hzsB, amoA*, and *ppk1-IA, ppk1-IIC, ppk1-IID, phaC, narG, nirK, nirS*, and *rpoN* (RNA polymerase sigma-54 factor, reference gene) genes using primer sets listed in Table S1. For both 16S rRNA genes, the synthetic cDNA samples were diluted 100 times before adding to the qPCR reaction mixtures. Each reaction mixture (20 μL) contained 10 μL of PowerUp SYBR Green master mix (ThermoFisher, USA), 1 μL of each primer (forward and reverse, 10 μM), 2 μL of template, and the remainder (6 μL) was nuclease-free water. The qPCR conditions for each reaction were applied according to the references listed in Table S1. The expressed abundances of the target genes were calculated based on the comparative C_T (threshold cycle) method, also known as $\Delta\Delta C_T$. The C_T value of the gene of interest at a specific time was subtracted with the corresponding C_T value of its reference gene (*rpoN*). Then the difference values were subtracted again with the difference value of the first sample to obtain the $\Delta\Delta C_T$ of each sample. Gene expression (as fold change) was expressed as $2^{-\Delta\Delta CT}$. qPCR analyses were carried out in triplicate on QuantStudio3 (ThermoFisher, USA). All RNA samples had a 260/280 value of 2.09 or higher, while their 230/260 values were also high and averaged 2.1. According to the manufacturer's manual, a high-quality RNA sample typically has a 260/280 ratio of 2.0 or higher and 230/260 ranging from 1.8 to 2.2. Trace gDNA contamination in the RNA samples was evaluated by running qPCR on negative reverse transcription control (NRTC, RNA sample without reverse transcription) for Anammox 16S rRNA and Accumulibacter 16S rRNA in RNA samples from biofilms and flocs, respectively. The average difference in C_T value between cDNA and NRTC was about 18.5 cycles in the biofilm RNA samples, while NRTCs for the floc RNA samples did not amplify after 40 cycles, suggesting that gDNA contaminations were negligible.

2.6. Analytical methods

For the lab-scale reactor, NO_2^-, NO_3^-, and PO_4^{3-} were analyzed using an ion chromatographic method (930 Compact IC Flex, Metrohm) with an anionic separation column (Metrosep A Supp 4 – 250/4.0, Metrohm). NH_4^+ was quantified using another ion chromatograph (883 Basic IC Plus, Metrohm) with a cation separation column (Metrosep C 6 – 150/4.0, Metrohm). HACH kits were used to measure COD. Samples were filtered through 0.22 μm filters for all of the above chemical analyses except for tCOD.

Volatile fatty acids (VFAs, modified Method 5560D), TN (modified Method 4500-N C), TSS, and VSS were analyzed according to the Standard Method (APHA, 2005). Polyhydroxyalkanoates (PHAs) were quantified using a modified method developed by Werker et al., 2008. VFAs and PHAs were analyzed using an Agilent 7890A Gas Chromatograph-Flame Ionization Detector (Agilent, USA) equipped with EC-1000 (FFAP), capillary column (15 m × 0.53 mm with 1.2 μm film thickness). 2-Ethylbutyric acid and benzoic acid were used as an internal standard for VFA and PHA analyses, respectively. N_2O was measured with N_2O Analyzer T320U (Teledyne-API, USA). For detailed descriptions of the VFA, TN, PHA, and N_2O quantification methods, refer to Text S2-S5 in SI.

Microbial community characteristics of the biomass were analyzed using QIIME2 software based on the V4 gene region sequencing of the 16S rRNA gene (Bolyen et al., 2019; Kozich et al., 2013).

3. Results

3.1. Reactor performance

Reactor performance was subdivided into 4 stages based on the amount of the AOB/Anammox biofilms added. Stage I (days 1–9) was the startup period without biofilm media. In Stage II (days 10–49), the reactor was operated with 5% (by volume) biofilms. In Stage III (days 50–74), the reactor operated with 10% biofilms, and in Stage IV (days 75–236), the reactor was operated with 15% biofilm media. In stage I, the reactor was started only with flocs seeded from a stable EBPR reactor (operational in the PI's lab) and aerobic activated sludge from a WWTP (CVWRF) to test the ability of the reactor to perform EBPR. During this stage, consistent EBPR performance was obtained as effluent PO_4^{3-}-P was below the detection limit of 0.03 mg P/L (Fig. 1a). During this startup period in Stage I, nitritation also gradually improved as the effluent NH_4^+-N kept decreasing while the effluent NO_2^--N kept increasing (Fig. 1bc). NO_3^--N as $NaNO_3$ was added to the reactor at the beginning of the anoxic phase of each cycle to simulate internal recycling of NO_3^--N from the aerobic cycle/zone. NO_3^--N loading was gradually increased from about 5.6 to 10 mg N/L from the beginning towards the end of Stage I (Fig. 1d). Influent nitrate concentrations were calculated by dividing the mass of NO_3^--N loading in the anoxic phase by the volume of the feed fed to the reactor. Effluent NO_3^--N was also low during this startup period in Stage I leading to high TIN removal of about 85% on Day 7 (Fig. 1e). This high TIN removal efficiency could be partly due to denitrification by endogenous decay induced by the high MLSS concentration seeded to the reactor and the relatively long HRT (18 h). It could also partly due to a high amount of intracellular carbon in the seed sludge in the form of PHAs, which the cells could have used to denitrify.

