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Water Impact Statement

While the deammonification bioprocess allows for energy efficient nitrogen removal from sidestream wastewater flows, its application to mainstream flows remains stalled in the research phase. This study demonstrates improvement in a mainstream deammonification reactor by bypassing some primary effluent around the A-stage. Adjusting this bypass allows for a tunable C:N ratio and translates directly to processes with upstream A-stage reactors.

Optimization of the carbon to nitrogen ratio for mainstream deammonification and the resulting shift in nitrification from biofilm to suspension

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Abstract

Application of the deammonification process to mainstream wastewater promises energy-efficient nitrogen removal, but has been limited by unwanted activity of nitrite oxidizing bacteria and low anammox activity at moderate temperatures (<20 °C). In the present study, N removal in a mainstream integrated fixed-film activated sludge (IFAS) deammonification process increased by 27% to 73% total inorganic nitrogen (TIN) removal by diverting 10% of the primary effluent flow around the A-stage and directly into the deammonification reactor, thereby increasing the influent C:N ratio from 2.3 to 3.1 g sCOD/g NH_4^+-N. This change coincided with a dramatic shift in nitrification activity from the biofilm to the suspension, and the increased carbon enabled a higher suspended solids concentration at a realistic solids retention time of 7.3 ± 2.1 days. Anammox biomass and activity was retained over the entire study (>3 years) and was not negatively impacted by the increase in influent carbon. N isotope testing indicated that cross feeding between denitrifiers and anammox played an important role in N removal and that about 53% of N removal was ultimately routed through the anammox metabolism. The reactor temperature was controlled near 20 °C for most of the study, and 72% TIN removal was maintained during a temperature decline down to 12 °C (after which TIN removal reduced to an average of 58% from 12 down to 8 °C). Our work demonstrates the impact of small changes in C:N on performance, population structure, and aggregate type (biofilm vs. floc) in mainstream deammonification bioprocesses and provides a simple approach to control C:N in practice.

Introduction

Deammonification is a carbon and energy-efficient method for nitrogen (N) removal that combines the activity of aerobic ammonia oxidizing bacteria (AOB) and anaerobic ammonia oxidizing bacteria (anammox) to produce dinitrogen gas (N_2). Specifically, AOB oxidize a portion of the ammonium (NH_4^+) present in wastewater to nitrite (NO_2^-) with dissolved oxygen (DO) as an electron acceptor, and anammox oxidize the remaining NH_4^+ and reduce the resulting NO_2^- to produce N_2. Both metabolic pathways are autotrophic, meaning that organic carbon can be redirected to energy recovery or other metabolic processes. Full-scale applications of the deammonification process to sidestream flows in wastewater treatment plants (with process temperatures around 30 °C and influent ammonium > 300 mgN/L) have grown in recent years.[1] Application of deammonification to mainstream wastewater that harbors the vast majority of reactive nitrogen in typical municipal wastewater treatment plants is limited, but research efforts have continued[2–8] due its potential to contribute to the transformation of wastewater treatment from an energy intensive to an energy exporting endeavor.[9–11] Key challenges that have hindered implementation of deammonification under mainstream conditions include high nitrite oxidizing bacteria (NOB) activity (and thus low total N removal) and low anammox activity at low temperatures.[12,13]

Biofilm systems have gained attention under mainstream conditions for their ability to retain anammox biomass and activity,[4,14,15] but their impact on NOB activity is less clear. Sufficient concentrations of suspended solids in biofilm systems can may shift NOB off the biofilms and into the bulk[16] where they can be selectively washed out via solids retention time (SRT) control, as in Laureni et al. (2019).[17] However, with the low COD loading in that study, accumulation of

sufficient suspended solids to induce the shift of NOB from the biofilm required an SRT of >150 days,[17] which is unrealistic for practice.

For this reason and others, suppression of NOB has remained a vexing and persistent challenge in mainstream deammonification studies, leading some researchers to abandon efforts to completely suppress NOB activity and focus on systems for combined nitrification, anammox and denitrification.[18–21] The impact of organic carbon on competition between functional groups in mainstream processes is critical, given that certain methods for NOB out-selection are only available under sidestream conditions, such as high temperatures[22] and elevated free ammonia.[23–25] A minimal COD:N ratio has long been assumed to be advantageous in deammonification, as this should favor autotrophs like anammox and AOB over ordinary heterotrophs and denitrifiers.[26,27] However, under sidestream conditions, moderate levels of organic carbon can improve N removal, while excess carbon can lead to anammox suppression via out-competition by denitrifiers and process failure[28–35]. Though not required by key functional groups, organic carbon may improve mainstream deammonification by increasing residual N removal via denitrification,[6] improving cross-feeding to anammox via denitratation,[18] and increasing competition for (DO), thus reducing nitrate (NO_3^-) production and increasing N removal. However, the influence of influent COD:N on aggregate type (biofilm vs. floc), population segregation and activity of nitrifiers and anammox in mainstream deammonification systems is poorly understood. Furthermore, a practical means to adjust COD:N has not been demonstrated under real-world conditions.

The objective of this paper is to demonstrate a novel and cost-effective solution for tuning the relative organic carbon content of deammonification systems with upstream A-stage carbon removal, and to demonstrate its influence on aggregate type (biofilms versus flocs), microbial

activity, and population structure. N removal in a mainstream integrated fixed-film activated sludge (IFAS) deammonification process operated for >1,000 days improved by diverting 10% of the primary effluent flow around the A-stage and directly into the deammonification reactor. Importantly, this change marked a dramatic shift in nitrification activity from the biofilm to the suspension at a realistic SRT of 7.3 ± 2.1 days, a shift that was not observed during previous IFAS mode without 10% primary effluent in the influent. Anammox biomass and activity was selectively retained on the biofilm over the entire study (>3 years) and was not negatively impacted by the increase in influent COD. N isotope testing was performed to measure the relative contributions of denitrification and anammox to N removal.

