Assessing the Effects of RAS Fermentation on EBPR Performance and Associated Microbial Ecology

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ABSTRACT: Enhanced biological phosphorus removal (EBPR) is an engineered water resource recovery facility (WRRF) process configuration that can produce effluent P < 0.5 mg/L. To consistently achieve low effluent P concentrations, EBPR requires volatile fatty acids (VFAs) to induce requisite biochemical reactions. Moreover, returned activated sludge (RAS) nitrate concentrations must be minimized. Returned activated sludge fermentation can potentially address process needs. However, research detailed herein highlights concerns with RAS fermentation integrated with EBPR. Under 2 and 4 hours of RAS fermentation periods, no consequential VFA production was observed; similar results were observed in batch tests with RAS from a full-scale EBPR WRRF. More critically, EBPR performance was poor, with average effluent concentrations of 1.0 to 2.4 mg/L. Furthermore, the glycogen accumulating organism (GAO) fraction under RAS fermentation was 4.3 to 8.7 times higher than in a conventional EBPR mixed microbial consortium (MMC). Integrated RAS fermentation-EBPR only performed well under "high" RAS nitrate; thus, should RAS fermentation be implemented, careful control to prevent anaerobic conditions in the fermentation zone is required. *Water Environ. Res.*, **90**, 659 (2018).

KEYWORDS: RAS fermentation, enhanced biological phosphorus removal (EBPR), phosphorus accumulating organism (PAO), glycogen accumulating organism (GAO), mixed microbial consortium (MMC).

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Introduction

Enhanced biological phosphorus removal (EBPR) is an engineered water resource recovery facility (WRRF) process configuration that can be used to remove significant quantities of phosphorus (P) from wastewater. Indeed, EBPR can reliably produce effluent P concentrations of 0.5 mg/L (Tchobanoglous et al., 2014), with numerous full-scale municipal WRRFs reporting effluent near or below 0.1 mg P/L. Of the conventional liquid stream P removal technologies, EBPR is more environmentally sustainable than chemical treatment methods (Coats, Watkins, and Kranenburg, 2011), and also generates a product of agronomic value (P-rich biosolids).

Enhanced biological phosphorus removal theory centers on cyclically exposing a mixed microbial consortium (MMC) to anaerobic (first) and aerobic (second) environments while concurrently providing an influent substrate rich in volatile fatty acids (VFAs) (Fuhs and Chen, 1975). Within this engineered system, the MMC becomes enriched with polyphosphate accumulating organisms (PAOs) that are capable of excess P removal. Specifically, Candidatus Accumulibacter *phosphatis* (Accumulibacter) is the putative predominant PAO species (Crocetti et al., 2000; Hesselmann et al., 1999). Polyphosphate accumulating organisms uptake and store VFAs anaerobically as polyhydroxyalkanoate (PHA), with the energy required for the uptake and catabolism of VFAs theoretically derived from both hydrolysis of intracellular polyphosphate (polyP) and glycogen catabolism, the latter of which also provides a primary source of reducing equivalents for PHA synthesis (Lemos et al., 2003; Seviour et al., 2003). These anaerobic metabolisms ultimately result in a large increase in bulk solution P. In the subsequent aerobic environment, PAOs oxidize PHA for energy to grow and to replenish internal glycogen and polyP reserves; anoxic conditions induce similar, albeit potentially less efficient, reactions. Through this cyclical process, coupled with MMC/PAO growth, more polyP is stored than is released, resulting in a significant net decrease of P in the bulk solution. Enhanced biological phosphorus removal systems can also enrich for glycogen accumulating organisms (GAOs), which exhibit similar VFA/PHA/glycogen metabolisms but may not contribute to EBPR and thus may be detrimental to process performance.

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To consistently achieve low effluent P concentrations, the EBPR process theoretically requires an adequate quantity of VFAs to drive the series of biochemical reactions necessary for maximal P removal. Although acetate is the model substrate for EBPR (Fuhs and Chen, 1975; Smolders et al., 1995), a blend of VFAs—specifically including propionate—is more favorable to enhance and stabilize EBPR (Carvalheira et al., 2014; Shen and Zhou, 2016; Tchobanoglous et al., 2014). For example, two recent studies support the potential value of propionate: Winkler et al. (2011) observed enhanced EBPR metrics with a VFA blend versus just acetate, while Coats et al. (2017) observed that EBPR in a synthetic fed system could not be achieved until propionate was blended with acetate. For WRRFs receiving wastewater streams low in VFAs, some form of primary solids fermentation can be implemented, or supplementation with purchased VFAs may be considered. However, the addition of synthetically derived VFAs increases treatment costs considerably while concurrently increasing the WRRF carbon footprint. A potential alternative source of carbon that is readily available for VFA production through fermentation within a WRRF is the mixed liquor. Specifically, the return activated sludge (RAS) could serve as a co-substrate to potentially generate additional carbon and thus potentially enhance EBPR; VFAs would be generated via sidestream fermentation (i.e., inserting an anaerobic zone to ferment some or all of the RAS prior to re-introduction into the mainstream EBPR system).

In addition to ensuring available VFAs, the RAS nitrate concentration should be minimized to sustain EBPR. Most conventional EBPR process configurations, which incorporate pre-anoxic denitrification (Tchobanoglous et al., 2014), result in residual, parasitic nitrate in the effluent and thus in the RAS stream. Excess RAS nitrate introduced in the anaerobic zone of the EBPR system can induce process failure (i.e., anaerobic conditions becoming anoxic); indeed, excess RAS nitrate is commonly a cause of EBPR failure (Oehmen et al., 2007). Process configurations aimed at ameliorating RAS nitrate—namely the Johannesburg Process and the Westbank Process—incorporate a pre-anoxic zone upgradient of the EBPR anaerobic zone, where denitrification is achieved either through endogenous decay, or through addition of raw wastewater or primary solids fermenter liquor. Alternately, it has been suggested that RAS nitrate reduction could potentially be integrated with and achieved in concert with VFA production through a sidestream fermenter configuration (Barnard et al., 2017; Tooker et al., 2016; Tooker et al., 2017; Vollertsen et al., 2006). Yet another process variant, enhanced post-anoxic mainstream denitrification—which targets the entire mixed liquor suspended solids (MLSS), not just the RAS stream—can significantly reduce nitrate concentrations (Appel, 2015; Coats et al., 2011; Romenesko, 2017; Winkler et al., 2011) while concurrently maintaining stable EBPR.

Conceptually RAS fermentation would seemingly be beneficial to EBPR-coupled VFA production and RAS denitrification. However, there are some potential concerns with adopting such a process configuration. Relative to primary solids, RAS is less readily fermentable (Eastman and Ferguson, 1981; Grady et al., 2011); thus, VFA production could be limited, or could require excessive retention times (i.e., excess WRRF tankage) to achieve desired productivity. Regarding process performance, anaerobic secondary P release is a concern. Per Barnard (1984) and Barnard and Fothergill (1998), exposure of EBPR biomass to anaerobic conditions in the absence of VFAs—or under conditions that might facilitate MLSS fermentation (Barnard and Fothergill, 1998)—can induce hydrolysis of polyP stores metabolically delinked from EBPR metabolisms. Another potential concern relates to use of glycogen reserves for denitrification of RAS prior to introduction into the anaerobic zone; while research has shown that some glycogen use for post-anoxic denitrification will not impair EBPR (Coats, Mockos, and Loge, 2011; Mellin, 2017; Winkler et al., 2011), excess glycogen utilization could impair process performance. Finally, GAOs also store glycogen, and RAS denitrification will all but certainly utilize glycogen as the electron donor (if an exogenous carbon substrate is not added; PHA reserves are typically fully depleted in the EBPR process aerobically/anoxically); as such, it is possible that sidestream RAS denitrification might enhance this microbial population putatively detrimental to EBPR by creating a condition for denitrifying GAOs to become competitive.