Due to the high removals of PO_4^{3-}-P, NH_4^+-N, and NO_3^--N and improved NO_2^--N accumulation in the effluent, the exchange volume was increased from 1 L to 1.5 L per cycle (6 L/day) on day 7. However, on day 9, the PO_4^{3-}-P removal decreased as the effluent concentration of PO_4^{3-}-P kept increasing from below the detection limit on day 8 to 4.2 mg P/L on day 10. This is partly due to the increasing effluent NO_2^--N concentrations, which carried over to the subsequent cycle with the remaining volume, causing organic carbon limitation in the next SBR cycle's anaerobic phase. To minimize NO_2^--N carry-over, the working volume of the reactor was dropped from 3 L to 2.5 L on day 10. As a result, the HRT also decreased to 10 h. Anammox biofilms were introduced in the reactor on Day 10 by adding AOB/Anammox biofilms grown on Anox™K5 carriers from the PI's lab at 5% of the mixed liquor volume. From Day 11, effluent PO_4^{3-}-P gradually decreased to below the detection limit on Day 16, suggesting a full recovery of the EBPR process after NO_2^--N carry-over was minimized. In Stage II, the average effluent NH_4^+-N and NO_2^--N were 6.9 and 6.1 mg N/L, respectively, while NO_3^--N in the final effluent remained low at 0.55 mg N/L on average, suggesting that the activities of NOB were under control. Stage II saw the lowest TIN removal efficiency at about 70.6% on average due to the high effluent NH_4^+ and NO_2^-. On day 50 (Stage III), AOB/Anammox biofilms were increased to 10% of the mixed liquor volume. Stage III (days 50–74) saw a significant improvement in NH_4^+ and NO_2^- removal with an average effluent concentration of 2.4 and 2.5 mg N/L, respectively, while average effluent NO_3^- increased to 1.6 mg N/L. These data indicate towards an increase in anammox activities as more biofilms were added. As a result, TIN removal in Stage III also improved with an average removal efficiency of 85.3%. In Stage IV (days 75 – 236), AOB/Anammox biofilms were finally increased to 15% on day 75. As a result, a higher average TIN removal efficiency of 91.1% was obtained. EBPR performance remained consistently high during Stages III & IV. With this stable and desirable TIN removal, biofilm loading was no longer increased. However, the rate of biofilm loading to archive stable anammox activities also depends on the characteristics of the inoculum

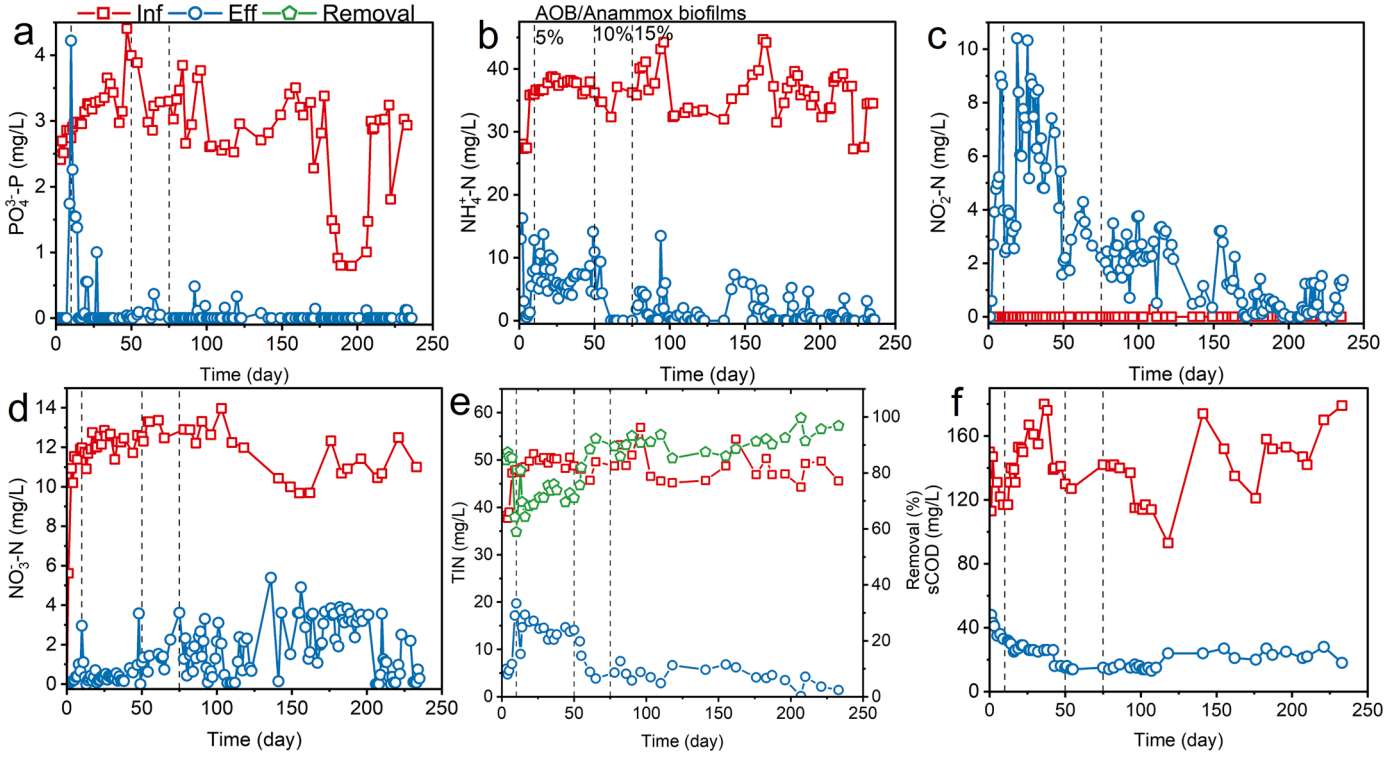

Fig. 1. Reactor performance, concentrations of (a) PO_4^{3-} (b) NH_4^+, (c) NO_2^-, (d) NO_3^-, (e) TIN with removal efficiency, and (f) sCOD in influent and effluent. Note that Influent NO_3^- concentrations were based on a calculation of the $NaNO_3$ addition in the anoxic phase. The three vertical dashed lines divide the reactor operation into 4 stages based on the amount of the biofilms added – 0, 5, 10 and 15% (v/v).

itself, which should be further investigated. The removal of sCOD (Fig. 1f) was consistent and not affected by the operational changes. A summary of the reactor performance for the last 100 days is provided in Table 2. During this period, the average TIN loading rate was 128.9 mg N/L·d, while the average removal rate was recorded at 117.7 mg N/L·d, which is within typical values (100 – 200 mg N/L·d) of mainstream applications (Metcalf and Eddy Inc et al., 2013).

