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Pushing the limits of solids retention time for enhanced biological phosphorus removal: process characteristics and Accumulibacter population structure

Paul Roots, Alex Rosenthal, Yubo Wang, Fabrizio Sabba, Zhen Jia, Fenghua Yang, Heng Zhang, Joseph Kozak and George Wells

ABSTRACT

Reducing the solids retention time (SRT) of the enhanced biological phosphorus removal (EBPR) process can increase organic carbon diversion to the sidestream for energy recovery, thereby realizing some of the benefits of the high rate activated sludge (HRAS) process. Determining the washout (i.e. minimum) SRT of polyphosphate accumulating organisms (PAOs), therefore, allows for simultaneous phosphorus and carbon diversion for energy recovery from EBPR systems. However, few studies have investigated the washout SRT of PAOs in real wastewater, and little is known of the diversity of PAOs in high rate EBPR systems. Here we demonstrate efficient phosphorus removal (83% orthophosphate removal) in a high rate EBPR sequencing batch reactor fed real primary effluent and operated at 20 °C. Stable operation was achieved at a total SRT of 1.8 ± 0.2 days and hydraulic retention time of 3.7–4.8 hours. 16S rRNA gene sequencing data demonstrated that Accumulibacter were the dominant PAO throughout the study, with a washout aerobic SRT between 0.8 and 1.4 days. qPCR targeting the polyphosphate kinase gene revealed that Accumulibacter clades IIA, IIB and IID dominated the PAO community at low SRT operation, while clade IA was washed out at the lowest SRT values.

Key words | A-stage, *Candidatus* Accumulibacter phosphatis, enhanced biological phosphorus removal (EBPR), high rate activated sludge (HRAS), polyphosphate accumulating organisms (PAO), wastewater treatment

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HIGHLIGHTS

- 84% orthophosphate removal from primary effluent was achieved via enhanced biological phosphorus removal at a 1.8-day solids retention time.
- The washout aerobic solids retention time of Accumulibacter polyphosphate-accumulating organisms was between 0.8 and 1.4 days at 20 °C.
- Short solids retention time operation selected for Accumulibacter clades IIA, IIB and IID.
- Sensor-based PID control of aeration was critical to successful phosphorus removal.

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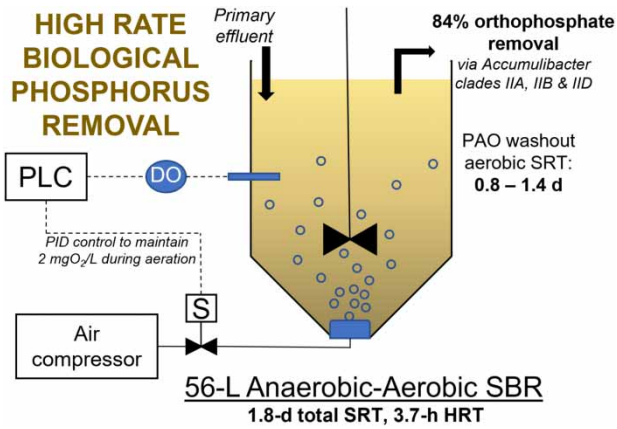
GRAPHICAL ABSTRACT



INTRODUCTION

Despite the large amount of energy typically used to treat wastewater, estimated at 3.2 kJ/g chemical oxygen demand (COD), the theoretical chemical energy contained in typical domestic sewage, 16.2 kJ/g COD, is about 5 times that amount (Wan *et al.* 2016). The high-rate activated sludge (HRAS) process (Chase & Eddy 1944) aims to reduce mineralization and increase the digestibility of COD diverted to anaerobic digestion by minimizing both HRT and SRT, thereby minimizing footprint and maximizing carbon diversion to sidestream anaerobic digestion for energy recovery (Ge *et al.* 2013). While the HRAS process has been widely applied in mainstream wastewater treatment, little research has focused on incorporating enhanced biological phosphorus removal (EBPR) into high rate A-stage processes. The potential for simultaneous recovery of COD and phosphorus (P) makes high rate EBPR processes an attractive option. It should be noted that the minimum SRT required to retain polyphosphate accumulating organisms (PAOs) in a high rate EBPR process may disqualify it as a true HRAS process, which typically operates with SRT < 1 day. Nonetheless, evidence has shown that a relatively low SRT (around 3 days) in an EBPR process achieved a higher biomass yield than with more typical SRTs of 10–20 days (Chan *et al.* 2017) and, by implication, higher energy recovery in anaerobic digestion. High rate A-stage EBPR in series with a B-stage autotrophic partial nitritation/anammox (PN/A) process for N removal could therefore enable energy efficient total nutrient removal (Gao *et al.* 2014). However, because conventional EBPR processes are typically operated with SRTs around 10 days or higher, knowledge about performance characteristics, population structure, and the limits of SRT for PAOs in high rate systems is limited.

Of the existing studies on low sludge age EBPR, many were conducted in conditions atypical of mainstream wastewater: Ge *et al.* (2015) established an optimal SRT of 2–2.5 days (aerobic SRT [SRT_{aer}] = 1.2–1.5 days) for P removal from a strong abattoir waste stream at a temperature of 20–22 °C, and Valverde-Pérez *et al.* (2016) found an optimal SRT of 3 days (SRT_{aer} = 1.5 days) at 16–19 °C for P removal from a primary effluent stream to which propionate had been added to simulate primary fermentation. Chan *et al.* (2017) found that EBPR performance was maintained at an SRT of 3.6 days (SRT_{aer} = 2.1 days) when treating synthetic wastewater at 25 °C, and Mamais & Jenkins (1992) found an optimal SRT of 2.3–2.6 days (SRT_{aer} = 1.7–2 days) at 20 °C in a bench scale process using primary effluent supplemented with acetate. Only a few studies have used real wastewater without supplemental carbon to investigate low SRT EBPR, which limits our understanding of the boundaries of SRT under real world conditions: Yang *et al.* (2017) used a 2–3 day SRT (SRT_{aer} = 1.5–2.25 days) for successful EBPR at 16–24 °C in an A-stage sequencing batch reactor (SBR) treating primary effluent, McClintock *et al.* (1993) demonstrated good EBPR at an SRT of 2.7 days (SRT_{aer} = 1.5 days) at 20 °C in a pilot scale process, and Shao *et al.* (1992) found an optimal SRT of 3 days (SRT_{aer} = 2.8 days) at 23 °C in a full scale process. Three of the above studies preceded the identification and classification of Accumulibacter (Hesselmann *et al.* 1999), and

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none of the more recent studies that selected for Accumulibacter investigated clade-level population dynamics. Fourteen clades of Accumulibacter have been identified (Mao *et al.* 2015; Camejo *et al.* 2016), and it remains to be investigated if low SRT operation exerts a selective pressure that leads to dominance of specific clades.