Ultimately, limited peer-reviewed data is available on the effects of RAS fermentation on VFA production, nitrate concentrations, PAO/GAO microbiology, and overall EBPR performance. Thus, in this study, research was undertaken to explore and interrogate this alternate process configuration. Research objectives were to (1) evaluate the fermentation potential of RAS obtained from full-scale WRRFs; (2) evaluate treatment performance effects of RAS fermentation integrated within bench-scale EBPR reactors; (3) characterize carbon production and/or cycling—specifically VFAs, PHA, and glycogen—under applied RAS fermentation conditions; and (4) evaluate the effect of RAS fermentation on PAO and GAO enrichment. All investigations used real municipal wastewater.

Materials and Methods

Operating Conditions and System Assessments. *Batch RAS Fermentation.* Batch RAS fermentation assessments were performed at the bench-scale using fresh RAS obtained from two WRRFs: Moscow, Idaho (operating a A2/O process, with the aerobic reactor being an oxidation ditch; average solids residence time [SRT] of 15 to 18 days) and Pullman, Washington (operating an MLE process; average SRT of 10 to 12 days). Samples were collected and tests performed in late August/early September. Both WRRFs receive wastewater from predominant residential communities, including midsized universities. The batch Moscow RAS fermentation tests involved open-air mixing of 2 L of RAS, with MLSS concentrations of 5590, 6740, and 7627 mg TSS/L for test nos. 1, 2, and 3, respectively; each test was performed on fresh RAS collected on different days. The batch Pullman RAS fermentation test used 1 L of RAS at a concentration of 18 123 mg TSS/L. Reactor temperature ranged from 20 to 22 °C; pH ranged from 7 to 7.3 throughout the testing.

Table 1—Summary details on EBPR reactor operational conditions.

Reactor	Operational period	SRT, days	HRT, hrs.	RAS fermentation period, hrs.	Anaerobic period, hrs.	Aerobic period, hrs.	VFA augmented?
V-EBPR	All	10 ± 1	18	–	1	5	Yes
R-EBPR	All		18	–	1	5	No
V2-EBPR	1		16	4	1	3	Yes
	2			2	1	5	Yes
R2-EBPR	1		16	4	1	3	No
	2			2	1	5	No

Integrated EBPR-RAS Fermentation. Four laboratory-scale EBPR sequencing batch reactors (SBRs) were evaluated for the integrated RAS fermentation-EBPR phase of the investigations (Table 1). Sequencing batch reactors were differentiated by substrate (raw wastewater vs augmentation with VFA-rich fermenter liquor) and operation (with or without RAS fermentation): R-EBPR (no RAS fermentation) and R2-EBPR (RAS fermentation) received only raw wastewater, while V-EBPR (no RAS fermentation) and V2-EBPR (RAS fermentation) received a substrate consisting of raw wastewater (90% by volume) and VFA-rich fermenter liquor (10% by volume). R- and V-EBPR were operated on a 6-hour cycle consisting of the following periods: feed (5 minutes), anaerobic (1 hour; incl. feed), aerobic (4 hours 10 minutes), de-oxygenate (10 minutes), settle (0.5 hour), and decant (10 minutes); operational assessment of these two EBPR reactors has been previously documented (Coats et al., 2017; Coats et al., 2015), although data presented herein was collected specific for this study. R2- and V2-EBPR, each inoculated from R- and V-EBPR, respectively, were operated on an 8-hour cycle. Period 1 (4-hour RAS fermentation) R2/V2-EBPR operations were conducted as follows: feed (5 minutes), anaerobic (1 hour; incl. feed), aerobic (2 hours 10 minutes), de-oxygenate (10 minutes), settle (0.5 hour), decant (10 minutes), and completely mixed RAS fermentation (4 hours). Period 2 (2-hour RAS fermentation) R2/V2-EBPR operations were conducted as follows: feed (5 minutes), anaerobic (1 hour; incl. feed), aerobic (4 hour 10 minutes), de-oxygenate (10 minutes), settle (0.5 hour), decant (10 minutes), and completely mixed RAS fermentation (2 hours). A programmable logic controller was used to maintain SBR operations. Sequencing batch reactors were operated at 2 L, with effluent decanted during each cycle and replaced with an equal volume of substrate to maintain the target hydraulic residence time (HRT); R- and V-EBPR were operated at an 18-hour HRT (667 mL decanted each cycle), while R2- and V2-EBPR were operated at a 16-hour HRT (1 L decanted each cycle). The SRT was controlled at ~10 days (periodic WAS measurements indicated ±10% variation) by automatically wasting 50 mL of mixed liquor per cycle at the end of each aerobic period prior to settling. All pumping was performed using peristaltic pumps (Watson Marlow, Wilmington, Massachusetts). Air was introduced through stone diffusors to create aerobic conditions (dissolved oxygen [DO] > 2 mg/L). To de-oxygenate the reactor, aeration was ceased while mixing continued; this phase was applied to ensure that anaerobic conditions prevailed at the beginning of the subsequent cycle (note: the more conventional application of an inert gas to induce anaerobic conditions can have the adverse effect of artificially increasing the pH by stripping CO_2 and shifting the carbonate cycle). Periodic DO checks throughout the operational cycle were used to confirm each SBR conformed to target DO concentrations (data not shown). Reactors were mixed with magnetic stir bars and operated at room temperature without pH control. Reactor temperature ranged from 22 to 24 °C; periodic measurements showed that pH varied from 7.5 to 8.4.

Sources of Wastewater. Raw wastewater was obtained from the Moscow WRRF, downstream of screening and grit removal. Fresh raw wastewater was obtained every 2 to 7 days, and filtered through a 55-μm sieve prior to use. Volatile fatty acid-rich fermenter liquor was recovered via centrifugation from a bench-top fermenter fed thickened primary solids from the Pullman, Washington, WRRF; details on fermenter operations and carboxylate speciation can be found elsewhere (Romenesko and Coats, 2018 [under review]; Romenesko, 2017). All wastewater was stored at 4 °C until use. Bioreactor substrate tanks were replenished daily. Nitrification was inhibited through the combined addition of thiourea and nitrapyrin ($ClC_5H_3NCCl_3$) into the daily substrate. Nitrapyrin was added because thiourea proved to be only partially capable of inhibiting nitrification. Nitrate was periodically measured to confirm nitrification was inhibited.

Analytical Techniques. Samples were monitored for soluble reactive phosphate (P), VFAs, nitrate (NO_3), MLSS, glycogen, and PHA as described in Coats et al. (2015). Measurement of pH was accomplished with a Thermo-Fisher (Waltham, Massachusetts) Scientific Accumet AP85 Waterproof pH/Conductivity Meter. Dissolved oxygen and redox measurements were collected using a Hach (Loveland, Colorado) HQ30d Meter with an LDO101 DO probe and an MTC101 redox probe.

Quantitative Polymerase Chain Reaction. Quantitative polymerase chain reaction (qPCR) was performed on genomic DNA, recovered during bioreactor performance assessments, to estimate the abundance of Accumulibacter (the model PAO) and GAOs relative to the total eubacterial population. Genomic DNA was extracted using the MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California). Genomic DNA yield and purity was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, Delaware) Primer sets used to quantify total bacteria ([Muyzer et al., 1993]; average gene copy number

of 4.1), PAOs ((targeting 16S rDNA (average gene copy number of 2) and the PPK gene (average gene copy number of 1); (Crocetti et al., 2000; He et al., 2007)), and GAOs (by targeting *Candidatus* Competitbacter phosphatis, a model GAO; average gene copy number of 1) (Crocetti et al., 2002) are described elsewhere (Coats et al., 2017); the lone modification was the use of primer 651f, replacing 518f, for quantifying PAOs based on 16S rDNA, consistent with recommendations of Albertsen et al. (2016). qPCR conditions were in accordance with Winkler et al. (2011). qPCR melting curves were evaluated to confirm a single melting peak, and agarose gel analysis confirmed a single band for each primer set; qPCR controls included a negative control with sterile water. Amplification efficiencies were calculated for each primer set using baseline-corrected fluorescence data (StepOne software v2.0) and the LinRegPCR program (Ramakers et al., 2003). Mean amplification efficiencies for the total bacterial, PAO, and GAO primer sets were 99.4 ± 2.84% ($n = 238$), 94.4 ± 4.45% ($n = 214$), and 84.4 ± 5.79% ($n = 119$), respectively. The cycle threshold was set at a constant value of 0.1 within the log-linear region across all samples for determination of quantification cycle (Cq) values. Relative abundances were calculated in accordance Winkler et al. (2011).