3.2. Microbial ecology

DNA samples of the flocs and biofilms were extracted on day 213 of reactor operation. The microbial community composition of the flocs and biofilms are displayed in Fig. 2a & b, respectively. In the flocs, the most abundant genotype was aerobic heterotrophic bacteria accounting for at least 32.7% of the total population. Among them, the most abundant group was hydrolyzers in the family of *Saprospiraceae*, accounting for about 16.5% of the relative abundance. Members in this family can hydrolyze and utilize complex organic carbon sources and are typically found in high abundances in activated sludge (McIlroy and Nielsen, 2014). Well-identified canonical denitrifiers in the genus *Thauera, Rhodobacter, Denitratisoma,* and *Zoogloea* collectively accounted for 3.7% of the relative abundance in the flocs. The most abundant PAO/DPAO-related bacteria belonged to the genus *Candidatus* Accumulibacter (hereafter referred to as Accumulibacter), accounting for 5% of the relative abundance. Results from the qPCR analysis showed that Clade IA, IIC, and IID (15.1, 16.5, 43.2%, respectively, compared to PAO 16S rRNA) were the most abundant Accumulibacter in the reactor . A similar distribution of Accumulibacter clades was found in other studies when real primary effluent was used as the feed (Camejo et al., 2016; Roots et al., 2020). *Tetrasphaera*, another potential PAO/DPAO group, which could use complex carbons other than VFAs for P-release, was found in small abundance at 0.3%, suggesting its potential involvement in the EBPR process of the reactor (Kristiansen et al., 2012). Glycogen accumulated organisms (GAOs) are the main competitors for PAOs as they also can anaerobically assimilate VFAs without contributing to the P removal of the reactor. *Candidatus* Competibacter was the only group of GAOs found in the flocs, where their population in the reactor was low (0.16%) and would have not significantly affected the performance of the reactor. AOBs in the genus *Nitrosomonas* and anammox bacteria in the genus *Candidatus* Brocadia were found in both flocs and biofilms. The AOB population in the flocs was 1.5% and 1.1% in the biofilms. Anammox bacteria population in the flocs was meager at only 0.7%, whereas their presence in the biofilms was 29%. NOBs in the genus *Nitrospira* were only found in the flocs at 0.6% abundance. The microbial ecology of the flocs was comparable to that of a full-scale EBPR plant (Albertsen et al., 2012).

3.3. P and N removal during the anoxic and aerobic phases

Samples were also collected regularly at the start and end of the anoxic phase in addition to the final effluent to check the activities of DPAOs. Fig. 3a & b show the contributions of PO_4^{3-}-P uptake/removal during the anoxic and aerobic phases. Consistent P-uptake during the anoxic phase (15.9% of the total P-uptake on average) suggests the activity of DPAOs. The remaining PO_4^{3-}-P was taken up almost completely during the subsequent aerobic phase. Fig. 3c & d show both the removal and removal efficiency of TIN in the anoxic and aerobic phases, respectively. TIN removal in the anoxic phase was 5.9 mg/L on average, contributing to about 22.5% of the overall TIN removal. In the aerobic phase, about 17.5 mg/L (or 66.7% of TIN) was removed on average, increasing the overall TIN removal efficiency to 89.2% on average. From Fig. 3e, NO_x^--N was removed during the anoxic phase at the expense of sCOD and PO_4^{3-}-P. These results demonstrate the removal of NO_x^--N via denitrification through canonical denitrifiers (which used COD) and DPAOs (which used PO_4^{3-}-P). However, whether the DPAOs used NO_2^--N or NO_3^--N for anoxic P-uptake remains unclear. A batch experiment was then conducted to test the P-uptake activities of DPAOs/PAOs by O_2 (air), NO_2^--N, and NO_3^--N. Results (Fig. 3f) show that the enriched DPAOs could use both NO_2^--N and NO_3^--N in addition to O_2, albeit the rate was about 7 times higher in the case of O_2. These findings agree with previous studies that found that Accumulibacter clade IA could use NO_3^--N and NO_2^--N for P-uptake (Flowers et al., 2009). NH_4^+-N removal in the anoxic phase, at 0.66 mg N/L on average, can be used as a surrogate for anammox activities. However, since the feed (primary effluent) also contained organic N (part of TN in Table 1), the activities of the canonical denitrifiers could produce NH_4^+-N as a byproduct of organic carbon degradation, as suggested by the observed sCOD removal. At the same time, canonical denitrifiers and DPAOs could also assimilate NH_4^+-N for cell synthesis, making the slight NH_4^+ removal alone inconclusive for the anammox activities in the anoxic phase.

Table 2
Reactor performance for the last 100 days in averages.

	Feed (mg/L)	Effluent (mg/L)	Removal (%)
PO_4^{3-}-P	2.43	0.04	98.35
NO_2^--N	BDL	0.71	–
NO_3^--N	10.81	2.16	79.97
NH_4^+-N	36.46	1.24	96.61
TIN*	47.27	4.11	91.3
TN*	51.82	5.36	89.65
DON	1.45	1.04	28.27
sCOD	153	23.8	84.44

BDL, below the detection limit.
* After adding $NaNO_3$ in the anoxic phase. DON, dissolved organic nitrogen.

3.4. Anammox activities in the micro-aerobic phase

In order to prove and estimate the anammox activities for TIN removal during the micro-aerobic phase, about 250 mL of mixed liquor containing only flocs were withdrawn and run in parallel to the reactor containing both flocs and biofilms at the beginning of the aerobic phase. The microbial community analysis in Section 3.2 showed that the anammox bacteria population in the flocs (0.7%) was negligible compared to the anammox bacterial population in biofilms. Two tests were conducted on days 219 and 226 during Stage IV. The results are shown in Fig. 4a & b. PO_4^{3-}-P was undetected at the end of the tests for both flocs only and flocs with biofilms. These results agree with the microbial community data where the majority of the PAOs were found in the flocs. However, NH_4^+-N and NO_2^--N in the effluents from the flocs-only assays, on average, were 4.2 and 5.9 mg N/L, respectively, higher than those of the flocs with biofilms assays, indicating a higher Anammox activity in the biofilm faction. The difference between TIN removal in the flocs only and in the flocs and biofilms can be used to distinguish TIN removal by flocs and biofilms. On average, the biofilms contributed to about 44.5% of TIN removal on an average in the aerobic phase, while the flocs accounted for about 37.9%. The TIN removal from the biofilm fraction could not be attributed to anammox activities alone but rather to the cumulative action of anammox and heterotrophic denitrification. Nevertheless, the lower effluent NH_4^+-N and NO_2^--N and TIN concentrations in the mixed liquor containing both flocs and anammox biofilm carriers proved the anammox activities and their contributions to the TIN removal in the reactor during the aerobic phase.