Materials and Methods

Reactor Operation

A 12-L sequencing batch reactor (SBR, but hereafter referred to as "reactor") for mainstream deammonification treatment was operated at the Metropolitan Water Reclamation District of Greater Chicago (MWRDGC) Terrence J. O'Brien Water Reclamation Plant (WRP) in Skokie, Illinois for 1,128 days. The reactor was seeded to a fill ratio of 30% on May 24, 2016 ("day 0" of operation) with anammox-enriched K5 carriers from the Kruger/Veolia Biofarm at James River, VA (equivalent to ~3400mg VSS/L) and ~340 mg VSS/L suspended growth biomass from the full-scale sidestream DEMON® process at the York River treatment plant (Hampton Roads Sanitation District) (equivalent to 10% of the estimated VSS on K5 carriers). Upstream treatment of real wastewater included primary settling tanks and a 56-L activated sludge SBR ("A-stage") for biological COD and phosphorus removal. From days 0 – 899, 100% of the reactor influent was A-stage effluent, and from day 900 to the end of the study (day 1,128) 90% of reactor influent was A-stage effluent and 10% was untreated primary effluent. Because

of the notable change in reactor performance after bypassing 10% of the influent around the A-stage, data reporting in this study is split into Phase 1 (days 0 – 899) and Phase 2 (days 900 – 1,128).

Aside from reactor inoculation on day 0, only 2 bioaugmentation events occurred throughout the study. First, on day 849 additional anammox-enriched K5 carriers were supplemented from the MWRDGC Egan WRP ANITA™ Mox process (Schaumberg, IL, USA) to a final volumetric fill ratio of 38% (up from the original 30%) to increase the anammox population. Second, on day 900, 3 liters of mixed liquor from a 56-L nitritation-denitritation SBR[36] (selected because it demonstrated robust selective nitritation) was added to increase the suspended biomass concentration (by 310 mg VSS/L). The biomass contained *Nitrosomonas* AOB, *Nitrotoga* and *Nitrospira* NOB and other bacteria according to 16S rRNA gene amplicon sequencing; see Roots et al. (2019) for further details.[36]

From days 0 to 335 SBR control was managed with ChronTrol programmable timers (4-circuit, 8-input XT Table Top unit, ChronTrol, San Diego, CA, USA), and from days 336 to the end of the study with code-based programmable logic control (PLC) (Ignition SCADA software by Inductive Automation, Fulsom, CA, USA, and TwinCAT PLC software by Beckhoff, Verl, Germany). Online sensors included the ammo::lyser™ eco + pH ion-selective electrode for ammonium and pH and the oxi::lyser™ optical probe for dissolved oxygen (DO) (s::can, Vienna, Austria). The reactor was operated in IFAS mode aside from days 132 – 314, when mixed liquor wasting was performed for operation in MBBR mode. Temperature was controlled to about 20 °C from days 0 to 951, gradually reduced to 8 °C from days 952 to 1,076 to stress test performance after achieving optimized N removal in Phase 2, and immediately increased back to around 20 °C from days 1,077 to the end of the study, day 1,128.

SBR control from day 0 to 357 consisted of the following fixed cycle lengths resulting in a fixed 9-hour hydraulic retention time (HRT) not including settling or decant:

- 6-L (50% volume) reactor fill (~2 min)
- Intermittently aerated reaction period (270 min)
  - (peak DO 0.2 – 2 mg O_2/L, aeration intervals 8 – 60 min long)
- Settling (40 min)
- 6-L decant (5 min)

The HRT is defined as the reactor volume divided by the flow rate out. In SBR operation the flow rate is intermittent and can be interpreted as the decant volume divided by the cycle time. However, to facilitate comparison to the HRT of plug flow configurations with a separate settling tank, the settling and decant times were not included in the flow rate calculation. In this case, the HRT = $\frac{reactor\ volume}{decant\ volume/reaction\ time} = \frac{12\ L}{6\ L/270\ minutes}$ = 9 hours.

On day 358 ammonia-based control was implemented, wherein the aerated portion of the cycle was terminated when the target effluent ammonium concentration of 2 mg NH_4^+-N/L was reached. SBR control from day 358 to the end of the study, day 1,128, consisted of the following cycle times resulting in a variable 6.2 ± 2.5-hour HRT not including settling or decant:

- 6-L (50% volume) reactor fill (~2 min)
- Anoxic reaction period (no aeration, 20 min)
- Aerated reaction period (variable length: 30 – 500 min)
  - Days 358 – 413: Intermittent aeration (peak DO 1 – 2 mg O_2/L, aeration intervals 8 – 60 min long)
  - Days 414 – 1,128: Low constant aeration (0.05 – 0.2 mg O_2/L)
- Anoxic reaction period (no aeration, 20 – 30 min)
- Settling (30 – 50 min)
- 6-L decant (4 – 5 min)

Reactor Sampling, SRT Control, and Batch Activity Assays

Composite influent and effluent samples (with approximately 24-hour composite times) were collected 3 to 5 times per week and refrigerated at 4 °C after filtration and preservation per

analysis requirements according to Standard Methods.[37] Analyses included total and soluble chemical oxygen demand (COD), total phosphorus, orthophosphate, total and volatile suspended solids (TSS and VSS), alkalinity, total Kjeldahl nitrogen (TKN), ammonium (NH_4^+-N), combined nitrate + nitrite (NO_3^- + NO_2^- = NO_X^--N), and NO_2^--N (one time per week) per Standard Methods.[37] Carrier biomass was scraped off of whole K5 carriers in duplicate once per month and analyzed for total and volatile dry solids per Standard Methods.[37]

The SRT of suspended biomass in the reactor was calculated by accounting for solids losses through both mixed liquor wasting (which occurred only from days 132 – 314, during which the reactor was effectively a MBBR) and in the effluent. The presence of floating biocarriers often prevented effective settling of suspended biomass, so settled solids were occasionally returned from the effluent (composite sampling) tank to the reactor when high suspended SRT values were targeted. In these cases, effluent VSS concentrations were measured from the overflow of the composite sampling tank for use in SRT calculations.

Batch kinetic assays were performed to determine maximum activities of anammox, AOB, and NOB functional groups under non-limiting substrate conditions as previously described[8,38], and maximum activity of AOB and NOB was measured separately for suspended and carrier biomass. See Supporting Information for details.

Nitrogen Isotope Testing

Nitrogen stable isotope testing was performed on days 1,100, 1,112 and 1,128 to estimate the relative contributions of anammox and denitrification to N removal following Wang et al. (2015).[39] Isotopes of $^{15}NH_4^+$, $^{15}NO_3^-$ and $^{15}NO_2^-$ were spiked separately under initially anaerobic conditions (i.e. with no O_2, $^{14}NO_3^-$, or $^{14}NO_2^-$ present), with $^{14}NH_4^+$ already present in solution, to quantify the percent contribution of anammox and denitrification by measuring the relative

amounts of $^{29}N_2$ and $^{30}N_2$ produced, respectively. Further details can be found in the Supporting Information.