Results and Discussion

Fermenting Return Activated Sludge. It has been postulated (Barnard et al., 2011; Tooker et al., 2016; Yuan and Oleszkiewicz, 2010) that RAS fermentation of EBPR-derived biomass can produce all-important VFAs. As a first assessment to vet this VFA production hypothesis, batch investigations were performed on RAS obtained from two full-scale WRRFs; one WRRF is configured as, and performs, EBPR, while the second WRRF does not (Modified Ludzack–Ettinger [MLE] configured). The MLE system was chosen as a control, and a contrast, against data obtained from the EBPR system.

In interpreting results, both P release and VFA synthesis were assessed, as both would be relevant to downstream EBPR performance. As would be expected, fermentation of EBPR RAS did result in an increase in bulk solution P (Figure 1a)—increasing from near zero up to 33 to 48 mg P/L over the tested time periods. For the first 500 minutes in each test, the rate of P release ranged from 0.025 to 0.047 mg P/L·min. Average redox over the duration of the respective tests ranged from –206 mV to –289 mV. As a contrast to the EBPR sludge, RAS from an MLE-configured WRRF was evaluated for fermentation potential; bulk solution P data is shown in Figure 1a (average redox of –362 mV). The bulk solution P increased to over 70 mg P/L, at a rate of approximately 0.124 mg P/L·min for the first 500 minutes; comparatively, these values were unexpectedly higher than realized by the EBPR biomass. However, normalizing P release to gTS shows that the release rates were relatively comparable (Figure 1b). Specifically, for the EBPR RAS, on an inocula total solids (TS) basis, the P release ranged from 1.97 (test no. 3) to 3.62 (test no. 2) mg P/g TS, ultimately increasing to 4.92 (test no. 3) to 7.04 (test no. 2) mg P/g TS over the full period assessed; the MLE RAS the P release peaked at approximately 4 mg P/g TS. While a significant fraction of the observed increase in bulk solution P for the EBPR RAS was most likely polyP hydrolysis from PAOs, the similar P increase in the MLE RAS was more likely associated with biomass decay and hydrolysis.

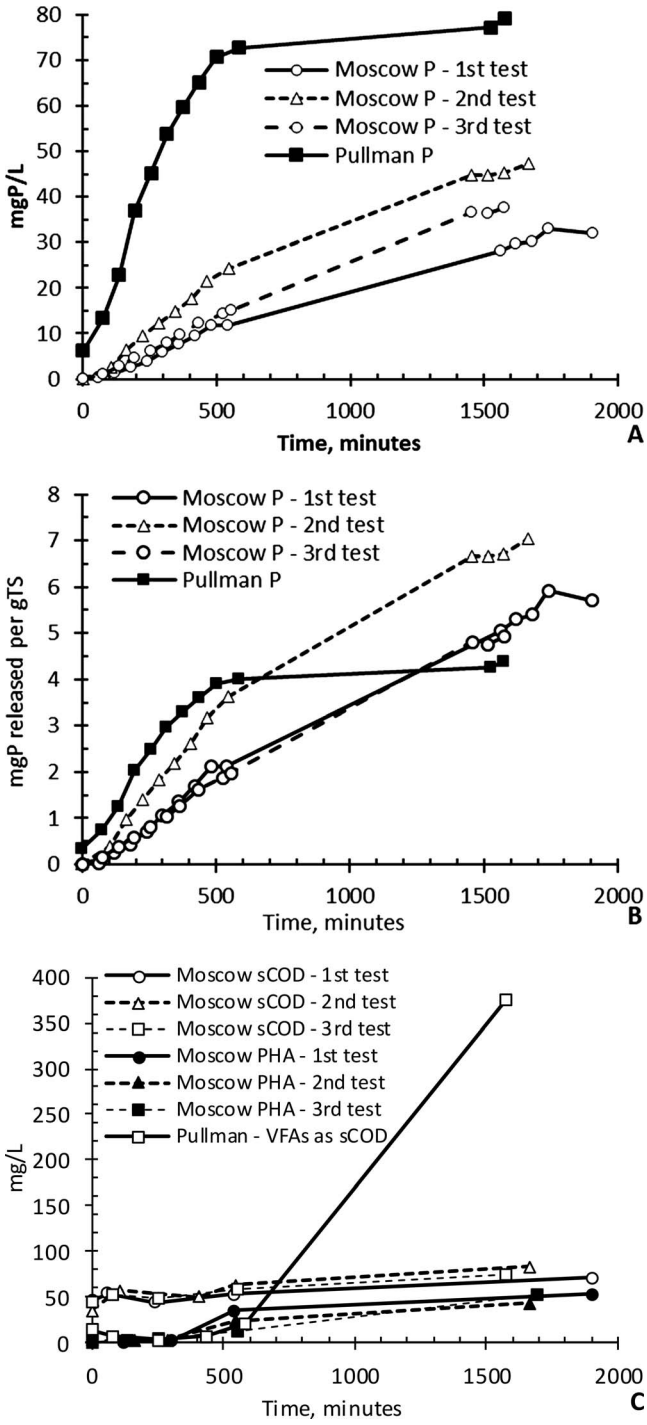

Figure 1—Batch RAS fermentation results showing (a) bulk solution soluble phosphorus; (b) phosphorus release normalized to gTSS; (c) and sCOD, PHA, and VFA concentrations over time for RAS obtained from Moscow, Idaho, EBPR WRRF and Pullman, Washington, MLE WRRF.

Although P release must be assessed in RAS fermentation, the ultimate goal is VFA production. First considering the EBPR RAS (Figure 1c), soluble chemical oxygen demand (sCOD) increased modestly over the duration of the fermentation tests, with increases of 15 to 82% during the first 500 minutes and 56 to 140% over the full duration. However, no VFA production was detected in any of the tests. Recognizing that, in accordance with EBPR theory, VFAs made available to PAOs under anaerobic conditions would be rapidly catabolized and converted to PHA, the lack of detectable VFAs was not—and should not—be a surprise. Ultimately, to ascertain if VFAs had been produced, biomass was recovered and PHA quantified. For test no. 1, no PHA production was observed in the first 300 minutes; however, some PHA production was observed by 540 minutes (1.7 Cmmol/L; 35.2 mg/L) and at 1905 minutes (2.57 Cmmol/L; 52.3 mg/L). Similar results were observed for test no. 2 (1.2 Cmmol/L (23.6 mg/L) at 545 minutes; 2.1 Cmmol/L (42.5 mg/L) at 1665 minutes), while PHA production was observed earlier for test no. 3 (0.25 Cmmol/L (5.1 mg/L) at 255 minutes; 0.57 Cmmol/L (11.6 mg/L) at 555 minutes; 2.55 Cmmol/L (51.9 mg/L) at 1695 minutes). Polyhydroxyvalerate (PHV) was the dominant form of PHA synthesized, suggesting both odd and even VFAs were generated via fermentation. Presuming the observed PHA production was associated with VFA synthesis achieved via RAS fermentation, PHA values can be conservatively assumed to represent VFA production (1:1 VFA-to-PHA conversion, Cmmol basis). Utilizing this theoretical VFA production, the resultant P released to VFA uptake ratio (P:C) would range from 0.22 to 0.40, 0.67 to 0.73, and 0.5 to 1.05 Pmol:Cmol for test nos. 1, 2, and 3, respectively. The P:C ratio is an indicator of EBPR metabolic activity (Filipe et al., 2001; Smolders et al., 1994), since anaerobic P release is metabolically linked to VFA catabolism for adenosine 5′-triphosphate (ATP) production. Although the extrapolated VFA production values are conservatively high (glycogen catabolism likely contributed to PHA synthesis; see Murnleitner et al. [1997]), results nonetheless suggest EBPR metabolic behavior was potentially pre-induced (Coats et al., 2017)—albeit not for the first 400 to 500 minutes.