Depending upon effluent end-use, an additional motivation to maintain low SRT in the high rate EBPR process is to prevent nitrification by washout of ammonia and nitrite oxidizing bacteria (AOB and NOB). This is the case in the present study, where the effluent of the high rate EBPR (A-stage) reactor fed a mainstream PN/A (B-stage) process that benefits from high ammonia and low COD concentrations. Such an A-B process has promise as an energy saving method for total nutrient removal, with no exogenous chemical additions required. However, growth parameters indicate that maintaining an SRT that retains PAOs and expels nitrifiers may be difficult: typical reported maximum growth rates for AOB and NOB at 20 °C are 0.76 and 0.81 d^{-1} (Rittmann & McCarty 2001), respectively, which corresponds to a theoretical minimum SRT_{aer} of 1.2–1.3 days. While this disregards microbial decay and variable reactor conditions, it clearly indicates that selectively washing out nitrifiers while maintaining PAOs will be difficult in practice where precise SRT control is a challenge.

This study addresses the knowledge gap of low sludge age EBPR performance and PAO population diversity in real mainstream wastewater without VFA addition. Our specific objectives were to determine the limits of SRT and HRT for robust EBPR, and to evaluate if low sludge age impacts clade level diversity of Accumulibacter. Additional attention was paid to conditions that allowed for simultaneous P removal and nitrification suppression.

MATERIALS AND METHODS

Reactor operation

A 56-L working volume sequencing batch reactor (hereafter called 'reactor') was enriched with suspended growth biomass intended to facilitate high rate COD and biological P removal. The reactor was inoculated with EBPR biomass from the James C. Kirie Water Reclamation Plant (WRP) of the Metropolitan Water Reclamation District of Greater Chicago (MWRD) on May 2, 2016 (day 0). The James C. Kirie WRP operated with an 8-day average SRT in April 2016. The reactor was then fed with primary effluent (PE) (Table 1) of the Terrence J. O'Brien WRP of the MWRD and temperature controlled to 19.7 ± 1.2 °C (average ± standard deviation over the entire study). SBR operation initially consisted of a ≈3-minute gravity fill, 45 minutes of anaerobic mixing, a 135-minute aerobic reaction phase, 30–40 minutes settling (0.41 m/h critical settling velocity), and 62.5% volume decantation (≈5 minutes), yielding an estimated 4.8-hour hydraulic retention time (HRT) excluding settling and decant. On day 416, reaction times were shortened to 35 minutes of anaerobic mixing and 105 minutes of aerobic

Table 1 | Reactor influent (O'Brien WRP primary effluent) average values over the entire study and reactor effluent values averaged over the three phases

	Influent (PE)		Reactor effluent		
	Days 0–499	Number of samples	Phase 1: Days 0–357	Phase 2: Days 358–486	Phase 3: Days 487–499
Total phosphorus (mgP/L)	2.5 ± 0.7	250	1.3 ± 1.2	0.55 ± 0.77	1.5 ± 0.5
Orthophosphate (mgP/L)	1.9 ± 0.6	186	1.0 ± 0.7	0.31 ± 0.22	1.3 ± 0.3
Total nitrogen (mgN/L)	21 ± 5	184	17 ± 5	17 ± 4	23 ± 1
NH_4^+ (mgN/L)	15 ± 4	184	14 ± 4	15 ± 4	20 ± 1
COD (mgCOD/L)	143 ± 45	249	44 ± 34	32 ± 10	50 ± 11
sCOD (mgCOD/L)	85 ± 23	249	30 ± 11	27 ± 8	41 ± 10
rbCOD (mgCOD/L)	39 ± 25	27	NA	NA	NA
TSS (mg/L)	44 ± 23	59	17 ± 38	7 ± 5	18 ± 0
Reactor HRT (hr)			4.9	4.1	3.8
Reactor SRT (d)			2.2 ± 0.5	1.8 ± 0.2	1.1 ± 0.2
Reactor MLVSS (mg/L)			434 ± 151	528 ± 15	255 ± 73

Arithmetic mean shown with standard deviation.

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reaction, yielding an estimated 3.7-hour HRT excluding settling and decant.

SBR control of reactor equipment from inoculation to day 358 was managed with on-off circuit switching via ChronTrol programmable timers (4-circuit, 8-input XT Table Top unit, ChronTrol). Aeration prior to day 358 was controlled manually by throttling a rotameter downstream of an air compressor, with a target dissolved oxygen (DO) concentration of 2–4 mg O_2/L. Starting on day 358 and continuing to the end of the study (day 499), reactor equipment was controlled with code-based Programmable Logic Control (PLC) (Ignition SCADA software by Inductive Automation and TwinCAT PLC software by Beckhoff). Upon PLC implementation, aeration control was switched to proportional-integral-derivative ('PID' is the conventional designation, although derivative control was not utilized in this study) control based on the online oxygen sensor (S::CAN oxi::lyser™ optical probe) signal to target 2 mg O_2/L. For the purposes of this paper, data is split into three time ranges, or phases. Phase 1, which occurred before implementation of PLC control, was an optimization phase to identify ranges of SRT for the dual goals of nitrifier suppression and PAO retention. Because reactor performance improved upon implementation of the PLC system on day 358, Phase 2 (after PLC implementation) was designated as the optimized testing phase. Finally, after allowing for sufficient time to demonstrate good performance at low SRT, Phase 3 was implemented to test the lower limits of SRT for PAO retention. In summary:

- **Phase 1**: days 0–357 before PLC control
  - Description: Optimization phase to identify ranges of SRT for the dual goals of nitrifier suppression and PAO retention.
- **Phase 2**: days 358–486 with PLC control
  - Description: Optimized testing phase.
- **Phase 3**: days 487–499 with PLC control
  - Description: Tested the lower limits of SRT for PAO retention.