Biomass Sampling, DNA Extraction, qPCR and 16S rRNA Gene Sequencing

Suspended (floccular) and carrier (biofilm) biomass was sampled once or twice per month for 16S rRNA gene sequencing analyses. Suspended biomass was washed with Tris-EDTA buffer before archiving at -80 °C, and whole K5 biocarriers were sampled and archived directly at -80 °C. DNA extraction was performed in duplicate with the FastDNA SPIN kit for soil (MPBio, Santa Ana, CA, USA) per the manufacturer's instructions, and DNA concentration of the extracts was measured via 260 nm wavelength light absorption on an Eppendorf BioSpectrometer® fluorescence (Hauppauge, NY, USA).

Quantitative polymerase chain reaction (qPCR) was used to quantify anammox in carrier biomass via the hydrazine synthase (*hzsA*) gene. The 1597f/1829r primer set was used with reaction conditions described in Harhangi et al. (2012)[40] *HzsA* gene copy numbers were normalized to ng DNA of the extracts.

For 16S rRNA gene sequencing, the V4–V5 region of the universal 16S rRNA gene was amplified on biological replicates for each sample via the 515F-Y/926R primer set[41] as previously described.[42] Raw sequence reads were deposited in GenBank with accession number PRJNA599569. Further details on biomass sampling, qPCR, and 16S rRNA gene sequencing can be found in the Supporting Information.

Results and Discussion

Phase 1 Reactor Performance

In the first 78 days of reactor operation, after biomass inoculation from a sidestream deammonification process on day 0, good performance of 69% total inorganic nitrogen (TIN) removal was observed (Figure 1). After day 78, effluent NO_3^- concentrations increased and TIN removal reduced to an average of 46% over Phase 1. This reduction in N removal performance after day 78 coincided with an increase in the relative abundance of *Nitrospira* NOB (Figure 2 A&B) on both the carriers and in the suspended biomass according to 16S rRNA gene sequencing. Given the advantages that high N concentrations can lend to NOB suppression (i.e. elevated free ammonia), the challenge presented by dilute wastewater in this study (Table 1) was only exacerbated by dilution from frequent wet weather events (see the variable influent NH_4^+ concentration in Figure 1-C, shown as a 2-week rolling average). Various efforts at NOB remediation during Phase 1 (outlined below) proved unsuccessful, and TIN removal did not improve until part of the influent was routed around the A-stage carbon removal reactor at the beginning of Phase 2 (day 900).

Due to the dilute in-reactor NH_4^+ concentrations (usually between 1 and 10 mg NH_4^+-N/L) and moderate pH values (7.2 ± 0.5), in-reactor free ammonia concentrations were likely insufficient to suppress NOB (which were predominantly *Nitrospira*). Using average in-reactor values (5 mg NH_4^+-N/L and 7.2 pH) and an acid-disassociation constant pK_a for NH_4^+/NH_3 of 9.25,[43] the average free ammonia concentration was 0.045 mg NH_3-N/L. This value is on the lower end of the free ammonia inhibition range for *Nitrospira* reported by Blackburne et al. (2007)[44] and is nearly two orders of magnitude below the inhibition range for *Nitrospira* reported by Simm et al. (2006).[45]