Although no VFA synthesis was observed on the EBPR RAS, the opposite occurred on the MLE RAS (Figure 1c). However, significant retention time was required to realize VFA production, consistent with recent RAS fermentation research (Tooker et al., 2016; Tooker et al., 2017). Negligible VFA production occurred for the first 585 minutes; thereafter fermentation was initiated, with bulk solution VFA exceeding 10 Cmmol/L (265 mg VFA/L) at 1575 minutes. Volatile fatty acids were dominated by acetic acid at 42% (Cmmol basis), although propionic, butyric, and valeric acid were also produced. Ultimately, VFA production exceeded that observed by Tooker et al. (2016). Contrasted with the EBPR RAS, no PHA production occurred.

Return activated sludge fermentation could potentially affect the relative fraction of PAOs and GAOs present in the MLSS; relative concentrations could increase if other fractions of the TSS—including ordinary heterotrophic organisms—are lysed and hydrolyzed, or they can decrease if said microbes become fermentation substrate. Both PAOs and GAOs could survive RAS fermentation conditions by relying on internal carbon storage reserves (Winkler et al., 2011). Polyphosphate accumulating organism and GAO fractions were estimated using qPCR; only the Moscow RAS was evaluated, because the Pullman WRRF does not perform EBPR. The PAO fraction of MLSS ranged from 2.9 to 5.75% in test no. 1, and 5.49 to 7.49% in test no. 2; conversely, the GAO population in both tests was <0.27% of the MLSS. The PAO population for test nos. 1 and 2 remained stable or increased (by a factor of 2) in the first 4 hours of batch operation; by the end of the test the PAO fraction was estimated at a 16% increase (test no. 1) to a 27% decrease (test no. 2). The GAO population decreased throughout test no. 1 (ultimately by 83%), while in test no. 2 the population increased by about 50% in the first 4 hours, then decreased to 85% of the inocula by the end of the test. Ultimately, qPCR results suggest that RAS fermentation could potentially affect both PAO and GAO fractions of the MLSS. However, the true measure must ultimately consider population variability with RAS fermentation fully integrated within the EBPR scheme.

Return Activated Sludge Fermentation Integrated with EBPR. With putative EBPR activity and potential fermentation observed in the batch RAS fermentation evaluations, coupled with data potentially indicating positive effects on PAO and GAO fractions, investigations were extended to integrated RAS fermentation-EBPR systems. Investigations were extended, in part, over questions about potentially misinterpreting the value of the batch RAS fermentation results. Neither full-scale WRRF included a RAS fermentation stage, and thus the MMC were not conditioned for such a metabolic environment; incorporating such an environment would be expected to enrich for bacteria within the MMC more uniquely suitable and better able to function and thrive in the imposed fermentative conditions. Bench-scale EBPR reactors (V2, R2-EBPR) were subjected to two different RAS fermentation periods over the course of the study: initially a 4-hour period was imposed (111 operational days), followed by a 2-hour period for 299 days. The RAS fermentation period was ultimately decreased due to poor EBPR performance, discussed in the subsequent sections. Periodic oxidation-reduction potential (ORP) measurements indicated that the ORP exceeded –300 mV under 4 hours. Return activated sludge fermentation conditions, and approached –200 mV under 2 hours of RAS fermentation conditions. Coupled with DO data, these ORP measurements affirmed that necessary anaerobic conditions were realized in the SBRs; research indicates that such "deep anaerobic" conditions are requisite to potentially achieve EBPR (Barnard et al., 2017).

Carbon Cycling: RAS Fermentation and EBPR. The integrated RAS fermentation-EBPR system was first assessed to determine if this alternate process configuration did, indeed, generate critical carbon substrate. Consistent with the batch fermentation evaluations, all pertinent forms of EBPR carbon—VFAs, PHA, and glycogen—were quantified. First examining the RAS fermentation part of the operational cycle, as shown (Figure 2a; Table 2), except for the first tested operational day of the investigations for V2-EBPR, negligible VFA production was

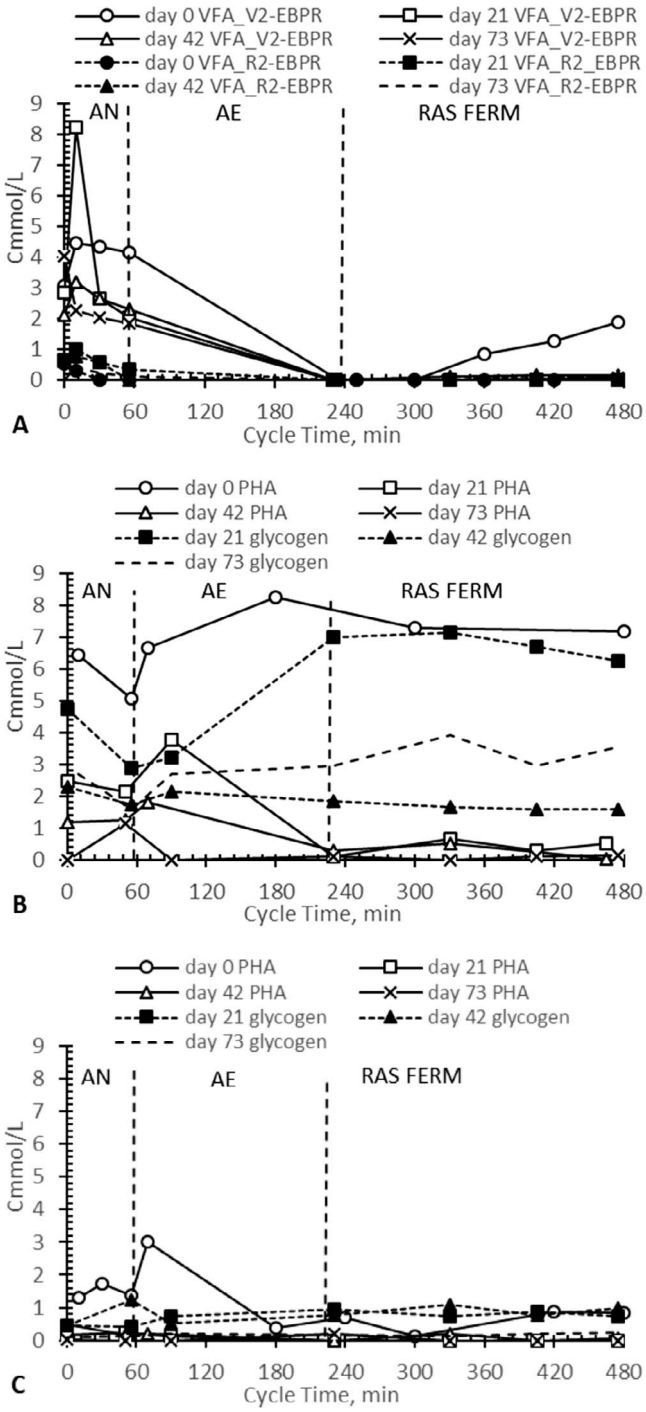

Figure 2—(a) VFA patterns for V2- and R2-EBPR, 4-hour RAS fermentation period; (b) PHA and glycogen patterns for V2-EBPR, 4-hour RAS fermentation period; and (c) PHA and glycogen patterns for R2-EBPR, 4-hour RAS fermentation.

detected under 4 hours of RAS fermentation conditions; generally similar results were observed under 2 hours of RAS fermentation conditions (Table 2), although R2-EBPR did realize some VFA production on one operational day. The limited VFA synthesis was markedly lower than observed in the Pullman RAS fermentation evaluation, and also notably lower than observed by Tooker et al. (2017), although the applied RAS fermentation HRT was also comparatively lower.

As with the batch investigations, lack of VFAs observed during RAS fermentation of EBPR biomass does not necessarily indicate no VFA synthesis, as the PAOs and GAOs would likely store VFA as PHA. However, interrogation of PHA data (Figures 2b, 2c; Table 2) shows that no consequential PHA production was realized by either V2- or R2-EBPR MMC. Regarding glycogen, the V2-EBPR MMC ultimately consumed this carbon reserve under RAS fermentation conditions (Figure 2b; Table 2); generally similar results have been observed in a post-anoxic EBPR configuration (Coats, Mockos, and Loge, 2011; Mellin, 2017; Winkler et al., 2011). The R2-EBPR MMC exhibited no consequential glycogen use or consumption (Figure 2c; Table 2); consistent with the low-VFA substrate, the MMC also exhibited very low concentrations of intracellular glycogen. In summary, the carbon data does not suggest that, for the applied RAS fermentation HRTs, EBPR metabolisms were pre-induced in either the applied RAS fermentation period, nor that substantive carbon was generated that would support EBPR.