SRT control

The goal of SRT control was to maintain PAOs and EBPR activity while washing out nitrifiers and was controlled by wasting a known volume of mixed liquor near the end of the aerobic phase. Mixed liquor suspended solids were measured twice per week and effluent suspended solids were measured once per week, and the overall SRT was calculated as in the following formula:

$$SRT = \frac{X_R V_R}{X_E Q_E + X_R Q_W} \quad (1)$$

where

SRT = Solids retention time (d)
X_R = Volatile suspended solids concentration in the reactor (mgVSS/L)
V_R = Volume of reactor (L)
X_E = Volatile suspended solids concentration in the reactor effluent (mgVSS/L)
Q_E = Effluent flow rate (L/d)
Q_W = Wasting flow rate (L/d)

Q_E and Q_W above were calculated via the reaction time, which excludes settling and decant. The aerobic SRT was calculated as follows:

$$SRT_{aer} = SRT\frac{t_{aer}}{t_C} \quad (2)$$

where (in addition to above)

SRT_{aer} = Aerobic solids retention time (d)
t_{aer} = Aerobic reaction time (min)
t_C = Cycle time excluding settling and decant (min)

P assimilation calculation

P removal via non-EBPR biomass assimilation was estimated following methods detailed in Rittmann & McCarty (2001), as follows:

$$P_{assm} = \frac{0.0267 \times Y(1 + (1 - f_d)b\theta_x)\Delta sCOD}{1 + b\theta_x} \quad (3)$$

where:

P_{assm} = P removed through non-EBPR biomass assimilation
0.0267 = Typical ratio of phosphorus mass to volatile dry mass of activated sludge
Y = typical biomass yield for ordinary heterotrophs, 0.46 mgVSS/mgCOD
f_d = biodegradable fraction of new biomass, 0.8
b = endogenous decay rate, 0.1 d^{-1}
θ_x = solids retention time, days
$\Delta sCOD$ = soluble COD removal, mgCOD/L (as a proxy for BOD removal)

Reactor sampling

Total and soluble COD, total suspended solids (TSS), volatile suspended solids (VSS), alkalinity, total and soluble

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Kjeldahl nitrogen (TKN, sTKN), NH_4^+-N, combined $NO_3^- + NO_2^-$-N (NO_X-N), total P, and orthophosphate were monitored 3–5 times/week in influent and effluent daily composite samples as per *Standard Methods* (APHA 2005). Volatile fatty acids (VFA) in the influent were not measured during the study period, but later measurements of the same influent revealed an average VFA:sCOD ratio of 0.16 ± 0.07 ($n = 143$, measured October 2017–May 2019). This translates to an estimated influent VFA concentration of 14 ± 6 mg VFA-COD/L during this study.

In situ batch activity assays

In-cycle tests to observe phosphate and carbon dynamics were performed on days 65, 79, and weekly to bi-weekly after day 253. No chemical dosing occurred before or during the tests in order to observe typical *in situ* rates of carbon removal and phosphate uptake and release. Grab samples were taken every ~11 minutes during the anaerobic reaction period and every ~20 minutes during the aerobic reaction period and analyzed for orthophosphate and (for certain tests only) readily biodegradable COD (rbCOD) and VFAs. In-cycle rbCOD was defined as the floc-filtered COD (ffCOD, following the method of Mamais *et al.* (1993)) for a given time point in the cycle minus the ffCOD in the effluent. Phosphate release and uptake rates were measured via a least-squares linear regression of the linear portion ($R^2 > 0.8$) of the phosphate uptake and release curves.

Reactor biomass archiving and DNA extraction

Reactor mixed liquor biomass was archived weekly to biweekly with the following procedure: 6 mL of mixed liquor was pipetted from each reactor and separated into six 1-mL aliquots in 1.5 or 2.0-mL centrifuge tubes. Each tube was centrifuged at 10,000 g for 3 minutes, after which the supernatant was removed and replaced with 1 mL of TE buffer. The tubes were centrifuged again at 10,000 g for 3 minutes, after which the supernatant was removed, leaving only the biomass pellet. All samples were archived at −80 °C. DNA extraction was performed with the FastDNA SPIN Kit for Soil (MPBio), as per the manufacturer's instructions.

16S rRNA gene amplicon sequencing

16S rRNA gene amplicon library preparations were performed using a two-step multiplex polymerase chain reaction (PCR) protocol, as previously described (Griffin & Wells 2017). All PCR reactions were performed using a Biorad T-100 Thermocycler (Bio-Rad, Hercules, CA). The V4-V5 region of the universal 16S rRNA gene was amplified in duplicate from 20 dates collected over the course of reactor operation using the 515F-Y/926R primer set (Parada *et al.* 2016). Thermocycling conditions were 95 °C for 5 minutes, 28 cycles of {95 °C for 30 seconds, 50 °C for 45 seconds, and 68 °C for 30 seconds}, followed by a final extension of 68 °C for 5 minutes. Specificity of amplification was checked for all samples via agarose gel electrophoresis.

All amplicons were sequenced using a MiSeq system (Illumina, San Diego, CA, USA) with Illumina V2 (2×250 paired end) chemistry at the University of Illinois at Chicago DNA Services Facility and deposited in GenBank (accession number for raw data: PRJNA599575). For amplicon sequence analysis, sequence quality control was performed through DADA2 (Callahan *et al.* 2016) integrated in QIIME2 version qiime2–2018.8 (Bolyen *et al.* 2018). Taxonomy was assigned to each unique sequence variant using the Silva database, release 132.

Quantitative polymerase chain reaction (qPCR)

qPCR was used to quantify the relative abundance of the 14 known *Ca.* Accumulibacter clades throughout the study via specific primer sets targeting the polyphosphate kinase (*ppk1*) gene developed by Camejo *et al.* (2016). Total bacterial (universal) 16S rRNA genes were quantified via the Eub519/Univ907 primer set (Burgmann *et al.* 2011). All assays employed 20 µl reaction volumes with thermocycling conditions and primer concentrations reported in the reference papers and were performed on a Bio-Rad C1000 CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA). For the total bacteria 16S rRNA assay, 10 µl of the Bio-Rad SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used. For assays targeting *ppk1* genes, 10 µl of Epicenter FailSafe™ PCR 2X PreMixF (5 µl of 200x SYBR green in DMSO and 2.5 uL of 10% Tween-20 were added to 2.5 mL of PreMix F to facilitate use in qPCR) and 1.25 U of Epicenter FailSafe™ PCR Enzyme Mix (Lucigen Corporation, Middleton, WI, USA) were used. Each sample date was analyzed with 2 technical replicates of 2 biological replicates (total of 4 replicates) for each assay. Standard series were generated in duplicate by tenfold serial dilutions (10^2–10^8 gene copies/µl) using cloned plasmid DNA or synthesized DNA (IDT Inc, Coralville, IA, USA). The amplification specificity of each qPCR assay was verified with melt curve analysis

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and agarose gel electrophoresis. For relative abundance quantification, 4.2 copies/cell of the 16S rRNA gene was assumed for total bacteria (Větrovský & Baldrian 2013) and 1 copy/cell of the *ppk1* gene was assumed for Accumulibacter (Mao *et al.* 2015; Camejo *et al.* 2016).