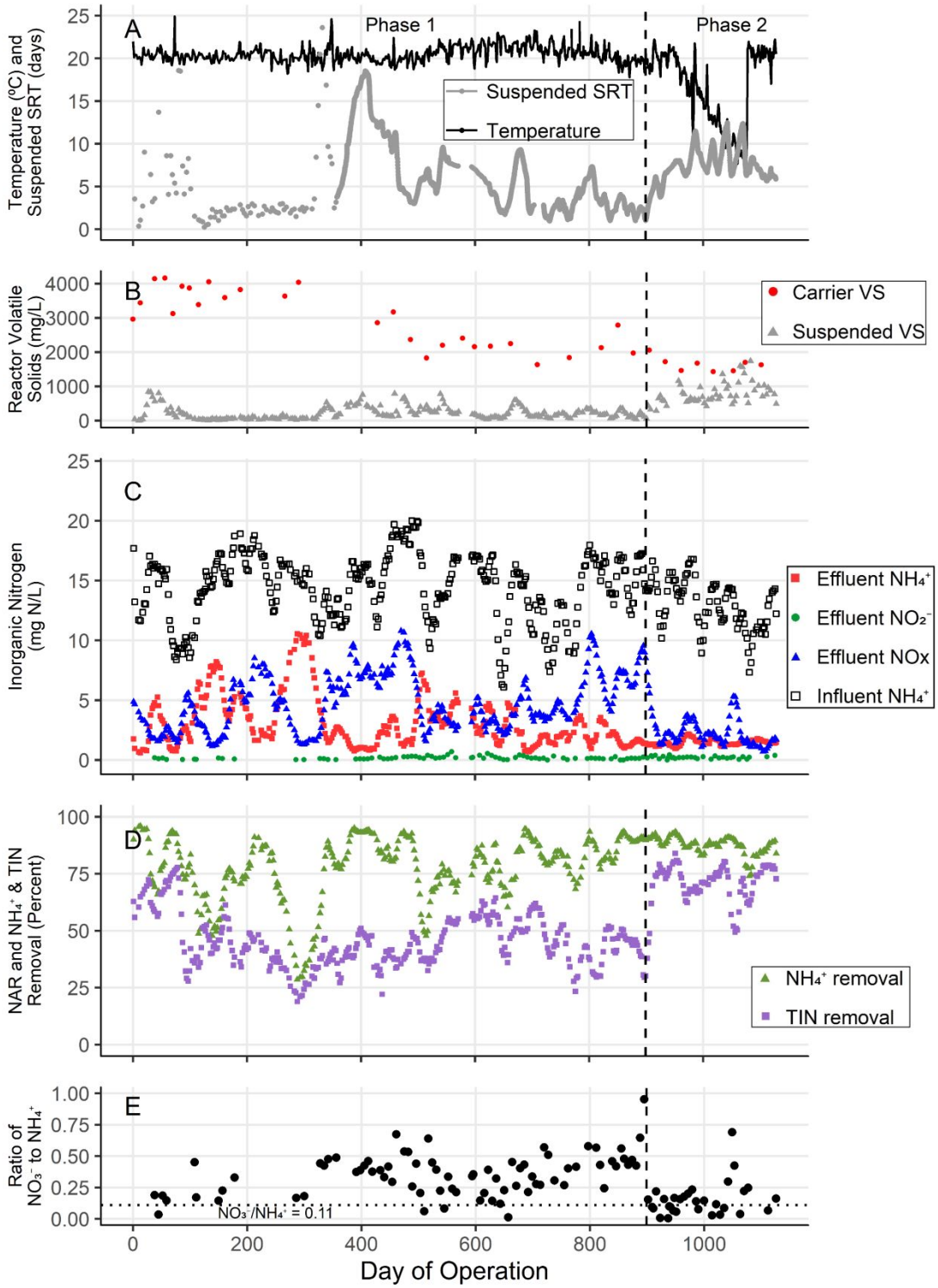


Figure 1. Reactor temperature and performance over 1,128 days of reactor operation. **A:** Average daily reactor temperature and suspended SRT. The higher frequency of SRT data points beginning on day 358 is due to implementation of variable reaction length based on the online ammonia sensor, at which point the SRT value was calculated on a per-cycle basis. **B:** Reactor volatile solids (VS) on the carriers and in

the suspension. **C:** Influent NH_4^+ and effluent NH_4^+, NO_X (NO_2^- + NO_3^-) (all shown as ~2-week rolling average) and NO_2^- (shown as discrete measurements) concentrations as measured from composite sampling. **D:** NH_4^+ and total inorganic nitrogen (TIN) removal (both shown as ~2-week rolling averages) calculated from composite sampling measurements. **E:** Ratio of NO_3^- produced to NH_4^+ removed as calculated from composite sampling measurements. The ratio of 0.11, shown in the graph, represents the theoretical combined stoichiometry of nitritation-anammox as reported in Vlaeminck et al. (2012).[46]

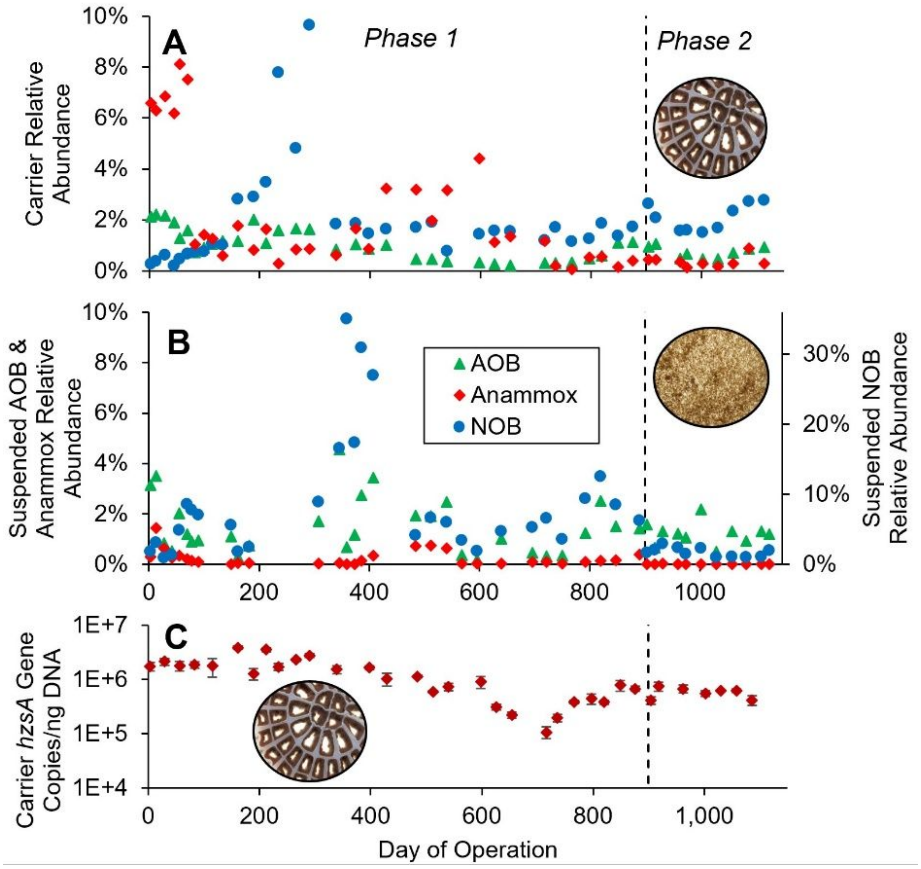


Figure 2. Relative abundance of key functional groups in the attached growth biomass (panel A) and suspended growth biomass (panel B) according to 16S rRNA gene sequencing. Genera detected included *Nitrosomonas* for AOB, *Nitrospira, Nitrolancea* and *Nitrotoga* (in order of abundance) for NOB, and *Candidatus* Brocadia for anammox. Panel C shows the anammox hydrazine synthase (*hzsA*) gene copy number from carrier biomass according to qPCR, normalized to ng DNA.

Table 1. Average reactor influent and effluent concentrations over the two phases.

	Phase 1: 0 - 899 d		Phase 2: 900 - 1128 d	
	Influent (A-stage effluent)	Reactor Effluent	Influent (90% A-stage 10% PE)	Reactor Effluent
TKN (mgN/L)	16.5 ± 5.0	4.4 ± 3.3	14.1 ± 4.1	2.3 ± 0.8
NH_4^+ (mgN/L)	14.4 ± 4.1	3.2 ± 2.7	12.9 ± 3.6	1.5 ± 0.5
NO_X^- (mgN/L)[a]	0.4 ± 0.5	4.9 ± 2.9	0.3 ± 0.2	2.1 ± 1.5
NO_2^- (mgN/L)[b]	*not measured*	0.2 ± 0.1	*not measured*	0.2 ± 0.1
Total COD (mgCOD/L)	45 ± 30	27 ± 14	56 ± 17	32 ± 13
Soluble COD (mgCOD/L)	33 ± 13	21 ± 8	40 ± 12	26 ± 11
sCOD:NH_4^+ (gCOD/gN)	2.3	*not applicable*	3.1	*not applicable*
alkalinity (meq/L)	4.7 ± 0.5	3.4 ± 0.6	5.0 ± 0.6	4.1 ± 0.6

Values shown as arithmetic mean ± standard deviation
[a]*$NO_X^- = NO_2^- + NO_3^-$*
[b]*NO_2^- was measured less frequently (n = 110) than NH_4^+ and NO_X^- (n = 425)*