Next considering the EBPR anaerobic period, VFAs, glycogen, and PHA utilization/synthesis patterns in V2-EBPR were generally consistent with EBPR theory (Oehmen et al., 2007) (Figures 2b, 2c; Table 3). Conversely, the R2-EBPR MMC exhibited carbon patterns less well aligned with EBPR theory; however, this result was not necessarily unexpected, given the low concentration of substrate VFAs (Tables 3 and 4) as well as the performance of the comparative control reactor R-EBPR (results both in this and previous studies [Coats et al., 2017; Coats et al., 2015]). Notably, VFA quantities available in the influent substrate and consumed anaerobically by the V2- and R2-EBPR MMC greatly exceeded the low quantities produced during the RAS fermentation period; similar differences can be seen with PHA synthesis and glycogen utilization, anaerobically versus RAS fermentation. More critically, the anaerobic carbon data would suggest that the effect of RAS fermentation, for the applied RAS fermentation HRT, might be potentially negative. Specifically, VFAs were not fully depleted anaerobically by the V2-EBPR MMC (Figure 2a). Incomplete VFA utilization is indicative of inadequate polyP and/or glycogen reserves (Mino et al., 1987; Oehmen et al., 2007), potentially expended under RAS fermentation conditions; both provide necessary ATP energy for VFA catabolism.

Comparatively for the control EBPR reactors—V-EBPR, which received VFA-rich substrate, and R-EBPR, which received just raw wastewater—VFAs were depleted during the anaerobic period (Table 3); the observed response is consistent with previous investigations on this reactor (Coats et al., 2017; Coats et al., 2015), and suggests sufficient polyP and glycogen reserves were available to generate the necessary ATP. Commensurate with anaerobic VFA consumption was PHA production (Table 3); as would be expected given the greater quantity of VFAs in the substrate, the V-EBPR MMC synthesized greater quantities of PHA than the R-EBPR MMC.

Table 2—RAS fermentation period phosphorus and organic carbon data. For Period 1, both reactors were subjected to a 4-hour RAS fermentation period, while for Period 2 the RAS fermentation period was reduced to 2 hours.

Parameter	Units	Reactor	Period	Average	SD	*n*
RAS fermentation phosphorus release	mgP	V2-EBPR	1	2.8	1.8	8
			2	1.3	1.9	12
		R2-EBPR	1	4.5	3.6	8
			2	0.5	0.6	8
RAS fermentation VFA synthesis	mg COD	V2-EBPR	1	16.8	29.3	4
			2	0.68	1.79	7
		R2-EBPR	1	2.04	2.50	4
			2	4.33	6.13	2
	Cmmol	V2-EBPR	1	0.50	0.90	4
			2	0.02	0.05	7
		R2-EBPR	1	0.06	0.07	4
			2	0.13	0.18	2
RAS fermentation glycogen consumption	Cmmol	V2-EBPR	1	0.13	0.69	3
			2	0.44	1.7	8
		R2-EBPR	1	–0.30	0.60	4
			2	0.01	0.02	3
RAS fermentation PHA synthesis	Cmmol	V2-EBPR	1	–0.44	1.20	4
			2	0.00	0.00	8
		R2-EBPR	1	0.00	0.30	4
			2	0.00	0.00	3

Table 3—Anaerobic period organic carbon and phosphorus data for all tested reactors. For Period 1, V2- and R2-EBPR were subjected to a 4-hour RAS fermentation period, while for Period 2 the RAS fermentation period was reduced to 2 hours.

Parameter	Units	Reactor	Period	Average	SD	*n*
Anaerobic VFA uptake	mgVFA (as COD)	V-EBPR	all	117.2	73.2	32
		V2-EBPR	1	214.5	59.4	4
			2	144.2	97.3	13
		R-EBPR	all	30.4	25.9	17
		R2-EBPR	1	26.0	20.6	4
			2	60.9	50.2	8
	Cmmol	V-EBPR	all	3.33	2.11	32
		V2-EBPR	1	6.05	1.62	4
			2	4.09	2.76	13
		R-EBPR	all	0.90	0.75	17
		R2-EBPR	1	0.77	0.62	4
			2	1.78	1.45	8
Anaerobic glycogen consumption	Cmmol	V-EBPR	all	0.68	1.44	9
		V2-EBPR	1	2.32	1.28	3
			2	4.30	1.52	6
		R-EBPR	all	0.00	0.12	6
		R2-EBPR	1	–0.52	0.96	3
			2	0.00	0.06	3
Anaerobic PHA synthesis	Cmmol	V-EBPR	all	1.90	1.60	9
		V2-EBPR	1	0.54	1.24	4
			2	1.28	1.32	5
		R-EBPR	all	0.20	0.86	7
		R2-EBPR	1	0.72	1.82	4
			2	0.00	0.00	3
Anaerobic P release	mgP	V-EBPR	all	9.0	8.3	36
		V2-EBPR	1	3.6	7.8	6
			2	9.5	5.1	18
		R-EBPR	all	1.6	2.8	13
		R2-EBPR	1	7.2	4.9	8
			2	1.5	0.7	8

Table 4—Influent and effluent phosphorus data for the EBPR reactors. Additionally, the influent VFA:P ratio is presented. For Period 1, V2- and R2-EBPR were subjected to a 4-hour RAS fermentation period, while for Period 2 the RAS fermentation period was reduced to 2 hours.

Parameter	Units	Reactor	Period	Minimum	Maximum	Average	SD	*n*
Influent phosphorus	mgP/L	V-EBPR	all	1.3	12.7	6.4	2.1	63
		V2-EBPR	1	9.2	4.4	7.5	1.4	8
			2	8.7	1.0	5.2	2.0	38
		R-EBPR	All	8.1	1.0	3.9	1.5	48
		R2-EBPR	1	6.2	3.7	5.1	0.9	8
			2	6.0	1.0	3.5	1.2	32
Effluent phosphorus	mgP/L	V-EBPR	all	0.00	6.6	2.9[1]	1.6	170
		V2-EBPR	1	0.2	6.2	2.4	1.8	17
			2	0.04	7.2	1.9	1.7	111
		R-EBPR	all	0.3	5.8	2.7	0.8	139
		R2-EBPR	1	0.02	2.9	1.0	1.1	17
			2	0.01	5.1	2.0	1.0	70
Influent VFA:Influent P	mgVFA (as COD)/mgP	V-EBPR	all	7.7	73.6	29.2	17.1	32
		V2-EBPR	1	17.1	65.8	35.4	21.1	4
			2	9.3	88.1	37.5	24.5	13
		R-EBPR	all	0.0	74.4	15.4	17.9	17
		R2-EBPR	1	0.0	10.8	5.4	4.8	4
			2	2.6	74.4	22.3	24.3	8

[1] For the first 91 days of the investigations, effluent P was 0.43 ± 0.54 mgP/L ($n = 16$).

RAS Fermentation Effects on EBPR Anaerobic Phosphorus Release. Polyphosphate accumulating organisms that are consuming VFAs should exhibit a marked P release anaerobically, measured via bulk solution P; the anaerobic P release is associated with ATP synthesis in support of the VFA uptake and catabolism. Ultimately, anaerobic phosphorus release is an important macro-level indicator of potential EBPR performance, in that the response indicates successful induction of EBPR metabolisms that should lead to low effluent P from the WRRF process. Thus, it is important to analyze P release during the RAS fermentation period (i.e., preceding the EBPR anaerobic period where requisite P release occurs), as potentially related to improved or deteriorated EBPR performance.

First considering the RAS fermentation period, similar to results observed in the batch evaluations, a P release was observed under both 4-hour and 2-hour conditions. The V2-EBPR MMC averaged an increase in bulk solution P of 2.8 and 1.3 mg P for the 4- and 2-hour RAS fermentation periods, respectively (Table 2; Figures 3a, 3c). Comparatively, R2-EBPR—which did not receive the model EBPR VFA-augmented substrate provided to V2-EBPR—experienced a much higher RAS fermentation P release for the 4-hour period: 4.5 mg P (Table 2; Figure 3b); however, for the 2-hour RAS fermentation period, the R2-EBPR MMC P release was lower than V2-EBPR at 0.5 mg P (Table 2; Figure 3d). Normalized to TS, the V2-EBPR MMC (4-hour, 2-hour fermentation periods) realized a P release of 0.58 and 0.15 mg P/g TSS, while R2-EBPR was 0.68 and 0.26 mgP/gTSS. In all cases the normalized P release was markedly lower than observed in the batch fermentation tests.