3.5. Cycle analysis with biochemical transformations and gene expression patterns

3.5.1. Cyclic biochemical transformations

Intra and extracellular chemical transformations and gene expression data during a typical cycle were conducted on day 230. Fig. 5a shows the changes of N, P and C concentrations during the cycle. During the anaerobic phase, PO_4^{3-}-P concentration increased at a linear rate (33.8 mg P/L·h, $R^2 = 0.997$) in the first 25 min and accounted for about

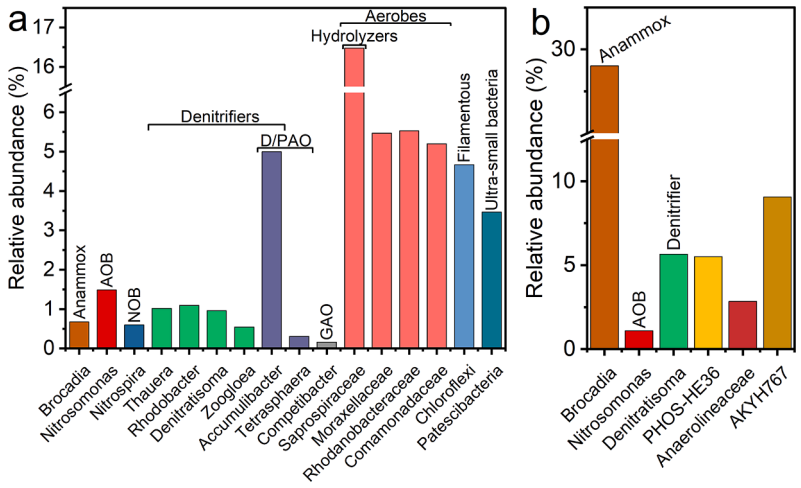

Fig. 2. Microbial profiles of (a) the flocs and (b) the biofilms on day 213 of operation. For detailed microbial profiles at the phylum and genus levels, refer to Fig. S1 and S2 for the flocs and Fig. S3 and S4 for the biofilms in SI.

86% of the total PO_4^{3-}-P released. The other 14% was released during remainder of the anaerobic time period. This P-release coincided with the removal of sCOD (from 137 in the feed to 45 mg/L after 50 min) and the accumulation of intracellular PHAs, indicating the activities of DPAOs/PAOs. The anoxic phase was introduced by adding NO_3^--N as $NaNO_3$ to simulate the internal nitrified recycling of the conventional A2O process. After the nitrate feeding, the concentration of NO_3^--N in the reactor was 6.1 mg N/L. At the end of the anoxic phase, NO_3^--N was almost completely removed (95.4%), along with further sCOD removal (12 mg/L) and anoxic P-uptake (2.61 mg/L) by DPAOs. NO_2^--N accumulation at the end of the anoxic phase was 0.98 mg N/L, and NH_4^+-N removal was 1.25 mg N/L, making the overall TIN removal in the anoxic phase 6.37 mg N/L, which was more than the externally provided of NO_3^--N. These anoxic chemical transformation patterns were similar to the findings observed in routine reactor sampling described in Section 3.3. Again, the removal of NO_x^--N at the expense of sCOD and PO_4^{3-}-P was due to both denitrification by canonical denitrifiers and DPAOs. About 86.5% of PO_4^{3-}-P was taken up in the first 70 min of the aerobic phase (160 min of the cycle), as opposed to about 13% taken up in the anoxic phase. The aerobic uptake rate was 14.33 mg P/L·h compared to 4.03 mg P/L·h for the anoxic uptake rate. PO_4^{3-}-P concentrations were below the detection limit at 220 min and remained there until the end of the cycle. PHA content was also quickly utilized with about 63% drop in the first 70 min of the aerobic phase and slowly depleted from about 4 (at 160 min) to 1.62 mg/g TSS (at 360 min) at a constant rate of 0.69 mg/g TSS·h. NH_4^+-N was removed at a linear rate of 6.72 mg N/L·h ($R^2 = 0.999$) throughout the 190 min of the aerobic phase despite the decreasing concentration of nearly 13 folds. The aerobic removal rate of TIN was also linear until the end of the aerobic phase (330 min of the cycle), though at a slightly lower rate of 5.51 mg N/L·h ($R^2 = 0.998$). This difference resulted from the slight accumulation of NO_2^--N throughout the aerobic phase, during which it increased from 0.98 to 4.28 mg N/L. At 330 min (240 min of the aerobic phase), NH_4^+-N was further removed to below the detection limit while NO_2^--N concentration decreased to 2.304 mg N/L. NO_3^--N concentration stayed below 0.279 mg N/L throughout the aerobic phase (90 – 330 min of the cycle) and was below the detection limit in the effluent. At the end of the cycle, only NO_2^--N was detected as TIN with a concentration of 2.18 mg N/L. The TIN removal efficiency for the whole process from the feed and NO_3^- addition in the anoxic phase was 96%, whereas PO_4^{3-} removal efficiency was nearly 100%.

3.5.2. *Gene expressions during a complete reactor cycle*

The function gene expressions of key genes were also examined to study the responses of the microbial communities in the flocs and biofilms to support our data related to the dynamics of different chemical species measured in the reactor. *rpoN* (RNA polymerase sigma-54 factor) was chosen as a reference gene in biofilm and floc samples as it is a constitutive gene with a stable expression level across all time point samples. *rpoN* was also used as a reference gene for qPCR normalization for Accumulibacter and other bacteria (Camejo et al., 2019; Chang et al., 2009).

Fig. 5b shows the expression profiles of genes related to DPAOs/PAOs in flocs. DPAOs/PAOs possess *ppk1* and *phaC* genes that catalyze reversible reactions of the synthesis and degradation of polyphosphate and PHA, respectively. In general, the expression of *phaC* and *ppk1* (all clades) exhibited similar patterns, muted in the anaerobic phase – except for *ppk1-IA,* which slightly increased by 1.6 folds at the end of the phase. Their expression levels rose at the end of anoxic phase and kept increasing to the highest at 20 min of the aerobic phase (110 min of the cycle). After that, their expression levels dropped until the end of the aerobic phase to about the same levels as that of the first sample (10 min of the anaerobic phase). The expression level of the Accumulibacter 16S rRNA gene kept decreasing throughout the anaerobic phase until the middle of the anoxic phase and only increased towards the end of the anoxic phase. The gene transcription level kept increasing further into the aerobic phase compared to those of *ppk1* and *phaC*, with the highest at 70 min of the phase (160 min of the cycle). Accumulibacter 16S rRNA expression level remained high for another hour before declining gradually. Bacterial cells maintain their ribosome and rRNA contents corresponding to their growth (Bremer and Dennis, 2008; Nomura et al., 1984; Otto et al., 2019). The increase in 16S rRNA expression at the end of anoxic and throughout the aerobic phase suggests that DPAOs/PAOs (Accumulibacter) were preparing for cell growth in these periods. These findings agree with the EBPR metabolic models that suggest PAO growth only occurs in the anoxic and aerobic phases due to the low energy requirements when external electron acceptors are available (He and Mcmahon, 2011).

