Successful Implementation of Biofilm Anammox in IFAS A2O Process for Simultaneous N and P Removal in Mainstream Treatment Train

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SUMMARY
This research studied integrated fixed film activated sludge (IFAS) technology to simultaneously remove N and P in real municipal wastewater by combining anammox biofilms with flocculent activated sludge for enhanced biological P removal (EBPR). After a steady state operation, robust reactor performance was obtained with average P and total inorganic nitrogen (TIN) removal efficiencies of 98.4±2.4% and 91.3±4.1%. The average TIN removal rate was 118 mg/L·d, which is a reasonable number for mainstream applications. Batch activity assays, which showed that 44.5% of TIN were removed by the biofilms in the aerobic phase, along with gene expression data, confirmed anammox activities.

ABSTRACT
This research studied integrated fixed film activated sludge (IFAS) technology to simultaneously remove N and P in real municipal wastewater by combining anammox biofilms with flocculent activated sludge for enhanced biological P removal (EBPR). The study was conducted in a sequencing batch reactor (SBR) operated as a conventional A2O (anaerobic-anoxic-oxic) process with an 8.8 h hydraulic retention time. After achieving steady-state operation, the reactor showed robust performance, with average removal efficiencies of 91.3±4.1% for total inorganic nitrogen (TIN) and 98.4±2.4% for phosphorus (P). Denitrifying polyphosphate accumulating organisms (DPAOs) were responsible for 15.9% of P uptake during the anoxic phase, while biofilms showed anammox activity in the aerobic step. The IFAS configuration with a low solid retention time (SRT) of 5 days prevented the washout of biofilm anammox bacteria and allowed selective washout of unwanted organisms. The results demonstrated the successful coexistence of anammox bacteria with other bacteria for efficient nutrient removal in real wastewater conditions.

KEYWORDS
Anammox, Biofilm, EBPR, A2O, IFAS, Nutrient, Nitrogen, Phosphorus, Mainstream

INTRODUCTION

Anammox has emerged as a promising sustainable bioprocess for removing nitrogen from municipal wastewater due to its energy efficiency and lower carbon requirements than conventional nitrification-denitrification (Agrawal et al., 2018; Arora et al., 2021). However, implementing full-scale mainstream anammox, especially in the presence of low ammonium concentrations and fluctuating temperatures, remains challenging. Coupling partial nitritation and anammox (PNA) has been successful in sidestream applications but faces obstacles in mainstream conditions, mainly due to nitrite-oxidizing bacteria (NOBs) growth and the presence of heterotrophic denitrifying bacteria competing for available nitrite (Le et al., 2019). Effluents from PNA systems still contain significant amounts of total inorganic nitrogen (TIN) in the form of residual NH_4^+ and in-situ produced nitrate, making integration of anammox into the mainstream difficult, particularly for plants with strict TIN limits (Cao et al., 2017).

To address these challenges, various solutions have been proposed, including integrating partial denitrification (PDN) with anammox. PDN uses heterotrophic denitrifiers to reduce nitrate to nitrite, providing in-situ nitrite for anammox to convert to nitrogen gas (Ahmad et al., 2021; Ma et al., 2020). However, PDN still requires organic carbon dosage. Another concern is phosphorus (P) removal, achieved through enhanced biological P removal (EBPR). To achieve a holistic treatment of nitrogen (N) and phosphorus in municipal wastewater, innovative approaches can pair EBPR-driven denitrification with anammox, reducing organic carbon requirements while removing P (Yin et al., 2021). Denitrifying polyphosphate accumulating organisms (DPAOs) can efficiently perform denitrification without external organic carbon, and their aerobic P-uptake reduces dissolved oxygen demand in the system.

Another major challenge in mainstream anammox processes is retaining slow-growing anammox bacteria. However, this issue can be addressed by cultivating anammox bacteria on carriers as biofilms, which have shown better retention than granular biofilms (Gao et al., 2022). Biofilms offer a dedicated space for anammox bacteria to thrive and exhibit higher tolerance to factors like high dissolved oxygen concentrations, low temperatures, and chemical inhibition, making them ideal for mainstream applications (Gilbert et al., 2014; Jiang et al., 2020; Lackner et al., 2015). The integration of attached-growth biofilms with flocculent activated sludge in an IFAS technology further enhances process intensification, increasing reactor stability and output for nitrification and anammox processes (Bai et al., 2016; Regmi et al., 2011).

This study employed IFAS technology in a sequencing batch reactor (SBR) to simultaneously remove P and N from real municipal wastewater without organic carbon dosage. The biofilm biomass mainly comprised AOBs and anammox bacteria for PNA, while the floc biomass consisted of DPAOs/PAOs and other bacteria for EBPR, denitrification, and aerobic C removal. The objectives were to assess removal efficiency of P, N, and C, study interactions between microorganisms at substrate and molecular levels, and identify optimal conditions for their coexistence. The data can inform the optimization of integrated EBPR-based denitrification and anammox processes, advancing energy- and carbon-efficient nutrient removal in wastewater treatment.