Return activated sludge fermentation inducing P release—particularly by the V2-EBPR MMC—suggests EBPR metabolic activity was potentially pre-induced prior to blending the MMC with influent substrate. In this regard, pre-induction of EBPR metabolisms could be observed through (1) metabolically linked VFA uptake-PHA synthesis under RAS fermentation (a potentially positive response), (2) effects on subsequent EBPR anaerobic period P release (potentially positive or negative response), and/or (3) enhanced overall P removal (addressed in the subsequent section). Considering (1), as described, commensurate metabolically linked carbon metabolisms were not seemingly induced (e.g., VFA uptake and synthesis of PHA). Regarding (2), MMC hydrolyzing polyP reserves prior to blending with fresh wastewater (commonly referred to as secondary P release [Barnard, 1984; Barnard and Fothergill, 1998]) is not necessarily viewed as a positive metabolic response for EBPR processes. Return activated sludge fermentation P release could potentially deplete polyP energetic reserves necessary to drive EBPR metabolisms once the fermented RAS was blended with new substrate. In this regard, such a concern would be most pertinent for the V2-EBPR MMC, which received VFAs augmented in the substrate and thus would require more ATP energy anaerobically (from polyP —as well as glycogen) to uptake and catabolize the carboxylate substrate to PHA (Filipe et al., 2001). For V2-EBPR, the MMC did exhibit additional release P anaerobically when provided exogenous VFAs (Table 3; Figures 3a, 3c)—averaging 9.5 mg P (2-hour RAS fermentation) and 3.6 (4-hour RAS fermentation) mg P in bulk solution. Comparatively, the V-EBPR MMC, which received the same VFA-rich substrate as V2-EBPR but did not experience RAS fermentation, on average realized an anaerobic P release that was nearly the same as the 2-hour RAS fermentation (Figures 3c vs 3e; Table 3); however, extended RAS fermentation conditions (4 hours) did appear to partially deplete the polyP reserves in the V2-EBPR MMC, with markedly reduced anaerobic P release relative to V-EBPR (Figures 3a vs 3e; Table 3). In contrast to comparative observations between V- and V2-EBPR, enhanced

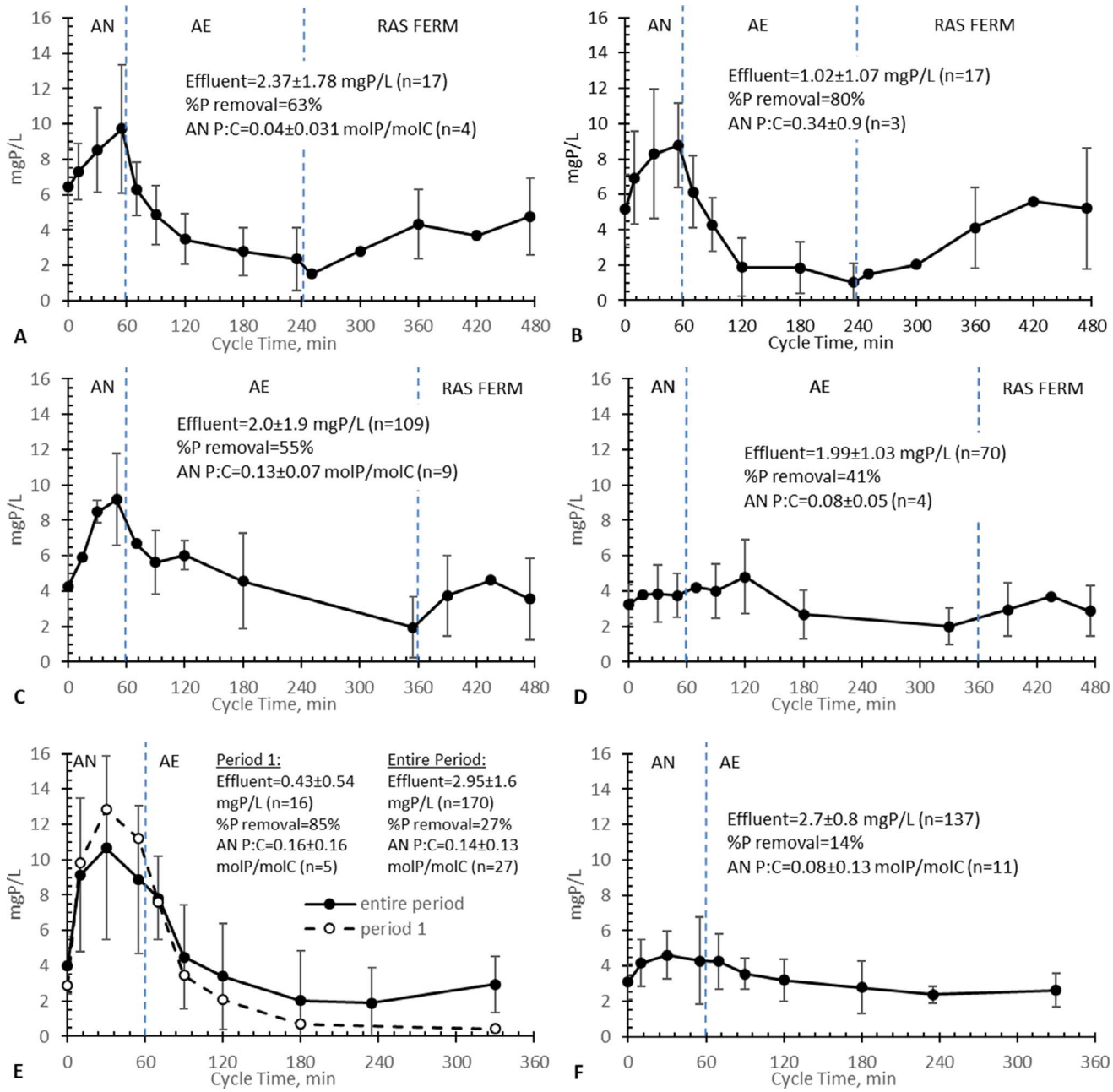

Figure 3—Average phosphorus cycling for (a) V2-EBPR, 4-hour RAS fermentation period; (b) R2-EBPR, 4-hour RAS fermentation period; (c) V2-EBPR, 2-hour RAS fermentation period; (d) R2-EBPR, 2-hour RAS fermentation period; (e) V-EBPR (for both Period 1 and for entire operational period; standard deviations only shown for entire operational period); and (f) R-EBPR. Error bars indicate standard deviation.

anaerobic P release was observed by the R2-EBPR MMC under 4-hour RAS fermentation conditions, relative to R-EBPR (Table 3; Figures 3b vs 3f). Under shorter RAS fermentation conditions (2 hours), though, the anaerobic P release was comparable (Figures 3d vs 3f).

Considering the theoretical importance of P release in EBPR success, a metric based on P release is useful in process monitoring and troubleshooting. Smolders et al. (1994) and later Filipe et al. (2001), in developing a PAO metabolic model, proposed an empirical metric known as the P:C ratio to encapsulate the relationship. The P:C ratio is calculated as the mass of P released divided by the mass of VFAs removed from bulk solution (mole basis); larger P:C ratios in theory suggest better overall EBPR performance, at least for VFA-fed systems. Applying the P:C concept to this study, consistent with the relatively low anaerobic P release under 4-hour RAS fermentation conditions, the V2-EBPR MMC exhibited a low average P:C ratio (0.04; Figure 3a); with increased anaerobic P release and decreased VFAs, the average P:C ratio under 2-hour RAS fermentation increased to 0.13 (Figure 3c). For the R2-EBPR

Table 5—Estimated fraction of PAOs and GAOs in the respective EBPR reactors. For PAOs, quantitation was made using both 16S rDNA primers and *ppk* primers. For Period 1, V2- and R2-EBPR were subjected to a 4-hour RAS fermentation period, while for Period 2 the RAS fermentation period was reduced to 2 hours.