Long term retention of robust anammox activity under low-concentration mainstream conditions

N removal performance issues during Phase 1 were related to excess NO_3^- production, and low TIN removal was not associated with low anammox activity. Retention of anammox biomass and activity was robustly maintained in this reactor for more than three years of operation in dilute mainstream conditions (average 16 mg TKN/L in the influent). After inoculation of the seeded K5 biocarriers on day 0 from a sidestream process with elevated ammonia concentrations, maximum anammox activity gradually declined as the biomass adapted to mainstream conditions (Figure 3). By day 435 the maximum activity had stabilized to an average of 129 ± 28 mgN/L/d (days 435 – 937, before the temperature decline), almost double the average N loading rate of 67 mgN/L/d over the same period. qPCR measurements of anammox abundance confirmed the initial decline in anammox on the carriers followed by long-term maintenance to >10^5 copies of the *hzsA* gene per ng DNA (Figure 2C). In contrast, 16S

rRNA gene sequencing suggested a greater decline in the only detected anammox genus of *Candidatus* Brocadia (Figure 2A), though it has been noted that so-called universal 16S rRNA primer sets underrepresent anammox and Planctomycetes in general.[40] Supplemental anammox biomass was added only once on day 849 in an attempt to increase N removal, but this had a minor impact on anammox activity and abundance and was not required to maintain process performance. In contrast, suspended or granular systems for mainstream deammonification have moved towards ongoing bioaugmentation from sidestream DEMON® processes to maintain anammox activity.[20,47] Taken together, these results indicate that anammox biomass and activity is robust and resilient to long term mainstream conditions, and with appropriate means for biomass retention (here, growth in biofilms on carriers) do not limit performance of mainstream deammonification processes.

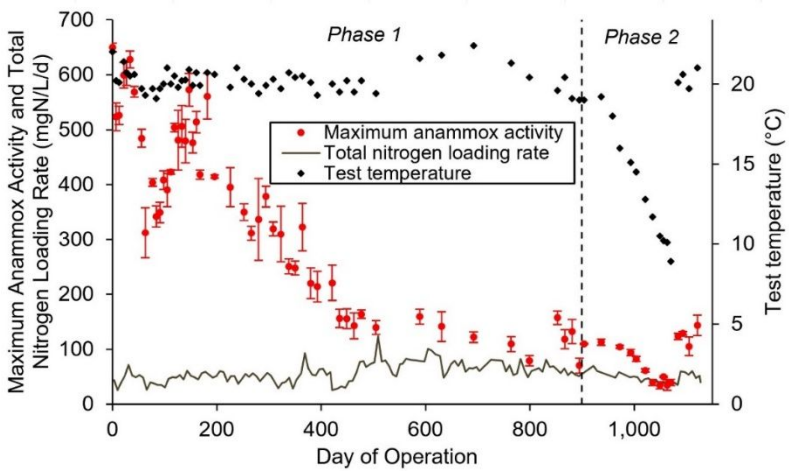
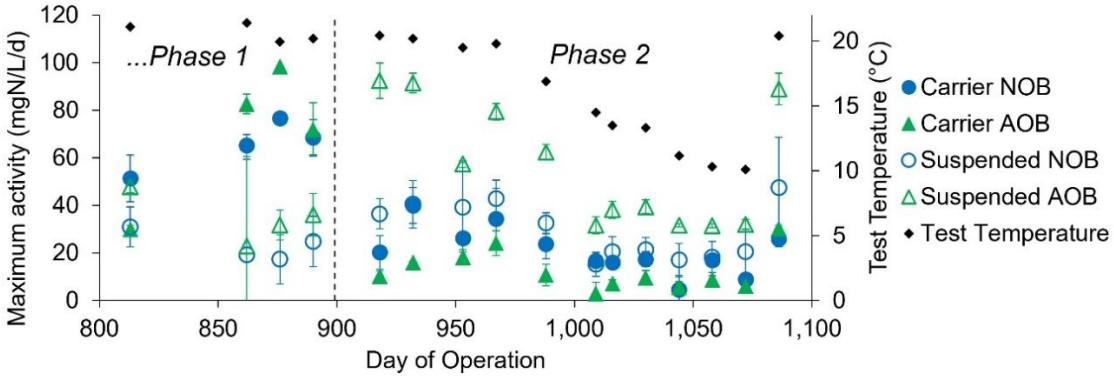


Figure 3. Maximum anammox activity from *in situ* activity tests along with associated test temperatures. The reactor total N loading rate was calculated as a weekly average.

Performance Improvement after Addition of 10% PE in Feed and Nitrifier Shift from Carriers to Suspension

On day 900 of operation, or the beginning of Phase 2, two operational changes were made: (1) 10% of the influent volume was sourced directly from the primary effluent (by bypassing the A-stage reactor) in order to increase the influent sCOD:NH_4^+-N ratio from 2.3 to 3.1 (Table 1) and (2) a one-time addition of 310 mg/L as VSS of suspended biomass was added from a bench-scale nitritation-denitritation reactor (as described in [36]) in an effort to increase AOB abundance and VSS concentration. TIN removal performance immediately increased and was sustained throughout Phase 2 (days 900 – 1,128) at an average of 73%, an improvement from 46% TIN removal during Phase 1 (Figure 1). Interestingly, an increase in maximum nitrification activity in the suspended biomass (Figure 4), while expected due to the increased suspended volatile solids (VS) concentration, was accompanied by a >70% decrease in maximum activity of both AOB and NOB on the carriers between activity tests on days 890 and 918 (Figure 4). Comparing the four maximum activity tests before and four maximum activity tests after day 900 (at 20 °C), both the AOB and NOB activity in the suspension was significantly higher after the influent change (t-test, $p = 0.005$ and 0.008, respectively), and both the AOB and NOB activity on the biofilm was significantly lower (t-test, $p = 0.04$ and 0.002, respectively). This shift of nitrification activity to the suspension ended a months-long domination of nitrification activity on the carriers.



284

285 **Figure 4.** Maximum AOB and NOB activities in the suspended biomass and on the carriers as
286 measured in *ex situ* batch activity assays from day 800 to the end of the project. The entire
287 dataset of AOB and NOB activity measurements is shown in Figure S1.