METHODOLOGY

This study applied IFAS technology by combining attached growth biofilms with suspended growth flocs in a 5-L sequencing batch reactor (SBR) to simultaneously remove P and N. The SBR operated in the A2O (anaerobic-anoxic-oxic) mode with a 6-hour cycle length (4 cycles/day). It consisted of a 50-minute anaerobic phase with 10 minutes of feeding, a 40-minute anoxic phase, a 4-hour aerobic phase, and a 30-minute settling/decanting phase. During the anoxic phase, NaNO3 was fed to the reactor with a total loading of 19 mg N/cycle (or 10 mg N/L in the feed of 1.9 L) to simulate internal nitrate recycling. The reactor was seeded with suspended biomass from a stable lab-scale EBPR reactor and activated sludge from a local wastewater treatment plant. Mature anammox/AOB biofilms grown on AnoxK^{TM}5 carriers from a PNA reactor were gradually added, reaching a final loading of 15% on day 75. The hydraulic retention time (HRT) was 8.8 hours, and the floc solids retention time (SRT) was maintained at 5 days. pH was monitored regularly but not controlled. Intermittent aeration (7 minutes on/off) was used during the aerobic phase, with a DO setpoint of 0.4 ± 0.1 mg/L. The reactor operated at room temperature (22±3°C).

Two batch assays were performed towards the end of the study (on days 219 and 226) to confirm anammox bacteria's activity and their contribution to overall TIN removal during the micro-aerobic phase. Due to possible variations in feed (real primary effluent), the assays were conducted a week apart using different feed batches. About 250 mL of mixed liquor containing only flocs was withdrawn from the reactor into a flask at the end of the anoxic phase, running in parallel to the reactor containing both flocs and AOB/anammox biofilms until the end of the aerobic phase. Air purging was provided in the flask from the same air pump used for the reactor to synchronize intermittent aeration. Manual control was used to manage DO in the flask. Samples were taken from both vessels for chemical analysis at the beginning and end of the tests.

Table 1. Reactor performance for the last 100 days in averages.
BDL, below the detection limit. *After adding $NaNO_3$ in the anoxic phase. DON, dissolved organic nitrogen.

	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

RESULTS

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 (**Figure 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 (**Figure 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 (**Figure 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 (**Figure 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^{TM}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 itself, which should be further investigated.

The removal of sCOD (**Figure 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 4.3. 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 & Eddy Inc et al., 2013).