RESULTS AND DISCUSSION

SRT optimization and reactor performance

Process optimization occurred during Phase 1 to characterize conditions for EBPR to occur with simultaneous nitrifier washout. A total SRT of around 2–3 days (SRT_{aer} around 1.5–2 days) (Figure 1) up to day 120 combined with a high target DO of 4 mg O_2/L in the aerobic phase resulted in $NO_2^- + NO_3^-$ (NO_X) detected in the effluent in days 67–150, with a maximum value of 5.1 mg NO_X-N/L on day 98 (Figure S1, Supporting Information). In order to wash out nitrifiers, on day 121 the target total SRT was lowered to 2.2 days (SRT_{aer} of 1.7 days) and the target DO level was lowered to 2 mg O_2/L, which eliminated NO_X in the effluent by day 150. While NO_X production and recycle to the anaerobic zone can be detrimental to the EBPR process, P removal did not improve upon washout of nitrifiers, and in fact temporarily worsened (Figure 2). During process optimization in Phase 1, P removal was variable (Figure 2), with an average orthophosphate removal of 48% in part due to poor DO control with manual adjustment of air flow.

PLC control of reactor equipment was implemented at the beginning of Phase 2 on day 358, which resulted in much more consistent DO control and an improvement in P removal performance (Figures 2 and 3 for in-cycle profiles). Phase 2, therefore, represented the optimized testing phase after Phase 1 had established ranges of SRT for simultaneous nitrifier suppression and PAO retention. Average orthophosphate and sCOD removal rates during Phase 2 were $83 \pm 17\%$ and $68 \pm 14\%$, respectively. Surprisingly, despite the low SRT_{aer} of 1.6 days at the beginning of Phase 2, nitrification was once again observed via NO_X in the effluent beginning on day 368 (Figure S1). Nitrification during Phase 2 at a lower SRT than Phase 1 may have been possible due to more consistent DO control. To wash out nitrifiers, the SRT_{aer} was reduced to around 1.3 days on day 399 (Figure 1), and by day 415 NO_X was no longer observed in the effluent (Figure S1). The HRT was also reduced to 4.1 hours (from 4.9 hours) on day 399, and eventually to 3.8 hours by day 414 (Figure 1). EBPR performance responded positively to the reductions in HRT and SRT beginning on day 399. The best P removal correlated with these reductions, and average effluent orthophosphate for Phase 2 (days 358–485) was 0.3 ± 0.2 mgP/L. The specific total P removal rate for Phase 2 was 18.3 ± 7.7 mgP/gVSS/d, up from 11.4 ± 9.5 mgP/gVSS/d for Phase 1. The average SRT during Phase 2 was 1.8 ± 0.2 days ($SRT_{aer} = 1.4 \pm 0.2$ days) with a MLVSS of 529 ± 155 mgVSS/L (Figure S2). This low SRT was necessary to wash out nitrifiers, as noted above, and was adequate to maintain stable P removal, as indicated by the low standard deviation ($\pm$ 0.2 mgP/L) in the effluent orthophosphate concentration during Phase 2. Sludge volume index (SVI) tests during Phase 2 indicated good settling performance of the biomass, with an average SVI_{30} of 83 ± 22 mL/g (see Supporting Information for further details).

In Phase 3 (day 487–499), the lower limit of SRT for PAO retention was explored. The average SRT during Phase 3 was 1.1 ± 0.2 days ($SRT_{aer} = 0.80 \pm 0.12$ days, see Figure 1). The resulting mixed liquor concentration was

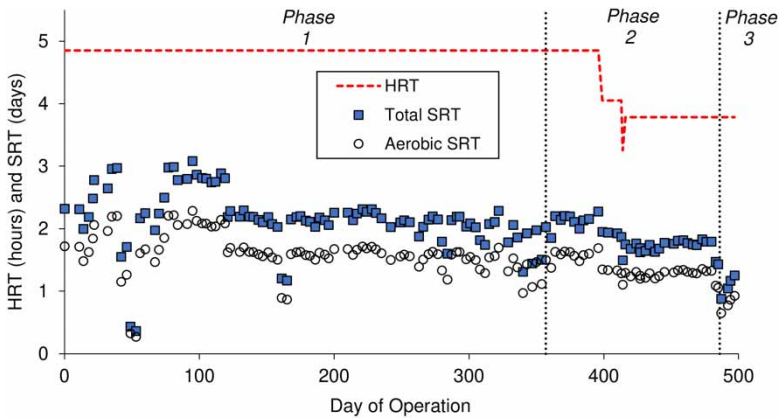

Figure 1 | Hydraulic retention time (HRT), total solids retention time (SRT) and aerobic SRT throughout the study.

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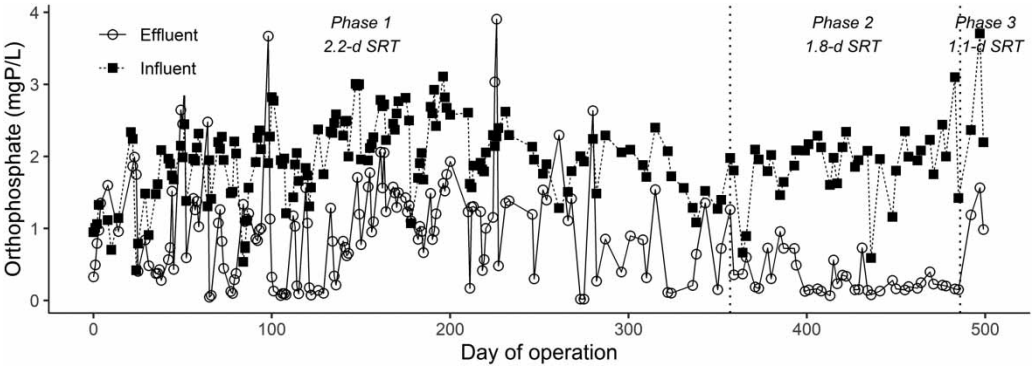

Figure 2 | Reactor influent and effluent orthophosphate from daily composite samples throughout the study.