288

289 Higher influent organic carbon was critical to increasing the suspended biomass
290 concentration to induce a shift of nitrification activity to the suspension and improving N
291 removal. During Phase 1 from days 370 – 460, a strategy of increased suspended SRT and
292 suspended VS was attempted (see in Figure 1-A&B) but with no associated process
293 improvement. In fact, the highest relative abundances of NOB (primarily *Nitrospira*) in the
294 suspended biomass was observed during that time (Figure 2). Despite a high suspended SRT of
295 14 ± 3 days, only 430 ± 158 mg/L of suspended VS was sustained in the reactor, presumably due
296 to low organic carbon in the influent. In contrast, with 10% primary effluent in the influent
297 during Phase 2 (days 900 – 1128), an average of 771 ± 310 mg/L suspended VS was achieved
298 with just a 7.3 ± 2.1 day SRT. This further suggests that the one-time addition of 310 mg VSS/L
299 of suspended biomass on day 900 played a minor role in process improvement compared to the
300 increase in organic carbon in the influent. Ultimately, both the increase in influent organic
301 carbon via the 10% primary effluent bypass and the resulting increased suspended solids
302 concentration contributed to the improved performance of the reactor, and the two metrics are

303 inextricably related. The long-term maintenance of good performance (Phase 2, 228 days)
304 suggests that the particular type of biomass augmented on day 900 was not critical to the
305 improved TIN removal.

306 The difficulty in accumulating high concentrations of suspended solids under low organic
307 carbon loading conditions is corroborated by Laureni et al. (2019);[17] in order to accumulate 3 g
308 TSS/L in their IFAS reactor loaded with pretreated primary effluent at a 2.3 sCOD:NH_4^+-N ratio,
309 a greater than 150-day SRT was required. This very high SRT was achieved via effluent
310 filtration and solids return to the reactor, which is unrealistic under full-scale operation.
311 Achieving high suspended solids to induce a shift of nitrification from the biofilm to the
312 suspension in an IFAS system,[16] then, may require increased organic loading in a full-scale
313 mainstream B-stage process (as in the present study, where a more realistic 7.3 ± 2.1 day SRT
314 was used). Moreover, MBBR systems that experience persistent NOB attachment and activity[15]
315 may benefit from such a transition to IFAS mode.

316 Certain methods for NOB suppression and process improvement that have been suggested in
317 the literature were found, in this study, to be at best only partially effective. The following
318 unsuccessful strategies were attempted during Phase 1 to improve reactor performance:

319 I. **MBBR vs. IFAS**:[6,15,48] The reactor was operated in MBBR mode from days 132 – 314
320 via mixed liquor wasting to facilitate a low suspended SRT of 2.1 ± 0.5 days and
321 suspended VS concentration of 63 ± 32 mg/L (Figure 1-A&B). While NOB were washed
322 out of the suspension, NOB activity proliferated on the carriers (Figure S1) and N
323 removal did not improve (Figure 1-D). IFAS mode was utilized from day 315 to the end
324 of the study.

II. **Aeration regime**: On day 414 the aeration regime was switched from intermittent aeration[49,50] with peak DO of 1 mgO_2/L to low constant aeration[8] to 0.05 – 0.2 mg O_2/L. N removal did not significantly improve (Figure 1-D), but low constant aeration was sustained for the remainder of the project due to simplicity of operation.

III. **Anammox bioaugmentation**: Ongoing anammox bioaugmentation has been proposed for suspended and granular mainstream deammonification processes.[20,47] To test this strategy in our IFAS process as a means to increase anammox biomass/activity and aid NOB out-competition, on day 849 K5 carriers from the Egan WRP sidestream ANITA™ Mox process were added up to a fill ratio of 38%. However, the biofilms were thin, with average volatile solids of 6.6 ± 2.4 mg/carrier, compared to 20.4 ± 3.9 mg/carrier for the original carriers from James River (measurements averaged over days 849 – 1100), and their effect on total anammox activity was minimal (Figure 3). No significant change in N removal or NOB suppression was observed following the addition of carriers (Figure 1-D&E).

IV. **Higher residual NH_4^+ concentrations:** Residual NH_4^+ has been shown to favor AOB activity over NOB activity.[51,52] Higher effluent NH_4^+ concentrations were therefore targeted on days 100 – 200 and 250 – 300 (see Figure 1.C). These varying effluent NH_4^+ concentrations in turn resulted in varying effluent NO_3^- concentrations, but the trend of low TIN removal (Figure 1.D) and high ratios of NO_3^- produced to NH_4^+ removed (Figure 1.E) due to NOB activity remained throughout Phase 1 after day 78**.**

Reactor Performance during the Phase 2 Temperature Decline

Robust N removal and anammox activity was demonstrated at 20 °C for 50 days after initiation of the A-stage bypass. To test process resilience under temperature stress typical of

348 temperate climates, the temperature was decreased from 20 °C on day 950 down to 8 °C on day
349 1,076 (about -0.7 °C per week). Good TIN removal of 72 ± 9% was sustained from days 950 to
350 day 1,044 down to around 12 °C, and dropped to 58 ± 18% for the remainder of the temperature
351 decline due to higher effluent NO_3^- (days 951 – 1076, 12 °C to 8 °C, Figure 1). 12 °C was also
352 the temperature at which the *ex situ* maximum anammox activity dropped below the N loading
353 rate to the reactor (Figure 3), suggesting that the lower specific anammox activity may have
354 limited N removal. A longer suspended SRT of 8.7 ± 1.7 days was maintained during the
355 temperature decline to facilitate a higher biomass concentration (Figure 1) to prevent long
356 reaction times caused by low metabolic rates at low temperatures. This strategy proved
357 successful, as the HRT of 7.6 ± 2.3 hours during the temperature decline was roughly equivalent
358 to the HRT of 7.5 ± 2.5 hours before (days 1 – 949, see Figure S2). The higher biomass
359 concentration also likely provided additional COD for denitrification via endogenous decay, thus
360 aiding N removal at low temperatures. The overall good performance of this reactor is
361 corroborated by other mainstream deammonification studies that have demonstrated the
362 resiliency of surface-attached anammox biofilms and process performance down to 10 °C,[14,53]
363 with superior performance at low temperatures compared to suspended/granular anammox
364 processes.[14,54]