		PAOs: 16S rDNA			PAOs: *ppk*			GAOs		
Reactor	Period	avg	SD	*n*	avg	SD	*n*	avg	SD	*n*
V2-EBPR	1	0.08%	0.09%	26	1.99%	1.80%	23	1.12%	1.33%	14
	2	0.07%	0.07%	20	0.85%	0.37%	20	1.24%	1.77%	20
R2-EBPR	1	0.15%	0.13%	24	1.80%	0.77%	22	0.18%	0.19%	14
	2	0.01%	0.02%	16	1.02%	0.66%	16	0.00%	0.00%	16
V-EBPR	All	0.35%	0.75%	45	1.75%	1.08%	42	0.67%	1.15%	33
R-EBPR	All	0.02%	0.03%	42	1.86%	1.07%	39	0.02%	0.03%	29

MMC, the P:C was 0.34 (4 hours; Figure 3b) and 0.08 (2 hours; Figure 3d). Comparatively, the P:C for V-EBPR was 0.14 (0.16 in period 1; Figure 3e), and 0.08 for R-EBPR (Figure 3f). Collectively, the observed P:C ratios for VFA-augmented systems were low relative to previously observed data for raw wastewater-based EBPR configurations (summarized in Coats et al. [2017]). Moreover, the P:C ratios might suggest potential enrichment of GAOs (Lopez-Vazquez et al., 2007; Oehmen et al., 2007), although real wastewater-based EBPR systems have been shown to exhibit P:C ratios as low as 0.15 to 0.2 while concurrently realizing successful EBPR (Coats et al., 2017).

Phosphorus Removal Performance—With and without RAS Fermentation. Ultimately, the value of any EBPR configuration is gauged by its ability to remove excess P from wastewater. As noted, EBPR can reliably produce effluent P concentrations of 0.5 mg/L (Tchobanoglous et al., 2014), with numerous full-scale municipal WRRFs reporting effluent near or below 0.1 mg P/L. Evaluating the performance of the RAS fermentation EBPR reactors, both V2- and R2-EBPR configurations performed quite poorly. Indeed, V2-EBPR effluent averaged 2.4 and 1.9 mg P/L (4- and 2-hour RAS fermentation periods, respectively; Table 4), while R2-EBPR effluent averaged 1.0 and 2.0 mg P/L. Notably, except for R2-EBPR, 4-hour RAS fermentation, the observed P:C ratios (being less than 0.15) would similarly predict poor effluent P (Coats et al., 2017).

The primary "control" reactor included in this study, V-EBPR, did not necessarily exhibit better overall effluent P characteristics (average 2.9 mg/L; Table 4 and Figure 3e). It might thus be argued that, relatively speaking, the RAS fermentation EBPR reactors performed well. However, the V-EBPR MMC actually performed quality EBPR until nitrification control was partially lost. Specifically, for the first 91 days of the investigations, nitrification control was near-complete; commensurately, effluent P averaged 0.43 mg/L, with a P:C of 0.16—aligning well with Coats et al. (2017). In addition to data collected and presented herein, the performance of reactor V-EBPR (with nitrification control) is documented elsewhere (Coats et al., 2017; Coats et al., 2015); this reactor has been operated continuously for more than 3 years, consistently producing effluent P of 0.25 ± 0.3 mg P/L ($n = 190$). However, after day 91 in this study, associated with inconsistent dosing of nitrification control chemicals, residual nitrate (average of 3.7 mg N/L) remained in the effluent; commensurately, effluent P averaged 3.2 mg/L. Residual effluent nitrate ultimately yielded pre-anoxic conditions during the designated anaerobic period, consuming critical VFAs and adversely affecting anaerobic P release. Indeed, excess RAS nitrate is a well-known and common cause of EBPR failure. Return activated sludge fermentation can, theoretically, remedy this problem; in this regard, RAS nitrate effects on the RAS fermentation configuration are discussed in a subsequent section.

Return Activated Sludge Fermentation and PAOs. Successful EBPR occurs when the MMC is sufficiently enriched with PAOs; in this regard, theoretically for V- and V2-EBPR, conditions applied in this research (wastewater temperature; blend of acetate and propionate) would enrich for PAOs over GAOs (Lopez-Vazquez et al., 2009). Although no target minimum fractional enrichment of PAOs, relative to the total eubacterial population, has been established to achieve and sustain EBPR, relative comparisons within and between EBPR-configured systems can inform useful interpretations. In this regard, qPCR was applied to estimate the fraction of PAOs in the respective MMC, using both 16S rDNA and *ppk*-based primer sequences. Although recent research suggests that conventional 16S rDNA-based primers might target PAOs other than *Candidatus* Accumulibacter phosphatis (Coats et al., 2017), research has nonetheless affirmed that these primers are indicative of a PAO-enriched MMC (Albertsen et al., 2016; He et al., 2010; McMahon et al., 2007). In addition to PAOs, the fractional GAO population was quantified.

First considering PAOs based on the 16S-based primers, as shown (Table 5) the average PAO fraction ranged from 0.01% (R-EBPR) to 0.35% (V-EBPR); overall the estimated fractional populations were generally lower than observed in similar, recent research (Coats et al., 2017; Coats et al., 2015). Notably, the fractional population estimated using the functional gene PPK suggested greater EBPR potential in all tested bioreactors. Examining PAO fractions against EBPR performance, the V2-EBPR MMC exhibited a low PAO population, with little difference between 4-hour and 2-hour RAS fermentation periods, while concurrently exhibiting poor overall P removal. For R2-EBPR, it is notable that the higher fraction of PAOs (0.15%, 4-hour RAS fermentation) was associated with the best overall average effluent P of all RAS fermentation reactors (1.0

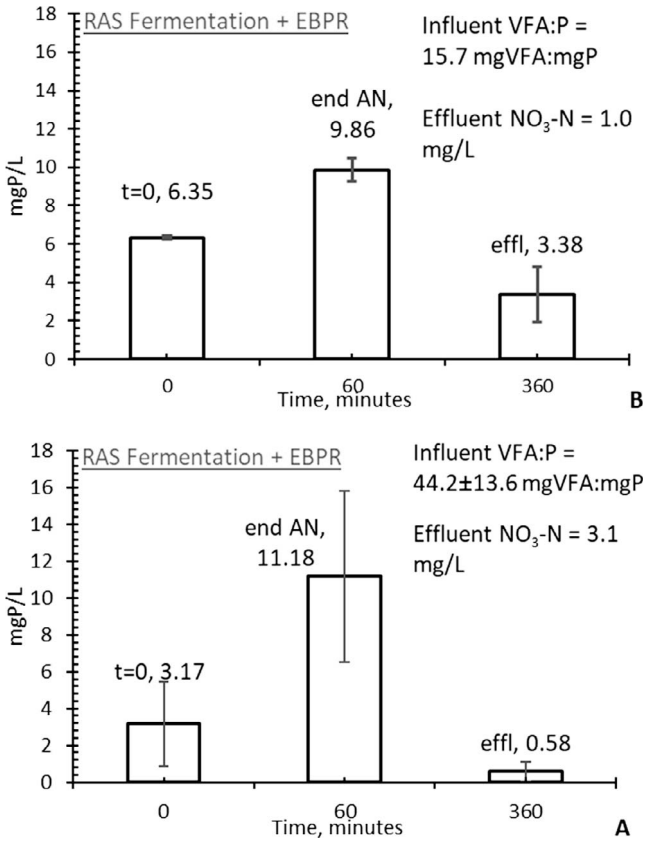

Figure 4—EBPR P cycling for V2-EBPR (2-hour RAS fermentation) with variable quantities of nitrate in the RAS. Error bars indicate standard deviation.

mg P/L; Table 4). Conversely, fractional PAOs for the 2-hour RAS fermentation R2-EBPR MMC were comparable to that of R-EBPR; average performance of the two reactors was poor and quite similar (Figures 3d, 3f). Regarding the V-EBPR MMC, during the first 91 days of operation, when good P removal was observed, the %PAOs was estimated at 0.63 ± 0.97% ($n = 25$)—markedly higher than observed over the entire operational period (Table 5).

