EBPR Using Crude Glycerol: Assessing Process Resiliency and Exploring Metabolic Anomalies

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ABSTRACT: Enhanced biological phosphorus removal (EBPR) is predicated on exposing bacteria to cyclical anaerobic/aerobic environments while providing volatile fatty acids (VFAs). Combined, this environment enriches for phosphorus accumulating organisms (PAOs) and induces metabolisms to ensure excess phosphorus removal. Crude glycerol (CG), a byproduct of biodiesel manufacturing, is an alternate waste stream that could be substituted to achieve excess phosphorus removal; research into the use of CG yielded unexpected findings. While CG was an excellent substrate to accomplish and/or help achieve excess phosphorus removal, CG-fed bacteria did not consistently exhibit theoretical EBPR metabolisms. Specifically, anaerobic phosphorus release was not required for successful EBPR; however, carbon cycling patterns were consistent with theory. Analysis of results suggests that PAOs will first leverage carbon to generate energy anaerobically; only as needed will the bacteria utilize polyphosphate reserves anaerobically. Results also demonstrated that excess phosphorus removal can be achieved with a small fraction of PAOs. *Water Environ. Res.*, **87,** 68 (2015).

KEYWORDS: enhanced biological phosphorus removal (EBPR), crude glycerol, polyphosphate accumulating organisms (PAOs), glycogen accumulating organisms (GAOs), volatile fatty acids (VFAs), polyphosphate.

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Introduction

Phosphorus (P) is an inorganic nutrient that can contribute to advanced surface water eutrophication if released into the aquatic environment in excess quantities and when phosphorus is the limiting nutrient. In this regard, water resource recovery facilities (WRRFs) are realizing increasingly stringent effluent phosphorus limitations in an effort to reduce point-source discharges. Enhanced biological phosphorus removal (EBPR) is an engineered wastewater treatment process configuration that can be used to successfully achieve low effluent phosphorus concentrations. Compared to chemical treatment methods, EBPR is a more environmentally sustainable alternative (Coats, Watkins, and Kranenburg, 2011) and should be considered a first line of defense in achieving wastewater phosphorus removal.

Successful EBPR is theoretically predicated 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). Within this engineered system, the MMC becomes enriched with polyphosphate accumulating organisms (PAOs) that are capable of excess phosphorus removal. The PAOs uptake and store VFAs anaerobically as polyhydroxyalkanoate (PHA). The energy required for the uptake and catabolism of VFAs is 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 can ultimately result in a large increase in bulk solution phosphorus. In the aerobic environment, PAOs oxidize PHA for energy to grow and to replenish internal glycogen and polyP reserves. Through this cyclical process, more polyP is stored than was released anaerobically (principally through PAO growth), resulting in a significant net decrease of phosphorus in the bulk solution (Oehmen et al., 2007).

To consistently achieve low effluent phosphorus concentrations, the EBPR process theoretically requires an adequate quantity of VFAs to drive the series of biochemical reactions necessary for maximal phosphorus removal. Although acetate is the model substrate for EBPR (Fuhs et al., 1975; Smolders et al., 1995), it has also been suggested that the three-carbon VFA propionate is a favorable EBPR substrate (Oehmen et al., 2007). To generate sufficient VFAs, EBPR WRRFs often incorporate some form of primary solids fermentation. For WRRFs without a fermenter or those receiving waste streams low in organic substrate, external VFA (typically acetate) addition may be considered. The addition of synthetically derived VFAs increases treatment costs considerably while concurrently increasing the WRRF carbon footprint (VFA manufacturing, transport, etc.).

As an alternative to augmenting the EBPR process with synthetic VFAs, some WRRFs may have ready access to other organic carbon-rich waste streams. One such waste source would be crude glycerol (CG), which is a byproduct of biodiesel production. Crude glycerol is produced at a rate of approximately 1 kg per 12.6 L of biodiesel created (Thompson et al., 2006). The primary components of CG are glycerol and residual ethanol or methanol–carbon sources that are direct precursors for EBPR-critical PHA synthesis (Ashby et al., 2004) and thus potential inducers of EBPR metabolisms. The combination of positive attributes associated with this waste stream—readily biodegradable carbon, minimal cost (potentially free), and availability in an existing industrial process (possibly at a local

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Table 1—Summary of bioreactor operational parameters.