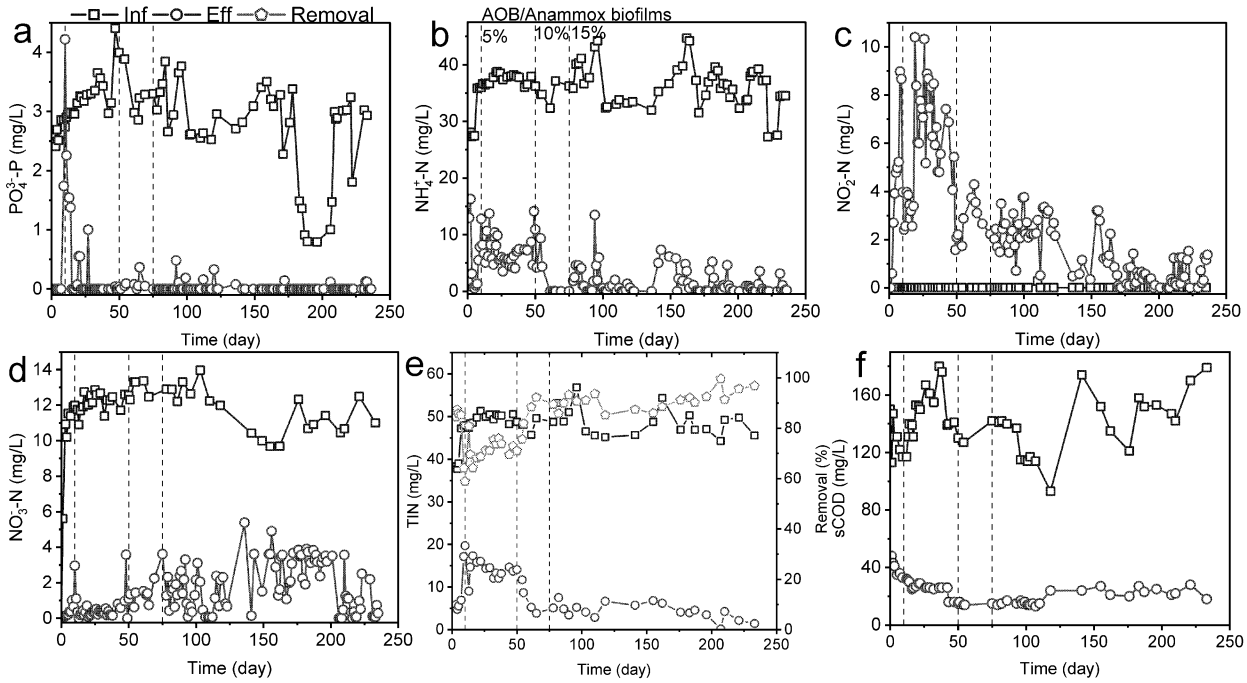

Figure 1 Reactor performance, concentrations of (a) PO_4^{3-} (b) NH_4^+, (c) NO_2^-, (d) NO_3^-, (e) TIN with removal efficiency, and (f) sCOD in influent and effluent. Note that Influent NO_3^- concentrations were based on a calculation of the $NaNO_3$ addition in the anoxic phase. The three vertical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

TIN removal during the anoxic and aerobic phases

NO_3^- was removed during the anoxic phase at the expense of sCOD and PO_4^{3-} (**Figure 2**), suggesting the removal by canonical denitrifiers and DPAO. NH_4^+ removal in the anoxic phase (0.66 mg N/L on average) can be attributed to anammox activities, which was modicum due to the NO_2^- limitation. 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, P-uptake during the anoxic phase was 15.9%, while the remainder was taken up almost completely during the subsequent aerobic phase.

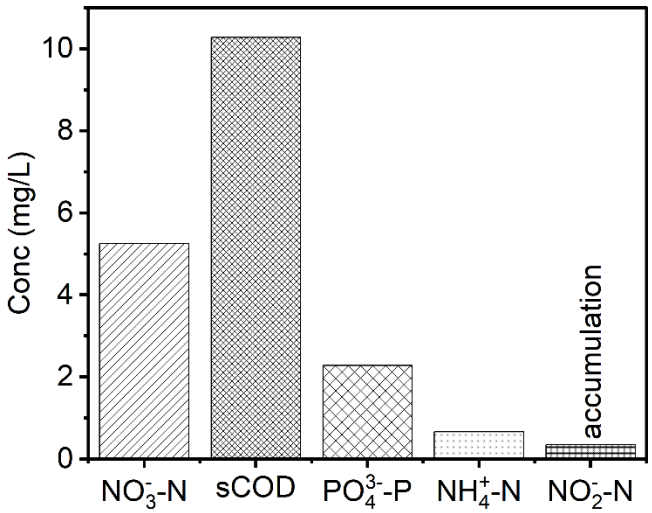

Figure 2 Average concentrations of NO_3^-, sCOD, PO_4^{3-}, and NH_4^+ removed and NO_2^- accumulated at the end of the anoxic phase

Anammox activities in the 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 to test for anammox activities. Two assays were conducted. PO_4^{3-} was undetected at the end of the tests for both flocs only and flocs with biofilms (**Figure 3a**). However, NH_4^+ and NO_2^- 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 fraction. On average, the anammox pathway contributed to about 44.5% of TIN removal in the aerobic phase, while simultaneous nitrification-denitrification (SNDN) accounted for about 37.9%.

Anammox functional gene expression (**Figure 3b**) confirms their activities in the aerobic and anoxic phases, complementing substrate data. The *hzsB* gene showed upregulation in the anaerobic phase, possibly due to in-situ NO_2^--N production within the biofilms. During the anoxic phase, *hzsB* expression increased and reached its peak (12.8 times higher than at 10 minutes) at 20 minutes into the aerobic phase (110 minutes of the cycle). Anammox bacteria remained active in deeper anaerobic layers of the biofilms throughout the aerobic phase, as evidenced by continuous *hzsB* expression, even during aeration at 20 minutes. This indicates that anoxic conditions persisted within the biofilms, protecting anammox bacteria from exposure to DO (0.4±0.1 mg/L) in the bulk liquid. Increased expression of anammox 16S rRNA in the anoxic and aerobic phases supports their role in TIN removal during both phases, as they were actively preparing for cell growth.