Fig. 5c shows the changes in transcription levels of genes associated with anammox (*hzsB* and anammox 16S rRNA) in the biofilms and AOBs (*amoA*) in the flocs and biofilms. Similar patterns of *amoA* expression in the flocs and biofilms were observed. The presence of trace DO in the feed at the beginning of the anaerobic phase could have triggered the upregulation of the gene seen in the middle of the anaerobic phase. However, despite the absence of DO (< 0.04 mg/L after feeding, 10 min) throughout the anaerobic phase, the expression of *amoA* from both floc and biofilm communities kept increasing. AOBs have been found to overexpress *amoA* in response to stress, such as low temperatures and chemical inhibition (Park and Ely, 2009; Radniecki et al., 2009; Wu et al., 2016). However, this overexpression has been observed in aerobic

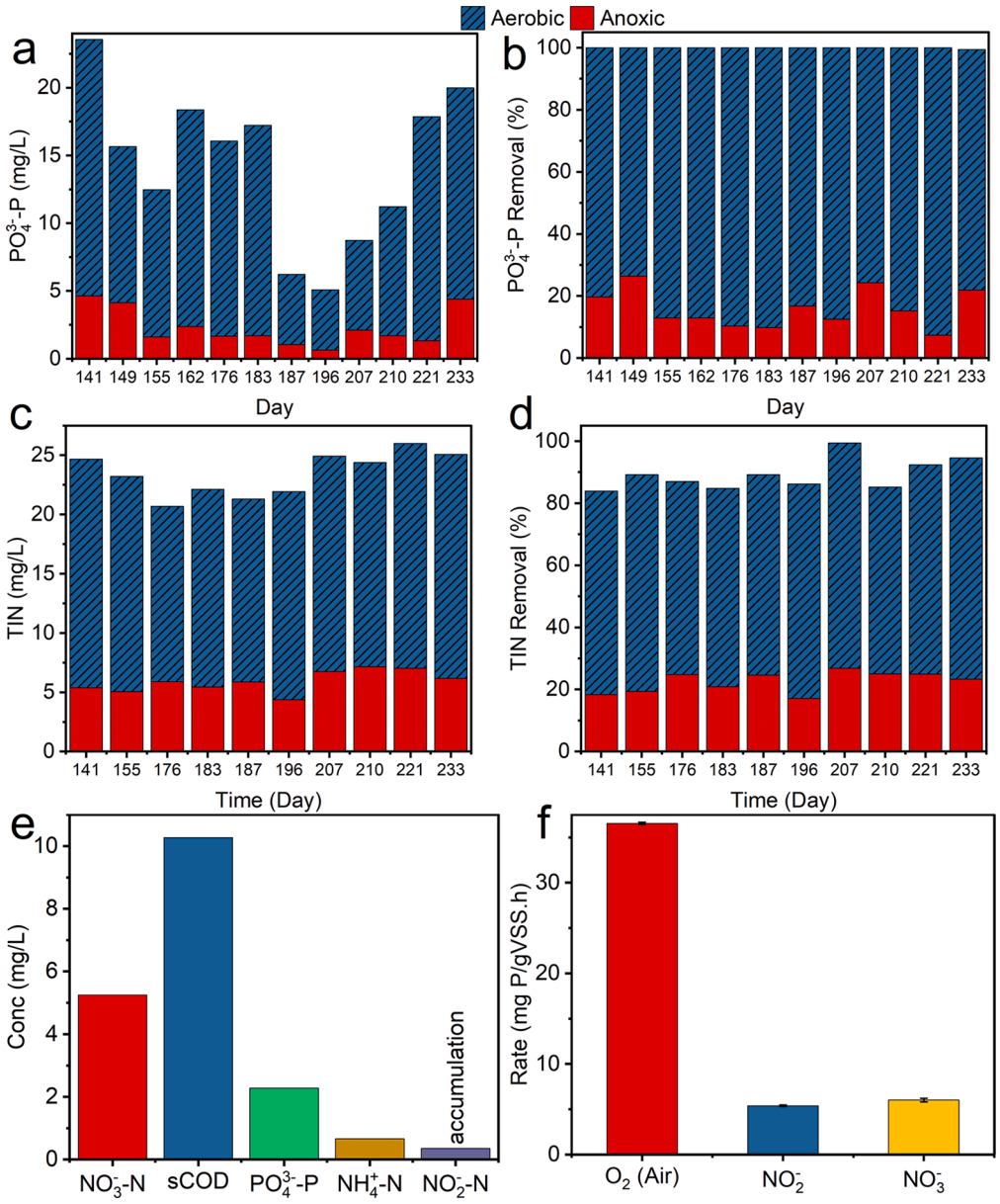

Fig. 3. Contributions of P-uptake as (a) concentration and (b) removal efficiency in the anoxic and aerobic phases. Contributions of TIN removal as (a) concentration and (b) removal efficiency in the anoxic and aerobic phases. (e) Average concentrations of NO_3^-, sCOD, PO_4^{3-}, and NH_4^+ removed and NO_2^- accumulated at the end of the anoxic phase for samples collected in (abcd). (f) Specific maximum P-uptake rates by PAOs based on different e^- acceptors.

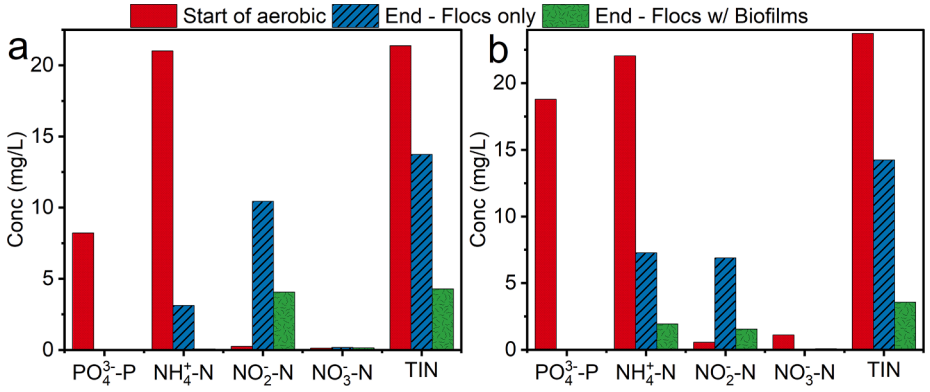

Fig. 4. Concentrations of PO_4^{3-}, NH_4^+, NO_2^-, NO_3^-, TIN at the start and at the end of the aerobic phase for flocs only and flocs with biofilms on (a) day 219 and (b) day 226 tests.