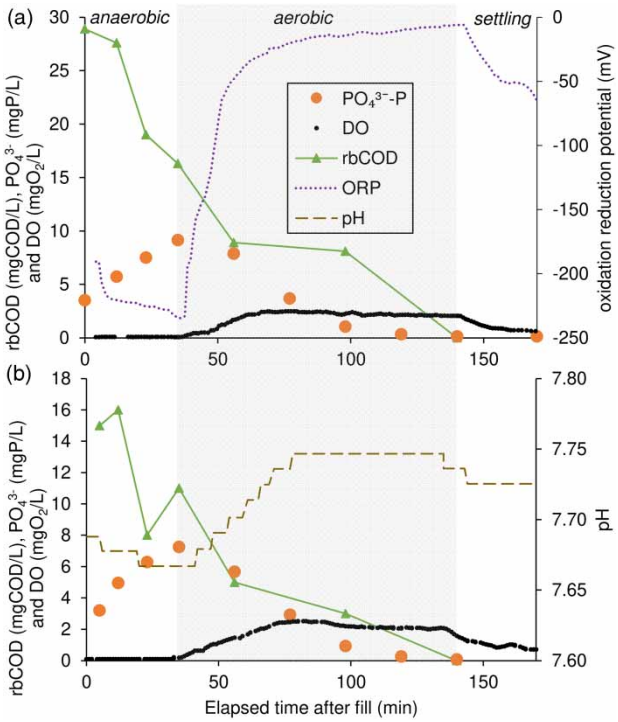

Figure 3 | Two representative in-cycle batch tests from Phase 2, (a) = day 457 and (b) = day 485.

255 ± 73 mgVSS/L, and P removal performance immediately suffered (Figure 2). This demonstrated that the Phase 2 SRT of 1.8 ± 0.2 days (SRT_{aer} of 1.4 ± 0.2 days) was nearing the lower limit of Accumulibacter PAO retention at 20 °C, and that the PAO washout SRT_{aer} lies between 0.8 and 1.4 days.

P removal via EBPR versus biomass assimilation

Because P comprises 2–3% of non-EBPR activated sludge by dry weight (Rittmann & McCarty 2001), some P removal can be attributed to typical biomass assimilation alone. An estimate of P removal from assimilation (Equation (3)) demonstrates that around 38% of orthophosphate removal during Phase 2 could be attributed to non-EBPR biomass assimilation. In other words, 62% of orthophosphate removal occurred via intracellular polyphosphate accumulation, indicating that EBPR was integral to the P removal performance of this process. Further evidence for EBPR activity included the orthophosphate release to anaerobic rbCOD uptake ratio during Phase 2. Although highly variable at 0.43 ± 0.41 g P/g COD ($n = 4$ in-cycle tests without dosing), the average ratio is indicative of the expected ratio from the PAO metabolism of 0.36 g P/g COD (Hesselmann *et al.* 2000) to 0.56 g P/g COD (Smolders *et al.* 1994), though further testing would be required to increase the confidence of our result. It should also be noted that the quoted studies used acetate as the sole carbon source, which may cause a discrepancy in the resulting P/COD ratio. Also measured during in-situ tests were P release and uptake rates (Figure S3), which for Phase 2 were 12.4 ± 4.1 mg P/g VSS/hr and 10.3 ± 4.8 mg P/g VSS/hr, respectively. Two representative in-cycle tests without exogenous dosing (i.e. with a typical fill of primary effluent without supplemental carbon) can be seen in Figure 3.

Accumulibacter vs. GAO abundance from 16S rRNA gene sequencing

Accumulibacter PAOs were highly abundant throughout the study according to 16S rRNA gene sequencing at a 15 ± 7% average relative abundance, while putative glycogen accumulating organisms (GAOs) were highly suppressed with a 0.7 ± 0.6% relative abundance (Figure 4). *Ca.* Competibacter was the most abundant GAO present at 0.4 ± 0.4% relative abundance. Other potential GAOs (Stokholm-Bjerregaard

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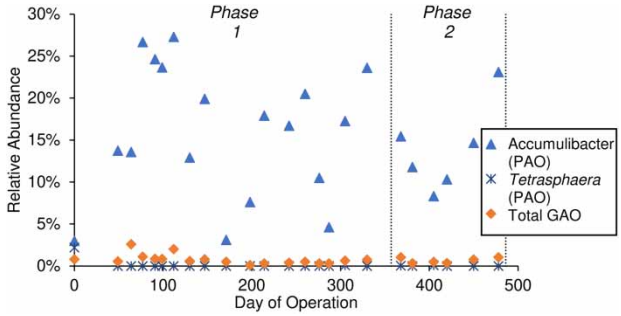

Figure 4 | Relative abundance of PAO and GAO taxonomic groups according to 16S rRNA gene amplicon sequencing. *Ca.* Competibacter was the most abundant GAO identified; other GAOs present at some time points and included in 'Total GAO' above were *Ca.* Contendobacter, *Micropruina*, *Propionivibrio*, and *Defluviicoccus*. Relative abundance of nitrifiers from 16S rRNA gene sequencing is shown separately in Figure S4 (Supporting Information).

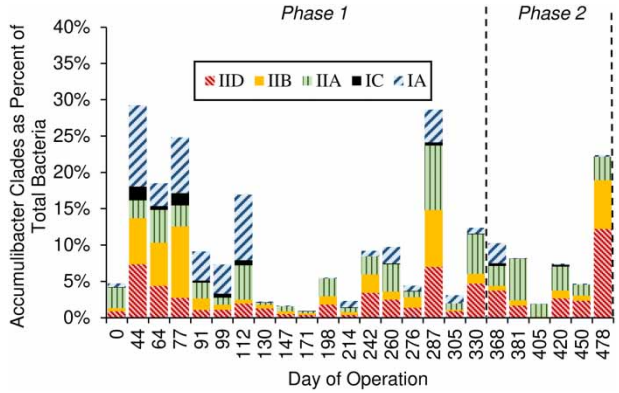

Figure 5 | Abundance of *Ca.* Accumulibacter clades IA, IC, IIA, IIB and IID relative to total bacteria according to qPCR. Clades detected but at negligible relative abundance (<0.3% at all time points) and not shown in this plot include clades IB, ID, IE, IIC and IIF. Standard deviations for clade-specific data points are shown in Figure S5 (Supporting Information).

et al. 2017) identified at certain time points but at low average relative abundance (<0.3%) included *Ca.* Contendobacter, *Propionivibrio*, *Micropruina*, and *Defluviicoccus*. The lack of a significant difference in total Accumulibacter abundance between the two phases of reactor operation (p value = 0.28 on t-test with hypothesis of no difference between means) indicates that the improvement in P removal observed in Phase 2 cannot be explained by better total PAO selection alone. Rather, the improvement in consistent anaerobic/aerobic conditions and DO control as enabled by PLC implementation and sensor-based aeration control at the beginning of Phase 2 likely facilitated consistent P uptake and release by PAOs, as well as selection for PAOs over GAOs and nitrifiers. Careful control of SRT to facilitate nitrifier washout on day 399, however, did correlate with the best P removal observed in Phase 2 (Figures 1 and 2).