365 To estimate the effect of temperature on anammox activity, maximum activity assays were
366 performed throughout Phase 2 between 21.0 and 8.9 °C to match the concurrent operating
367 temperature (Figure 3). An activation energy E_a of 71 ± 8 kJ/mol was calculated from a least-
368 squares linear regression of the Arrhenius plot of Phase 2 activity tests (Figure S3). This result is
369 not a direct measure of temperature sensitivity because the tests were performed over a seven-
370 month period and may reflect temperature adaptation and population shifts in the community;

however, it does allow a comparison point for anammox activation energies measured in the literature. 16S rRNA gene sequencing identified *Candidatus* Brocadia as the only known anammox genera in our reactor. Our activation energy of 71 ± 8 kJ/mol closely matches the 70 kJ/mol activation energy measured by Strous el al. (1999)[55] for *Candidatus* Brocadia anammoxidans, though that was measured with a temperature range of 20 – 43 °C. Lotti et al. (2015)[56] found that the Arrhenius coefficient of anammox increased with decreasing temperature, though temperature sensitivity was least pronounced in granular biomass dominated by *Candidatus* Brocadia fulgida with a 6 month-long cultivation at 10 °C, with activation energies of 61 kJ/mol at 15 – 20 °C and 95 kJ/mol at 10 – 15 °C. This and other research[57] demonstrates the importance of adaptation time for optimal anammox activity at low temperatures.

After the expected decline in anammox activity at low temperatures, recovery after resuming operation at 20 °C was rapid (Figure 3). The maximum activity test on day 1,084, seven days after the temperature increase, showed 123 mgN/L/d of anammox activity, close to the average of 129 ± 28 mgN/L/d from days 435 – 937, before the temperature decline. The average of the last four activity tests at 20 °C was 125 ± 16 mgN/L/d.

Community analysis via 16S rRNA gene sequencing and qPCR

Aggregate type significantly influenced population structure in this study; an analysis of similarities (ANOSIM) test on genus-level 16S rRNA gene sequencing data revealed a statistically significant difference between carrier and suspended biomass samples ($R = 0.78$, $p = 1E\text{-}4$). An accompanying non-metric multidimensional scaling (NMDS) ordination is shown in Figure S4. Influent carbon also greatly impacted community structure, as further ANOSIM tests revealed statistically significant differences between Phases 1 and 2 carrier samples ($R = 0.30$, p

394 = 6E-4), and Phases 1 and 2 suspension samples (R = 0.39, p = 1E-4). Together with the
395 performance and activity data between the two phases, this demonstrates that small changes in
396 influent organic carbon can induce significant and lasting changes in N removal and community
397 structure.

398 *Candidatus* Brocadia was the only anammox genus identified in our reactor according to
399 16S rRNA gene sequencing. qPCR demonstrated anammox biomass maintenance on the carriers
400 of $>10^5$ *hzsA* copies/ng DNA throughout the study (Figure 2C). The qPCR trend, which
401 demonstrated a 71% decline in relative abundance between the first four and last four sample
402 dates, roughly paralleled the maximum anammox activity trend, which demonstrated a 78%
403 decline between the first four and last four tests of the study (Figure 3), and a stable plateau over
404 the last ~1.5 years of the study. *Nitrospira* was by far the most abundant NOB present according
405 to 16S rRNA gene sequencing, and its very high abundance in both the suspended and carrier
406 biomass during Phase 1 reflects the challenges in NOB suppression faced during that time.
407 Interestingly, although nitrifier activity on the carriers was suppressed during Phase 2 (Figure 4),
408 neither AOB nor NOB relative abundance on the carriers declined during Phase 2 relative to
409 Phase 1. The average NOB (primarily *Nitrospira*) relative abundance in the suspension was
410 significantly lower during Phase 2 at 1.7 ± 0.6% than Phase 1 at 8.6 ± 8.6% (p = 0.0001), which
411 may be due to increased abundance of heterotrophs with higher COD in the influent during
412 Phase 2. *Nitrosomonas* was the only detected genus of AOB and its presence was consistent in
413 both the suspended and carrier biomass at 1.4 ± 1.0% and 0.9 ± 0.5% relative abundance,
414 respectively, over the entire study.

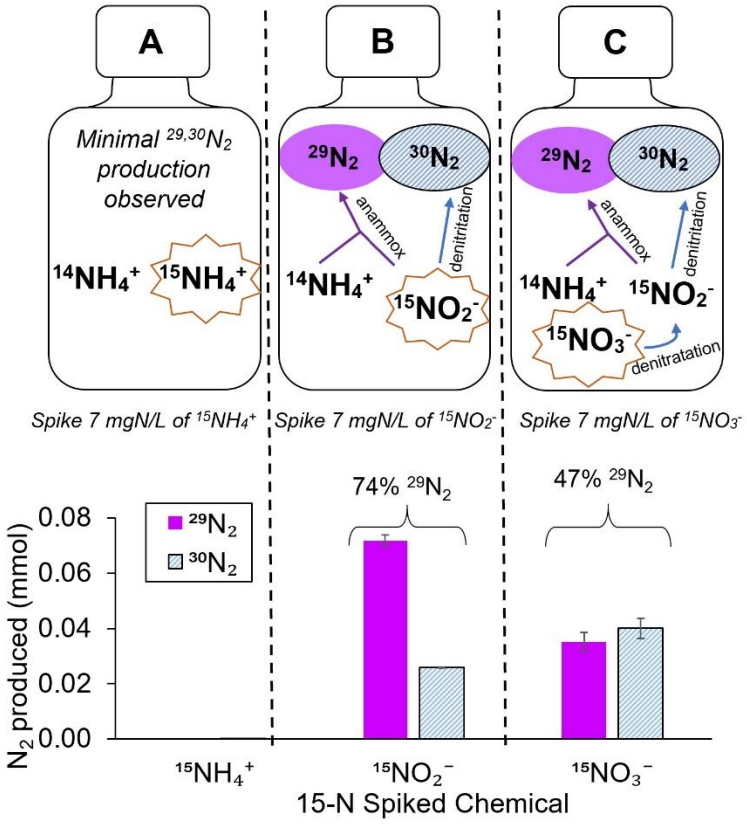
415 Given the importance of denitrification in this process (see next section), the
416 heterotrophic community was essential to nitrogen removal, and indeed comprised most of the

community according to 16S rRNA gene sequencing. On the carriers, the three most abundant amplicon sequence variants (ASVs) were of the class Ignavibacteria, one of which annotated to the genus *Ignavibacterium*, and together comprised an average of 23% relative abundance on the carriers (Figure S6). At least one species of *Ignavibacterium* is a known facultative denitrifier,[58] indicating a likely role in N removal in this reactor. Other abundant heterotrophic genera included "UTCFX1" of the family Anaerolineaceae (3.6%) and *Limnobacter* (2.2%). The classes Ignavibacteria and Anaerolineae have also been found in high abundance in anoxic anammox granules[59] and deammonification biofilms,[60] suggesting a functional role in anammox processes. In the suspended biomass, abundant heterotrophic ASVs included the genus *Trichococcus* (average relative abundance = 3.4%), the family Anaerolineaceae (2.4%), and the genus *Terrimonas* (2.2%), among others. *Trichococcus*, a few species of which are capable of NO_3^- reduction and filamentous growth,[61] were significantly higher during Phase 2 (8.6 ± 5.1%) than Phase 1 (1.5 ± 2.0%, t-test: p = 0.0009). Relative abundance plots of the most abundant genera according to 16S rRNA gene sequencing are shown in Figures S6 and S7.