Beyond the Accumulibacter 16S rDNA-based data, an interesting result was the seemingly larger enrichment of bacteria containing *ppk* across all bioreactors. For the 4-hour RAS fermentation conditions (both reactors), although the apparent functional potential did not translate into improved EBPR performance, the extended RAS fermentation period may have caused of the increased enrichment (relative to the 2-hour RAS fermentation period). Indeed, both the V2- and R2-EBPR MMC exhibited greater overall P release under extended RAS fermentation conditions; *ppk* is a bifunctional gene that can both synthesize and hydrolyze polyP (Kornberg et al., 1999). More generally, the *ppk*-based population data does not align well with EBPR performance, suggesting that imposed EBPR conditions (i.e., alternating anaerobic–aerobic environments) can enrich for a MMC with greater functional potential than actually realized. Finally, these results are consistent with that of Coats et al. (2017), where it was shown that amplicons based on the conventional Accumulibacter 16S rDNA primer set seemingly underrepresented the PAO population.

EBPR, RAS Fermentation, and RAS Nitrate. As noted, excess RAS nitrate is a primary cause of EBPR failure; RAS fermentation can serve to denitrify the RAS prior to blending with substrate (i.e., the Johannesburg process [Tchobanoglous et al., 2014]), thereby potentially stabilizing EBPR. While overall the RAS fermentation process configuration presented and discussed herein, for the RAS fermentation HRTs applied, did not exhibit any benefit to improved EBPR, closer examination of the RAS nitrate data revealed interesting insights related to MMC microbiology and potential process stability.

Investigations herein used real wastewater, not synthetic substrate; consequentially, vigilant nitrification control is required to sustain a cyclical anaerobic–aerobic condition, as the new substrate can introduce autotrophic bacteria every operational cycle. In this regard, during operational days 177 through 246, when incomplete nitrification control was realized, V2-EBPR (2-hour RAS fermentation) experienced greater nitrification than realized throughout the remainder of the investigations; average RAS nitrate was 3.1 ± 3.1 mg/L, ranging from 0.6 to 13.6 mg/L ($n = 45$), while the average was 1.3 ± 0.7 mg/L ($n = 46$) after this operational period (days 247–299). Commensurate with the higher RAS nitrate concentrations, effluent P was markedly lower—at 0.58 ± 0.52 ($n = 46$)—than observed over the whole of the V2-EBPR, 2-hour RAS fermentation operational period (1.9 mg P/L; Table 4) as well as after operational day 246 (2.6 ± 1.5 mg P/L [$n = 45$]). Similarly, P cycling over the SBR cycle (Figure 4a) was more typical of a system performing quality EBPR.

Recognizing the improved EBPR performance associated with increased nitrate, enhanced vigilance on nitrification control was imposed beginning on operational day 247; the purpose was to evaluate in greater detail RAS fermentation EBPR with low RAS nitrate. Interrogation of an SBR cycle under enhanced nitrification control was performed on operational days 261, 263, and 265. Compared to the "high" nitrate conditions, average RAS nitrate was 1.0 mg/L and effluent P was 3.38 mg/L; moreover, P cycling over the SBR cycle was less typical of a VFA-fed MMC (Figure 4b). It could be suggested influent VFA concentrations potentially confounded the interpretation of these results; however, it would appear that influent carbon relative to RAS nitrate was comparable between the two operational periods (Figures 4a, 4b). Moreover, while the VFA:P ratio under "low" RAS nitrate (Figure 4a) was theoretically sufficient to induce good EBPR performance (exceeding 15 mg VFA_{COD}/mg P; [Coats et al., 2017]), it would appear that other factors adversely impaired overall P removal.

To better understand and potentially explain the differences in EBPR performance, the anaerobic period for V2-EBPR and V-EBPR on operational days 261, 263, and 265 was interrogated, with an emphasis on carbon cycling and associated anaerobic period EBPR energetics. To quantify anaerobic energy production, ATP synthesis from polyP hydrolysis was estimated to be generated on a 1 mol ATP to 1 mol P(rel) ratio (Smolders et al., 1994). ATP is also generated anaerobically through substrate

Table 6—Summary of anaerobic period energetics analysis for V2-EBPR (2-hour RAS fermentation period) and V-EBPR for operational days 261, 263, and 265. The "% Energy" values represent the ATP fraction generated via glycogen catabolism or polyP hydrolysis relative to ATP demands for VFAs consumed.

	V2-EBPR	V-EBPR
VFA consumption, Cmmol	2.9	1.9
Glycogen Consumption, Cmmol	3.1	1.1
Phosphorus release, Pmmol	0.22	0.67
% Energy from glycogen	62.7	37.5
% Energy from PolyP	9.1	39.8

level phosphorylation associated with glucose (from glycogen) catabolism (Madigan and Martinko, 2006); a value of 0.5 mol ATP per Cmol was used, assuming the Embden–Myerhof pathway was used (which is consistent with Schuler and Jenkins [2003]). ATP requirements for VFA uptake were estimated using an equation developed by Filipe et al. (2001): α (mol ATP mol VFA^{-1}) = $0.16^{*}pH_{out} - 0.7985$. The α ranged from 0.47 to 0.50 (consistent with observed pH of 7.9 to 8.1). The estimated α values were consistent with prior investigations on similar EBPR reactors (Coats et al., 2015). For VFA uptake, estimates of required ATP (mol ATP mol VFA^{-1}) were 0.5, 0.33, 0.25, and 0.2 for HAc, HPr, HBu, and HVa, respectively (Braunegg et al., 1998; Zhang et al., 2008). Results from this anaerobic EBPR energetics analysis (under "low" RAS nitrate conditions) are summarized in Table 6, compared against V-EBPR on the same operational days. As shown, the V2-EBPR MMC exhibited a dominant use of glycogen for anaerobic ATP as compared to the V-EBPR MMC. Conversely, nominal ATP was derived from polyP hydrolysis (<10%), whereas the V-EBPR MMC generated four times the comparative energy from polyP.

The V2-EBPR MMC seemingly generating the dominant fraction of anaerobic ATP via glycogen catabolism is indicative of a GAO, not PAO, metabolism; GAOs are believed to be incapable of polyP storage (Oehmen et al., 2007). Returning to the qPCR data, the GAO fraction of the MMC for V2-EBPR was 8.71 times larger beginning on operational day 247 ("low" RAS nitrate) versus operational days 177 to 246 ("high" RAS nitrate). Moreover, the V2-EBPR contained 4.3 times the fraction of GAOs versus V-EBPR after operational day 247. For the entire period of operations for the 2-hour RAS fermentation V2-EBPR reactor (wherein generally poor EBPR performance was observed), GAOs numbered more than twice that of the V-EBPR MMC, while the PAO population (16S rDNA-based) was five times greater in V-EBPR (Table 5). Combined with the anaerobic carbon data, it would appear that RAS fermentation integrated with EBPR has the potential to enrich for a more GAO-dominated culture, depending on the RAS nitrate concentration. Indeed, research suggests the environmental state in the RAS denitrification basin should be predominantly anoxic, not anaerobic, aligning well with that of Winkler et al. (2011).

Conclusions

The purpose of this study was to evaluate the effects of integrating RAS fermentation within an EBPR scheme, with the principal aim of producing VFAs and reducing RAS nitrate to improve EBPR performance. Key conclusions are as follows:

- In general, for the applied RAS fermentation HRTs of 2 and 4 hours, RAS fermentation integrated with an EBPR scheme did not improve process performance. There was no observed improvement in VFA or related carbon production in the RAS fermentation period, under either batch fermentation or fed-batch operations.
- Under conditions of low influent VFA wastewater, results suggest that longer duration RAS fermentation (≥4 hours) could potentially improve EBPR performance—if no other VFA production options are available, and if effluent P requirements are not stringent.
- Critically, RAS fermentation appeared to potentially enrich for GAOs in the MMC—which is an outcome that could be quite detrimental to EBPR performance.
- The potential value in RAS fermentation appears to be RAS denitrification, not VFA production. However, even for this purpose, process failure can result if anaerobic conditions are achieved in the RAS fermentation basin associated with either low RAS nitrate or excess HRT.

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