Clade-specific Accumulibacter abundance via qPCR

In addition to evaluating the feasibility of very low SRT EBPR in an A-stage process, a key objective of this study was to characterize clade-level diversity of PAOs selected at low SRT. Of the 14 known clades (clades IA-E and IIA-I; Mao *et al.* 2015; Camejo *et al.* 2016), 10 were identified as present at some point during operation. The 5 most abundant clades were IA, IC, IIA, IIB and IID (Figure 5), and the other detected clades (IB, ID, IE, IIC and IIF) were at negligible relative abundances (<0.3% in all samples compared to total bacteria). The sum of the clades present relative to total bacteria via qPCR (average throughout the study = 10 ± 9%) confirmed the high relative abundance of Accumulibacter found via 16S rRNA gene sequencing (average throughout the study = 15 ± 7%). We observed higher clade-level Accumulibacter diversity during Phase 1 of reactor operation, followed by selection for clades IIA, IIB and IID at the lowest SRT values during Phase 2 (or in other words, washout of clades IA and IC, see Figure 5). An analysis of similarities (ANOSIM) statistical test performed in R (R Foundation for Statistical Computing, Vienna, Austria) of Accumulibacter clade-level abundance (relative to total Accumulibacter; that is, the sum of the clades, based on clade-specific qPCR) indicated a statistically significant difference in Accumulibacter population structure between phase 1 and phase 2 of reactor operation ($R = 0.24$, $p = 0.03$). An accompanying non-metric multidimensional scaling (NMDS) plot, using the same data as the ANOSIM test, visually demonstrates the shift in Accumulibacter population structure during reactor operation, as SRT was decreased (Figure 6). The abundances of clades IA and IC relative to total Accumulibacter were statistically significantly positively correlated with SRT ($p = 5E\text{-}6$ and 0.01, respectively); in other words, low SRT was correlated with low type I Accumulibacter relative abundance. The abundances of clades IIA, IIB and IID relative to total Accumulibacter were all negatively correlated with SRT (i.e. higher relative abundance at lower SRT values), but this correlation was only statistically significant for clade IID ($p = 0.008$). These results imply that low SRT operation may select for type II over type I Accumulibacter. This study is the first to investigate Accumulibacter clade abundance at low SRT values, and suggests that Accumulibacter clades IIA, IIB and IID may be better suited for high rate EBPR systems than others.

Exploring the limits of high rate EBPR

Current EBPR process variations generally employ total SRTs of 10 days or longer (although long SRTs are often

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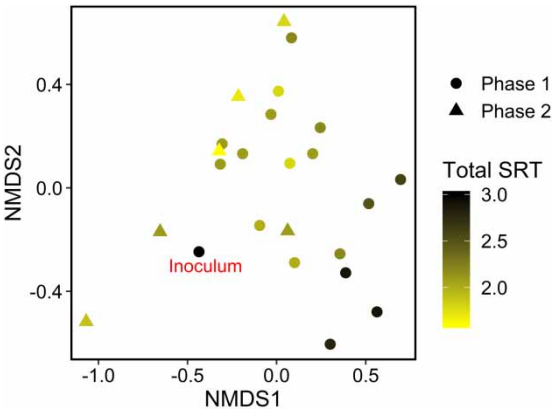

Figure 6 | Non-metric multidimensional scaling (NMDS) ordination of Accumulibacter clade population structure, calculated from clade-specific qPCR data. The SRT for each sample is the average of 3 measurements (taken over about 1 week) prior to the sample date. The inoculum (day 0) came from an EBPR process with an SRT around 8 days, which is not represented on the SRT scale bar. The significance of the ordination is represented by the stress value of 0.07.

chosen to retain nitrifiers), and recent work in the literature has demonstrated EBPR at total SRTs down to 2–4 days. Here we demonstrate robust and high rate EBPR at the low SRT of 1.8 ± 0.2 days ($SRT_{aer} = 1.4 \pm 0.2$ days) with real primary effluent. As discussed earlier, this A-stage process, which prioritizes P and organic carbon removal, can provide ideal effluent for a B-stage autotrophic N removal process. This provides evidence that the competing goals of low SRT for carbon diversion via HRAS and the carbon requirements of EBPR may be reconcilable, although some compromise in carbon diversion will always occur as compared to a true HRAS process with a < 0.5-day SRT. To maximize carbon diversion, therefore, knowledge of a washout SRT for PAO, and thus the minimum allowable SRT to maintain the EBPR process, is crucial. The total SRT of 1.8 ± 0.2 days as calculated in this study does not include settling and decant time (Equation (1)) in order to better facilitate comparison to plug-flow processes with a separate settling tank. Whether the settling and decant time is included in the SRT calculation of SBRs in other high rate EBPR studies is generally not specified, making direct comparison challenging. At any rate, the aerobic SRT (SRT_{aer}) is independent of the settling and decant time, so SRT_{aer} will be utilized in the following discussion of the washout SRT of PAOs.