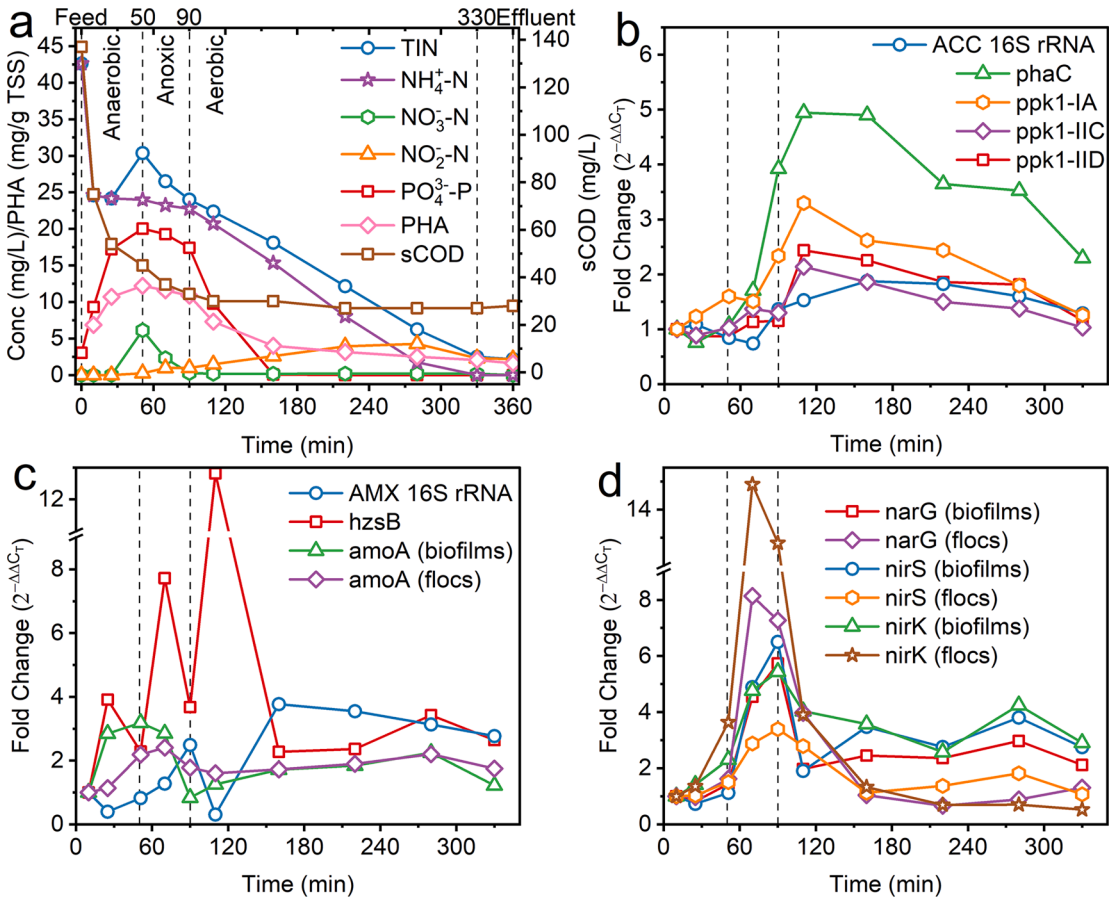

Fig. 5. Cycle analysis on day 225 of operation. (a) chemical concentrations at different times of the cycle. Gene expression profile patterns of (a) Accumulibacter (DPAOs/PAOs) in the flocs, (b) anammox bacteria in the biofilms (AMX 16S rRNA and *hzsB*), and AOBs (*amoA*) in both flocs and biofilms, and (c) of nitrate reducers (*narG*) and nitrite reducers (*nirS* and *nirK*) in both flocs and biofilms. Fold changes were calculated using the expression of *rpoN* as a reference at each time point sample and normalized to the first sample at 10 min of the cycle. The two vertical dashed lines divide the cycle into anaerobic, anoxic, and aerobic phases.

conditions, and a probable explanation given by the researchers was that AOBs may have tried to compensate for the loss of the enzyme activities. On the other hand, the increased expression of *amoA* in the anaerobic phase in this present study is interesting and warrants further investigation. During the anoxic phase, the expression of *amoA* in both communities decreased. The expression of *amoA* gradually increased throughout the aerobic zone except at the end of the phase. The increase in transcription levels of *amoA* coincided with the removal of NH_4^+-N in the aerobic phase. Likewise, the transcription levels also dropped when NH_4^+-N was exhausted at the end of the aerobic phase.

The expression of the anammox functional genes can further confirm their activities in the aerobic phase and, more importantly, in the anoxic phase when substrate data alone could not provide a definitive answer (Section 3.3). The anammox *hzsB* gene was also upregulated in the middle of the anaerobic phase, like *amoA*. It may have been due to the in-situ NO_2^--N production within the biofilms. Results show that the expression of *hzsB* increased in the middle of the anoxic phase and further increased to the highest level recorded (12.8 folds higher than in the first sample at 10 min) 20 min into the aerobic phase (110 min of the cycle). After that surge, the expression level of *hzsB* dropped but remained higher than in the first sample throughout the aerobic phase. Anammox bacteria consistently expressed *hzsB* in the aerobic phase even when the aeration was on, especially at 20 min (110 min of the cycle), proving that they were active in deeper anaerobic layers of the biofilms throughout the aerobic phase (Veuillet et al., 2014). Due to the biofilm structure, anoxic conditions were likely to be maintained inside the biofilms even when the aeration was on, protecting anammox bacteria from exposure to DO (0.4 ± 0.1 mg/L) in the bulk liquid. The increased expression of anammox 16S rRNA in the anoxic and aerobic phases indicates that anammox bacteria were active and preparing for cell growth, providing further evidence for their contributions to TIN removal in both phases.