Quantification of anammox vs denitrification contribution to N removal

Nitrogen isotope testing during Phase 2 revealed that under anoxic conditions approximately 74% of N removal is routed through anammox when only NO_2^- (and not NO_3^-) is present (Figure 5-B). However, in-cycle tests during Phase 2 revealed that NO_3^- was usually, though not always, at higher concentrations than NO_2^- (Figure S5), indicating that denitratation-anammox may play an important role in this process. Indeed, when 15-N labeled NO_3^- was dosed in the N isotope test, 47% was routed through the anammox metabolism (Figure 5-C), suggesting a substantial amount of cross feeding from denitratation to anammox. To translate *ex situ* N isotope tests to an estimation of the *in situ* anammox contribution to N removal, *in* situ concentrations of NO_2^- and

440 NO_3^- are needed. The eight in-cycle tests (Figure S5 shows 2 of these tests) with average
441 temperatures > 19 °C during Phase 2 (nitrogen isotope testing was performed at 23 °C) had an
442 average in-cycle NO_2^- concentration of 22% that of total NO_X^-. N isotope testing indicates that
443 74% of NO_2^- (which comprises 22% of the NO_X^- present) removal is routed through anammox
444 and 47% of the NO_3^- (which comprises 78% of the NO_X^- present) removal is routed through
445 anammox, such that 74%×0.22 + 47%×(0.78) = 53% of N removal occurred via anammox in
446 this process during Phase 2, excluding the temperature decline.



447

448 **Figure 5.** Results from nitrogen isotope testing, which was performed to quantify relative
449 contributions of anammox (which produce $^{29}N_2$ in this test) and denitrification (which produce
450 $^{30}N_2$) to N removal. The testing schematic is shown in the top panel and results from day 1,128
451 are shown on the bottom panel.

452

Nitrogen isotope testing was only performed during Phase 2 with higher organic carbon in the influent than during Phase 1, so while N removal was lower during Phase 1, a greater proportion was likely routed through anammox. Conversely, it is likely that more N removal was routed through denitrification during the temperature decline. Indeed, only when the reactor temperature dropped below 13 °C did the maximum anammox activity drop below the N loading rate to the reactor (Figure 3), although good N removal performance was maintained for most of this period (Figure 1-D). An increased proportion of denitrification was likely possible due to the increased suspended solids concentration during this time (Figure 1-B) and organic carbon from endogenous decay.

While an excess of organic carbon can lead to anammox failure from out-competition for NO_2^- by denitrifiers,[32] at least some anammox organisms, such as *Candidatus* Brocadia fulgida and *Candidatus* Anammoxoglobus propionicus, can use acetate or propionate to reduce NO_3^- to NO_2^-.[62,63] The addition of organic carbon to an anammox process, therefore, does not necessarily imply increased activity of heterotrophic denitrifiers over that of anammox.

A note on terminology: it would be a metabolic oversimplification to call this process "partial nitritation/anammox" (because nitratation/denitratation was demonstrated via in-cycle NO_3^- concentrations and isotope testing) or "partial denitrification/anammox" (because of the likely presence of nitritation/anammox due to transient NO_2^- accumulation observed from in-cycle tests [Figure S5]). The term "simultaneous partial nitrification, anammox and denitrification (SNAD)," as used by Zheng et al. (2016)[19] is the most general and closest to the truth, but "deammonification" has been chosen for simplicity with the stipulation that NOB and denitrification play key roles in the process.

Implications for Practice

The quantity of organic carbon relative to N in the influent is critical to the success of anammox processes, as too much can lead to the suppression of anammox[28,32] and too little can limit the overall N removal[6,29,31]. However, adjusting the influent COD:N ratio at full scale remains challenging. This study demonstrates a practical means for tuning the influent COD:N ratio of any anammox reactor with an upstream A-stage carbon removal process. Although the ratio of rerouted primary effluent to total reactor influent was fixed at 10% in this study, this ratio could be adjusted to optimize N removal performance of a given process. Moreover, this study demonstrated the importance of the COD:N ratio in tailoring aggregate type in mainstream deammonification processes, specifically by promoting the accumulation of suspended solids and the shift in nitrification activity from the biofilm to the suspension. This in turn improves our understanding of key controls and underlying mechanisms of IFAS systems for mainstream deammonification applications.

Conflicts of Interest

There are no conflicts of interest to declare.

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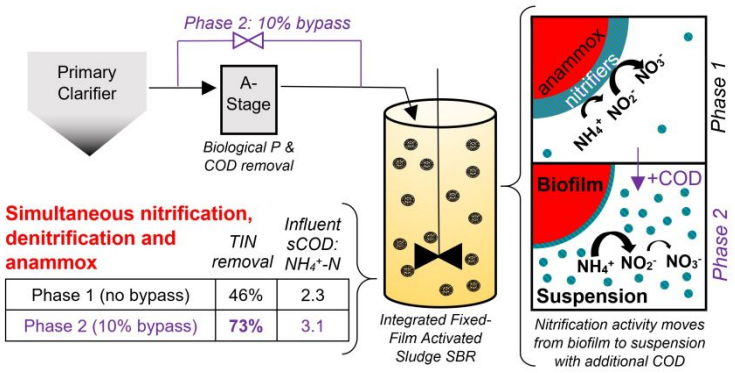

Mainstream deammonification performance in an Integrated Fixed Film Activated Sludge (IFAS) reactor improved from 46% to 73% TIN removal after routing 10% of the primary effluent around the A-stage reactor.