While washout SRT for PAOs depends on multiple factors, including temperature, pH, the influent COD/P ratio, and the anaerobic fraction of the HRT (Brdjanovic *et al.* 1998; Carrera *et al.* 2001; Erdal *et al.* 2006), the following discussion focuses on two of the most important factors: temperature and the influent COD/P ratio (Carrera *et al.* 2001). This study provides evidence that the washout SRT_{aer} for Accumulibacter PAOs is between 0.8 and 1.4 days at 20 °C (Table 2). Ge *et al.* (2015) found a PAO washout SRT_{aer} between 1 and 1.2 days at 20–22 °C with 28.2 mg TP/L and 1,000 mg sCOD/L in the influent. Under such high COD concentrations, which differ significantly from typical primary effluent or raw municipal wastewater, they selected for the previously unknown PAO Comamonadaceae. Chan *et al.* (2017) found a higher Accumulibacter PAO washout SRT_{aer} between 1.75 and 2.1 days at 25 °C given synthetic wastewater concentrations of 20 mg TP/L and 226 mg sCOD/L (all sCOD as propionic acid). Valverde-Pérez *et al.* (2016) demonstrated effective EBPR via Accumulibacter at $SRT_{aer} = 1.5$ days at 16–19 °C given substrate conditions of 8 mg TP/L and >200 sCOD/L (200 mg COD/L of supplemental propionic acid was dosed to the influent to avoid carbon limitation), but did not investigate the PAO washout SRT. Yang *et al.* (2017) achieved stable EBPR at a 1.5–2.3-day SRT_{aer} at 16–24 °C with real primary effluent (160 mg sCOD/L and 5.9 mg TP/L), although they also did not investigate the PAO washout SRT. Historical studies that investigated the washout SRT_{aer} of PAOs but preceded the classification of Accumulibacter (Hesselmann *et al.* 1999) include Mamais & Jenkins (1992), who found a washout $SRT_{aer} = 1.5$ days at 20 °C via primary effluent (8.5 mg TP/L and 230 mg sCOD/L, though 50 mg/L of that was supplemental acetate). McClintock *et al.* (1993) observed a reduction in P removal at $SRT_{aer} = 0.9$–1.5 days at 20 °C with primary effluent (255 mg tCOD/L, 19 mg TP/L, some phosphate supplemented), and Shao *et al.* (1992) found a washout $SRT_{aer} = 1.4$ days at 23 °C via primary effluent (144 mg BOD_5/L, 6.3 mg TP/L). The present study is the first to combine the washout SRT of PAOs fed real municipal wastewater with an investigation into the clade-level diversity of Accumulibacter selected under low SRT conditions.

Brdjanovic *et al.* (1998) noted that determining the minimum SRT for PAOs in a given EBPR system is not trivial, and is dependent upon (among other things) the biomass specific VFA to PHA conversion rate (not necessarily limited to acetate as a substrate), the biomass specific yield, and the PHA storage capacity of the biomass. Brdjanovic's model predicted a minimum SRT_{aer} for PAOs of 1.0–1.6 days, depending on the PHA storage capacity of the biomass, at 20 °C. This matches well both with the observed optimal SRT_{aer} of 1.4 ± 0.2 days during Phase 2 of the present study, and the observed washout SRT_{aer} of 0.80 ± 0.12 days during Phase 3. However, it should be noted Brdjanovic's study used acetate as the sole carbon

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Table 2 | Comparison table for key studies of low sludge age EBPR processes

Feed	Scale/reactor type	Optimal total SRT (d)	Optimal aerobic SRT (d)	Washout aerobic SRT (d)	Primary PAO	Temperature (°C)	Reference
Primary effluent	Bench/SBR	1.8 ± 0.2	1.4 ± 0.2	0.8–1.4	*Accumulibacter*	19.7 ± 1.2	Present study
Primary effluent	Bench/SBR	2–3	1.5–2.3	NA	NA	16–24	Yang *et al.* (2017)
Primary effluent	Full/plug flow	3	2.8	1.4	NA	23	Shao *et al.* (1992)
Abattoir wastewater	Bench/SBR	2–2.5	1.2–1.5	1–1.2	*Comamonadaceae*	20–22	Ge *et al.* (2015)
Primary effluent + suppl. propionate and phosphate	Bench/SBR	3	1.5	NA	*Accumulibacter*	16–19	Valverde-Pérez *et al.* (2016)
Primary effluent + suppl. phosphate	Pilot/continuous	2.7	1.5	0.9–1.5	NA	20	McClintock *et al.* (1993)
Primary effluent + suppl. Acetate	Bench/continuous	2.3–2.6	1.7–2	1.5	NA	20	Mamais & Jenkins (1992)
Synthetic wastewater	Bench/SBR	3.6	2.1	1.8–2.1	*Accumulibacter*	25	Chan *et al.* (2017)

NA, Not available.

source. In complex matrices such as mainstream wastewater, rbCOD fermentation may become the rate limiting step in the anaerobic zone. This further complicates efforts to quantify a minimum SRT for PAOs, as influent composition and process characteristics can greatly influence their apparent growth rate. Regardless, the importance of tight aeration control, as observed in the present study with the improvement of P removal upon implementation of PID aeration control, affirms Brdjanovic's observation that retention of PAOs is better determined by a sufficient aerobic time to completely oxidize intracellular PHA than by the more typically used specific growth rate. Conversely, analysis by Barnard *et al.* (2017) concluded that EBPR failure was often associated with inadequate anaerobic zone design, indicating that proper design of both zones is critical for successful EBPR performance; this may become challenging in full scale systems when HRT and SRT are pushed to the limits.

PAO abundance and GAO suppression in high rate EBPR

Low SRT operation in this study may have contributed to the suppression of GAOs, as GAOs are thought to compete more effectively at higher SRTs due to their observed lower growth rates than PAOs (Wang *et al.* 2001; Onnis-Hayden *et al.* 2020). Also, the low influent rbCOD:TP ratio in this study (16 gCOD:gP) is just above the lower limit of 15 gCOD:gP that has been suggested for EBPR in systems with minimal fermentation (Barnard *et al.* 2017). This may contribute to GAO suppression, as higher influent organic carbon levels have been shown to be associated with *Ca.* Competibacter abundance (López-Vázquez *et al.* 2008), likely due to the availability of rbCOD in the anaerobic zone beyond that required for PHA production via polyphosphate release.

Given the relatively poor EBPR performance during the optimization phase of this study (Phase 1: 48% average orthophosphate removal), the 16 ± 8% average relative abundance of Accumulibacter PAOs according to 16S rRNA gene sequencing is surprisingly high. It is important to note that the measurement of DNA-based abundance of PAOs indicates the potential for biological P uptake, but not actual function. This is in part due to the metabolic diversity of Accumulibacter, which have been observed to exhibit a GAO-like metabolism under certain conditions (Barat *et al.* 2006; Erdal *et al.* 2008; Zhou *et al.* 2008; Acevedo *et al.* 2012; Welles *et al.* 2014). The observed orthophosphate release to anaerobic rbCOD uptake ratio during Phase 1 was 0.24 ± 0.22 g P/g COD ($n = 11$ in-cycle batch tests without dosing), lower than the expected PAO ratio of 0.36 g P/g COD (Hesselmann *et al.* 2000) to 0.56 g P/g COD (Smolders *et al.* 1994) (although those two studies used acetate as the sole carbon source). While a GAO-like metabolism of Accumulibacter is one potential explanation for these observations, alternate mechanisms include unidentified PHA accumulators and/or noncanonical GAOs.