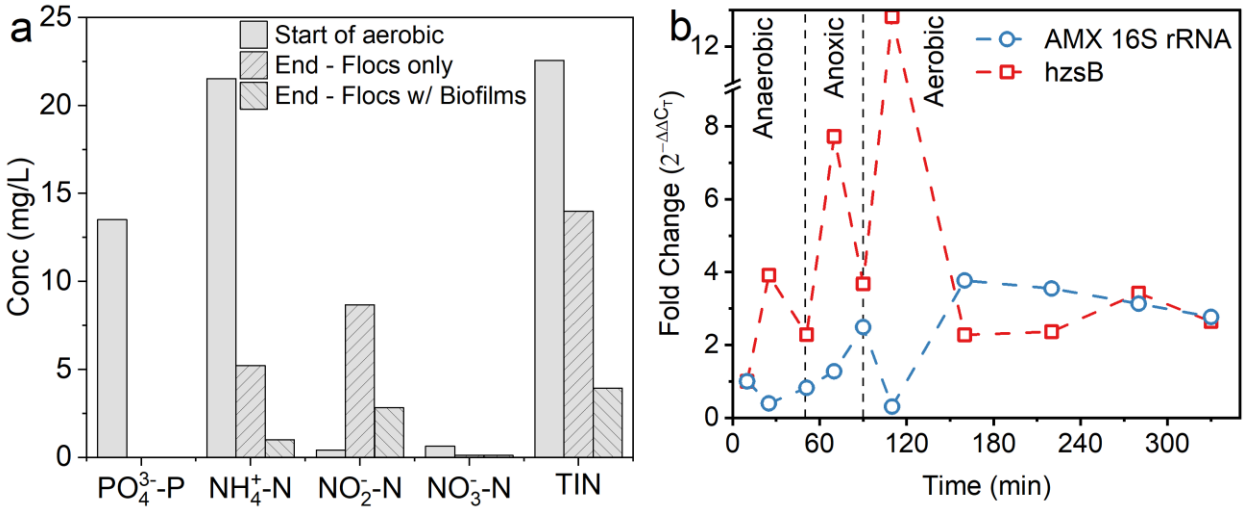

Figure 3 (a) Anammox activity assay test in aerobic phase and (b) Anammox *hzsB* and 16S rRNA gene expression during a cycle.

Microbial ecology

Figure 4 shows the microbial profiles of the flocs and biofilms at the genus level. In the flocs, the most abundant genotype was aerobic heterotrophic bacteria accounting for at least 32.7% of the total population, with hydrolyzers in the family of *Saprospiraceae* being the most abundant group, 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 & Nielsen, 2014). Well-defined 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, accounting for 5% of the relative abundance. *Candidatus* Competibacter was the only group of glycogen-accumulating organisms (GAO) 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. AOB in the genus *Nitrosomonas* and anammox bacteria in the genus *Candidatus* Brocadia were found in both flocs and biofilms (Fig. 2ab). 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%. NOB in the genus *Nitrospira* were only found in the flocs at 0.6% abundance.

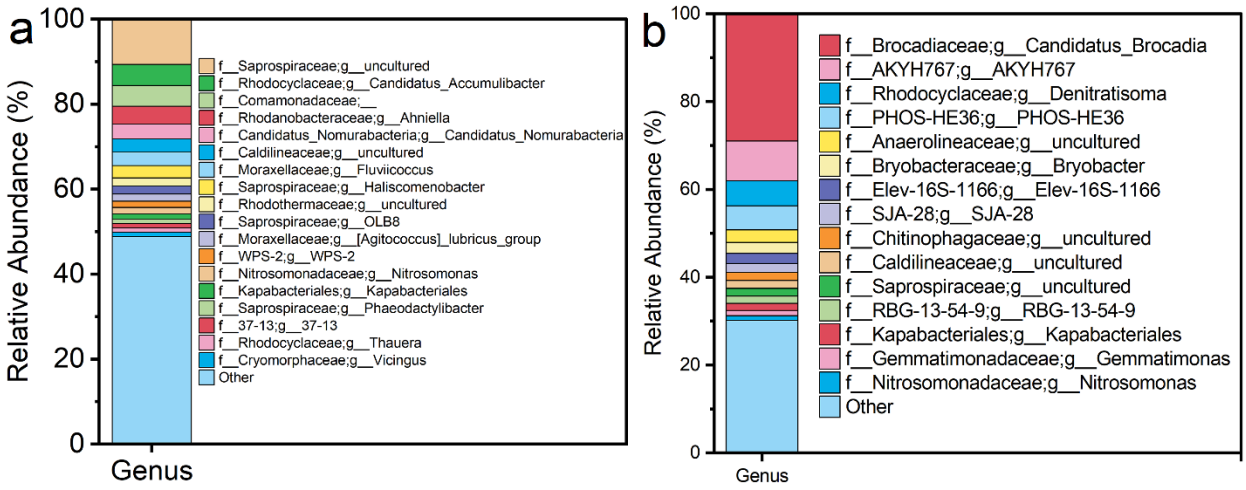

Figure 4 Microbial profiles at the genus level of the (a) flocs and (b) biofilms

DISCUSSION

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.

The suppression of NOB growth in the aerobic zone has been proven difficult under mainstream conditions (Han et al., 2016; Lotti et al., 2015). 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 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 N2O 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 & Eddy Inc et al., 2013). Effluent DON 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. 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.

CONCLUSION AND LESSONS LEARNED

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.

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