As anticipated, nitrate reductase (*narG*) and nitrite reductase (*nirS* and *nirK*) genes in floc and biofilm communities were very active in the anoxic phase, as shown in Fig. 5d. In the aerobic phase, the expression levels of these genes were higher in the biofilm community than their flocculent counterparts, partly due to anoxic conditions which could have been maintained in inner layers of the biofilms. The higher expressions of the *narG, nirS*, and *nirK* in the biofilms helps explain the absence of nitrate accumulation from the proven anammox activities in the aerobic phase. A previous study also found that heterotrophs inside anammox granules depended on organic secretions from anammox bacteria to denitrify (Lawson et al., 2017).

It is worth noting the expression of *hzsB* and denitrifying genes dropped significantly into the aerobic phase, despite the proven TIN removal through the anammox pathway and SNDN (Section 3.4) in the biofilm and floc sludges. During the aerobic zone the supply of substrates (NO_2^- for anammox and NO_2^-/ NO_3^- for denitrification) was stable but low as they were consumed once produced. It is postulated that this low supply may have slowed down the production of the corresponding enzymes resulting in the observed drop in the expression levels of *hzsB* and the denitrifying genes.

4. Discussion

4.1. IFAS configuration with low DO for mainstream anammox and EBPR

This study applied mainstream conditions such as using conventional A2O configuration and real primary effluent as feed. Challenges for mainstream anammox applications include retention of anammox bacteria, high C/N ratio, and suppression of NOB growth (Cao et al., 2017; X. X. Li et al., 2018; Trinh et al., 2021).

Traditionally, SRTs in activated sludge processes are governed by nitrification rates which in turn also depend on temperature and DO setpoint. It has been found that maintaining high SRT (> 10 d) for complete nitrification may negatively impact EBPR performance (Onnis-Hayden et al., 2020). The IFAS configuration of this reactor decoupled the autotrophic deammonification (PNA) process from other processes, including EBPR, denitrification, aerobic organic oxidation, and nitratation (oxidation of NO_2^- to NO_3^- by NOBs). This decoupling allowed operation at a low flocculent SRT (5 d) without washing out slow-growing AOBs and anammox bacteria as they were retained in the reactor through carriers. Also, with the proven anammox activities, only a portion of influent NH_4^+ needed to be removed through nitritation by AOBs.

The C/N ratio in the primary effluent in this study was 6.3 – 7 (tCOD/NH_4^+-N), which was higher than the optimal range of 0.5 – 2 for anammox processes (Daigger, 2014; Lackner et al., 2008). High C/N ratios allow ordinary heterotrophs to outcompete anammox bacteria for space and nutrients (Chamchoi et al., 2008; Güven et al., 2005). However, due to the attached growth, anammox bacteria were protected in the biofilms for space and from DO stresses. Furthermore, the pre-anaerobic phase and enrichment of PAOs/DPAOs competed out ordinary heterotrophic bacteria, which also favored anammox bacteria enrichment. After at least 138 days of inoculation in the reactor, the relative abundance of anammox bacteria remained high in the biofilms at about 29% (Fig. 2b) compared to about 34.5% in the original seed (SI, Figs. S5 and S6). This slight drop could be from decreased NH_4^+ loading in the seed reactor's filtrate to the present reactor's primary effluent.

The suppression of NOB growth in the aerobic zone has been proven difficult under mainstream conditions (Agrawal et al., 2018; Cao et al., 2017; Han et al., 2016; Lotti et al., 2015; Ma et al., 2020). In this study, with a combination of low flocculent SRT (5 d), low DO setpoint (0.4 ± 0.1 mg/L), room temperature operation (22±3 °C), and intermittent aeration, NOB growth was kept under control, indicated by their low abundance in the flocs (~0.6%), absence in the biofilms, and low NO_3^--N concentrations in the effluent. Laureni et al. (2019) also found that NOB growth can be controlled under mainstream conditions by segregating anammox bacteria in biofilms from AOBs and NOBs in flocs. PDN has been proven as an alternative pathway to consistently supply NO_2^--N for anammox when there are no effective measures for NOB suppression. In addition, endogenous denitrification has become increasingly popular in implementing PDN due to its high NO_2^- accumulation rates. In previous studies, however, endogenous denitrification mainly relied on GAOs by starting up with GAOs or ending up shifting from a DPAO dominant to a GAO dominant population (Ji et al., 2020b; Li et al., 2022; Wang et al., 2019, 2015; Yin et al., 2021). Using GAOs for endogenous denitrification would require more organic carbon dosage for EBPR, where its performance could also be destabilized in the long run. It may eventually make carbon-neutral nutrient removal, one of the main purposes for bringing anammox to the mainstream in the first place, unrealized. However, in this study, the need for partial denitrification was significantly reduced due to the effective strategies for NOB washout. Moreover, with efficient implementation of endogenous denitrification through DPAOs, rather than GAOs, the reactor was not just able to remove most of the in-situ N and P found in the feed but was also able to remove extra N added externally (as $NaNO_3$ in the anoxic phase) without external carbon dosage. There were several factors employed in this study that led to the suppression of GAO growth, including room temperature (19 – 26 °C) operation, presence of a mixture of acetate and propionate in the feed (primary effluent), low DO (0.4 ± 0.1 mg/L), short floc SRT (5 d), and pH (7.3 – 8.3) (Carvalheira et al., 2014; Chan et al., 2017; Lopez-Vazquez et al., 2009; Onnis-Hayden et al., 2020; Winkler et al., 2011).

There are some drawbacks to low DO operation, such as sludge bulking due to the proliferation of filamentous bacteria, high dissolved organic nitrogen (DON) formation, and high N_2O emissions (Kampschreur et al., 2008; Liao et al., 2022; Martins et al., 2003; Peng et al., 2014). However, filamentous bacteria were found at low abundance, and the sludge volume index (SVI) of the flocs during Stage IV of reactor operation ranged from 102 - 128 mL/g. These values were within SVI values for good settling biomass in the activated sludge process, which is below 150 mL/g (Metcalf and Eddy Inc et al., 2013). Effluent DON (Table 2) was found at a similar value to typical activated sludge processes (Zheng et al., 2021). These results suggest that DO at 0.4 ± 0.1 mg/L was not too low to cause bulking problems and DON formation. It may also have been partly due to the relatively low concentration of TSS (about 1.3 g/L) maintained in the reactor that balanced F/M (food to microorganism ratio) and SRT, thus reducing endogenous decay. During the cycle analysis (Section 2.6 and 3.5), N_2O emissions were also monitored. The average N_2O emission factor in the anoxic and aerobic phases was 0.19% and 3.85%, respectively, of the TIN removed. As such, the total emission factor was estimated to be 3.02%. The aerobic N_2O emission factor is similar to that of PNA and PAO based processes reported in the literature (Cavanaugh et al., 2022; Li et al., 2020).