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Accumulibacter diversity in high rate EBPR

The fact that this study selected for Accumulibacter PAOs as did the reactors run by Chan *et al.* (2017) and Valverde-Pérez *et al.* (2016), which used synthetic wastewater to mimic primary effluent and primary effluent with supplemental propionate and phosphate, respectively, suggests that Accumulibacter have a competitive advantage over other PAOs under mainstream conditions at low SRT. All low SRT EBPR studies that looked for *Tetrasphaera,* including the present study, have found near-negligible levels (Ge *et al.* 2015; Valverde-Pérez *et al.* 2016; Chan *et al.* 2017); *Tetrasphaera* has been occasionally identified as the dominant PAO in full scale (not high-rate) EBPR systems (Nguyen *et al.* 2011). Other bacterial genera that have been observed to act as PAOs in conventional (again, not high-rate) EBPR systems such as *Microlunatus* (Nakamura *et al.* 1995), *Pseudomonas* (Günther *et al.* 2009), *Dechloromonas* (Kong *et al.* 2007) and *Halomonas* (Nguyen *et al.* 2012; Nielsen *et al.* 2019), were either not detected or were at negligible average relative abundance (<0.3%) in this study according to 16S rRNA gene amplicon sequencing.

No other studies have investigated Accumulibacter clade abundance at low SRT values, but other investigations of Accumulibacter clades in wastewater treatment systems with higher SRT values have also observed high diversity (Mao *et al.* 2015; Camejo *et al.* 2016). Mao *et al.* (2015) reported that clades IIC and IID were the most abundant in a survey of 18 wastewater plants (SRT not reported), though other studies have demonstrated the dominance of clades IA (Gao *et al.* 2019; SRT not reported though likely high due to lack of biomass wasting), IB (Mao *et al.* 2014; SRT not reported), and IC (Camejo *et al.* 2016; SRT = 80 days). Little is known of what selective pressures operating conditions exert on the 14 known clades. Only one study to date has directly investigated the effect of SRT on Accumulibacter clade-level diversity (Onnis-Hayden *et al.* 2020), though it used a SRT range of 6–40 days and did not investigate very low SRT values. In that study, clade IIA dominated systems with both (relatively) short and long SRTs (6, 20 and 40 days) and clade IIB dominated at SRTs between 7 and 10 days. Clades IA and IIC were present but more abundant at the 10-day SRT than at longer or shorter SRTs. Mao *et al.* (2015) suggested that the Accumulibacter clades may represent distinct ecotypes, but little correlation beyond the expected association with influent levels of total P and COD was revealed. In contrast, studies by Camejo *et al.* 2019 and Gao *et al.* 2019 demonstrated the ability of clades IA and IC to use NO_2^- or NO_3^- as electron acceptors in denitrifying P uptake. While type II Accumulibacter have also been shown to harbor at least some denitrifying genes (i.e. clades IIA, IIC and IIF; see Camejo *et al.* 2019), the lack of periplasmic nitrate reductase (*nap*) and nitrous oxide reductase (*nos*) gene clusters in some type II genomes (and a more complete denitrifying pathway in type I genomes) suggested a potential advantage of type I Accumulibacter in denitrifying P uptake in work by Gao *et al.* (2019). Research by Flowers *et al.* (2009) and Oehmen *et al.* (2010) has also demonstrated the ability of certain type I Accumulibacter (and the inability of certain type II Accumulibacter) to use NO_3- as an electron acceptor. The relevance of this discussion to the present study is that low SRT operation results in nitrifier washout and a *lack* of NO_X available. In this study, nitrification (and thus NO_X) was present only at higher SRT values (Figures 1 and S1), and the lowest SRT values during Phase 2 were associated with the absence of clades IA and IC and other type I Accumulibacter (Figure 5). In other words, low SRT leads to a lack of selective pressure for denitrifying PAOs and may be correlated to the predominance of type II Accumulibacter. This study suggests, therefore, that Accumulibacter type II, particularly clades IIA, IIB and IID, may be better suited to low SRT operation. Whether the selection for type II in this study is due to higher growth rates (via oxygen and not NO_X as an electron acceptor) or other selective pressures is unknown, and further study is suggested.

Nitrification in low SRT EBPR systems

Washout of nitrifiers at low SRT operation can facilitate a nitrogen-rich and carbon-poor effluent that is ideal for a B-stage autotrophic PN/A process. An additional advantage of preventing nitrification, however, is to limit the production of NO_X. With lower NO_X in the return sludge, less rbCOD will be consumed by denitrifiers in the anaerobic zone, making more rbCOD available for PHA production by PAOs. Of the eight low SRT studies summarized in Table 2, including the present one, five of the eight were able to prevent nitrification with average SRT_{aer} values from 1.3 to 1.9 days with average temperatures of 17.5–21 °C (Mamais & Jenkins 1992; Ge *et al.* 2015; Valverde-Pérez *et al.* 2016; Yang *et al.* 2017). Chan *et al.* (2017) was unable to report on nitrifier washout due to the use of allylthiourea (ATU) to prevent nitrification, and that study utilized the highest SRT_{aer} (2.1 days) and the highest temperature (25 °C). McClintock *et al.* (1993) observed some nitrification even at the lowest SRT_{aer} tested at 1.5 days at

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20 °C. A small amount of nitrification was observed at an SRT_{aer} as low as 1.6 days at 20 °C in the present study. Given the difficulties in preventing nitrification in this and previous studies (even at very low SRTs in small-scale reactors) it is anticipated that a key challenge to scale up is careful balancing of high-rate EBPR with the prevention of nitrification.

CONCLUSIONS

- Stable P removal via EBPR from real wastewater (primary effluent) was achieved without chemical dosing at low SRT (1.8 ± 0.2 days) operation.
- Accumulibacter PAOs were highly abundant and clades IIA, IIB and IID were dominant at the lowest SRT values. GAOs were robustly suppressed with an average relative abundance of <1.0%.
- Successful A-stage EBPR performance at low SRT depended upon tight aeration and DO control via sensor based PID operation.
- A narrow SRT control window was needed to maintain EBPR and prevent nitrification; NO_X production was observed at an SRT_{aer} as low as 1.6 days.
- The low SRT EBPR process is a promising efficient technology with a small footprint for the diversion of carbon and P to the side-stream, and effluent can be routed downstream to a PN/A process for nitrogen removal.

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DATA AVAILABILITY STATEMENT

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

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