It has been found that biofilm AOB/Anammox required a relatively high DO setpoint in the bulk liquid at 0.5 – 1 mg/L due to resistance in mass transfer (Christensson et al., 2013). In this study, despite a lower DO setpoint at 0.4 ± 0.1 mg/L, ammonium removal remained consistently high. AOBs were found in substantial abundance while also active in both floc and biofilm communities, as revealed from the microbial ecology and gene expression data. The presence of AOBs in the flocs could be one of the reasons for the observed stable nitritation, as AOBs in the flocs required a lower DO setpoint. Veuillet et al., 2014 observed that in the IFAS reactor for sidestream PNA, most AOBs grew in the flocs while anammox bacteria predominantly occupied the biofilms. For this reason, the researchers found that the IFAS reactor required a lower DO setpoint (0.1 – 0.25 mg/L) than the biofilm-only reactor (0.6 mg/L), where AOBs and anammox bacteria were comingled in the biofilms.

4.2. Coexistence of anammox bacteria with DPAOs and other groups of microorganisms

Due to their ability to denitrify, DPAOs can potentially compete for NO_2^--N with anammox bacteria, especially in the anoxic phase when NO_2^--N was limiting. From gene expression data (Fig. 5c), anammox bacteria were active during the anoxic phase, however based on the average NH_4^+-N removal, their contributions to TIN removal in the anoxic phase was little (Fig. 3e). On the other hand, the enriched DPAOs/PAOs preferred O_2 over NO_x^--N as an electron acceptor for their P-uptake (Fig. 5abf). Camejo et al. (2016) found that NO_2^--N was a poor electron acceptor for DPAOs while P-uptake through NO_3^--N respiration was incomplete, and NO_3^--N removal also ceased when PO_4^{3-}-P was exhausted. Hence, just after 70 min into the aerobic phase, when 99.55% of PO_4^{3-}-P was already taken up, DPAOs' ability to denitrify was limited, making most of NO_2^--N from AOB respiration available for anammox bacteria.

Organic carbon was utilized efficiently in each phase of the cycle. Batch experiments showed that the enriched DPAOs/PAOs could only use VFAs (acetate and propionate) for anaerobic P-release (SI, Text S6 and Table S2). P-release/C-uptake for acetate and propionate was 0.59 and 0.45 Pmol/Cmol, respectively. These values were similar to the values reported in the literature for highly enriched PAOs with low-populated GAO sludges (Diaz et al., 2022). Hence, during the

anaerobic phase, most VFAs were consumed by DPAOs/PAOs, and by the end of the anoxic phase, sCOD was almost exhausted, suggesting that readily biodegradable carbon was already consumed. Hence, the only available organic carbon in the aerobic phase was mostly particulate, which also was supported by the high abundance of hydrolyzers in the family of *Saprospiraceae* in the flocs (Fig. 2a). TIN removal through SNDN in the aerobic phase (37.9% in the floc fraction) may be partly due to the breakdown of complex organic compounds by the hydrolyzers to simpler forms during aeration, making them available for the denitrifiers when the aeration ceased. Particulate COD has also been found to improve TIN removal through SNDN (Klaus et al., 2020). DPAOs could also have contributed to the SNDN in the aerobic phase. After P-depletion in the aerobic phase, DPAOs could use their stored PHA to denitrify to gain maintenance energy (Lu et al., 2007). PHA content was found to consistently drop after P-depletion occurred 160 min into the cycle (Fig. 5a).

In the cycle analysis test, the NH_4^+ removal rate in the aerobic phase (Fig. 5a) was constant. During the initial stage of the aeration phase oxygen was consumed by several functional groups such as PAO, aerobic heterotrophic bacteria, and AOB, once organic C was consumed from the bulk liquid more O_2 became available to AOB which is reflected by a sharp increase in ammonium oxidizing capacity as the aerobic phase progressed. Also, as more nitrite accumulated in the aerobic phase, anammox activities should have increased, hence providing another sink to NH_4^+ and compensating for the removal rate loss by AOBs with decreasing NH_4^+ concentration. High and stable removal of C, P, and N suggests the harmonious coexistence of autotrophic AOBs/anammox bacteria with heterotrophic DPAOs/PAOs, denitrifiers, and aerobes.

5. Conclusions

The integration of EBPR and PNA processes was proven successful in removing P and N without external carbon dosage. Sustained AOB and Anammox growth was enabled through attached growth on IFAS system while EBPR was predominantly occurring in the suspended sludge fraction. The following conclusions can be made from this study:

- Decoupling EBPR and nitritation/anammox into two sludges – flocs and biofilms – allowed the reactor to be operated at a low SRT without washing out slow-growing AOBs and anammox for reliable EBPR performance while maintaining high TIN removal efficiency.
- With a low DO setpoint (0.4 ± 0.1 mg/L) and intermittent aeration, this low SRT operation provided selective pressure against NOBs and GAOs under mainstream wastewater conditions. Low DO setpoint also maintained anammox activities and SNDN throughout the aerobic phase.
- Anammox activities and their contributions to TIN removal were well proven from the evidence at the substrate and genetics levels.
- DPAOs were enriched, and their activities for P-uptake in the anoxic phase helped remove the majority of TIN in the anoxic phase without organic carbon dosage, increasing the reactor's capability to remove TIN in a carbon-neutral manner.
- Incorporating anammox into aerobic zones would significantly reduce the internal recycle flow rates typically required by the traditional A2O process as a considerable amount of TIN was already removed by anammox activities. Low internal recycle flow rates result in small anoxic and aerobic reactor footprints, thus reducing capital and operational costs further.
- Microbial community dynamics along with metagenomics and metatranscriptomics information should be further investigated to better understand the interactions between each microbial group.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.119758.

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