Reactor	Substrate (% by volume)	Average MLSS (mg/L)
R-EBPR	100% raw wastewater	961
V-EBPR	90% raw wastewater, 10% fermenter liquor (v/v)	1894
G-EBPR	Raw wastewater plus 0.132 mL CG each cycle	1485
V2-EBPR	Initially: 90% raw wastewater, 10% fermenter liquor (v/v) After day 406: Raw wastewater plus 0.132 mL CG each cycle	1542
G2-EBPR	Initially: Raw wastewater with 0.2 mL CG L^{-1} After day 406: 90% raw wastewater, 10% fermenter liquor (v/v)	1789

facility)—makes CG an EBPR substrate worthy of further investigation.

Glycerol and methanol have proven successful as a carbon source for denitrification (Akunna et al., 1993; Grabinska-Loniewska et al., 1985). Regarding EBPR, pure glycerol (not CG) was evaluated by Yuan et al. (2010) in an EBPR system enriched on acetate. The authors concluded that glycerol was unable to induce EBPR metabolisms unless it was pre-fermented to VFAs. Guerrero et al. (2012) drew similar conclusions, and their recommendations focused on achieving fermentation within the anaerobic zone of the EBPR system (to produce VFAs) by employing long anaerobic hydraulic retention times (4+ hours). Regarding methanol, Puig et al. (2008) found that pure methanol fed to PAOs enriched on ethanol led to very low phosphorus release and uptake rates. Louzeiro et al. (2002) similarly concluded that pure methanol could not directly drive EBPR.

Although pure glycerol has been considered as an alternate EBPR substrate, to the authors' best knowledge the EBPR process has yet to be investigated using crude glycerol. Recognizing this data gap—and also the ready availability of this waste carbon source associated with a growing biodiesel industry worldwide—the objective of the research presented herein was to assess the potential of using CG as a direct substitute in accomplishing EBPR. Research was specifically focused on characterizing the performance and associated MMC of an EBPR-configured system augmented with CG in lieu of VFA-rich fermenter liquor. Stress testing was also performed to examine EBPR performance when MMC enriched on CG or VFAs experienced a switch in substrate.

Materials and Methods

Source of Microorganisms, Wastewater, and Crude Glycerol. Inocula were obtained from the Moscow, Idaho, WRRF, an A^2/O (the oxic element being an oxidation ditch) EBPR process. Raw wastewater was obtained from the same facility, downstream of screening and grit removal. The VFA-rich fermenter liquor was recovered from a bench-top fermenter fed thickened primary solids from the Pullman, Washington, WRRF. Crude glycerol was acquired from the Biological and Agricultural Engineering Department at the University of Idaho (Moscow, Idaho), generated from their pilot-scale biodiesel production system (Thompson et al., 2006). All wastewater was stored at 4 °C until used, and raw wastewater was obtained every 2 to 7 days. Bioreactor feed tanks were replenished daily.

Bioreactor Operating Conditions. Three 2L laboratory-scale sequencing batch reactors (SBRs) were operated at room temperature without pH control (the pH varied between 7.2 and 8.2, and never decreased below 7). One SBR (R-EBPR) received only raw wastewater (control). The second SBR (V-EBPR) received a traditional EBPR substrate of wastewater and VFA-rich fermenter liquor. The third SBR (G-EBPR) received raw wastewater augmented with CG. Substrate conditions and carbon speciation are summarized in Tables 1 and 2. On day 367, the V2-EBPR and G2-EBPR "child" reactors were established, each inoculated with biomass from and operated consistent with their "parents"; on day 406, the substrate provided to each "child" reactor was switched (i.e., V2-EBPR started receiving CG and vice versa).

Each 6-hour SBR cycle included the following periods: feed (5 minutes), anaerobic (1 hour), aerobic (4 hours, 15 minutes), settle (30 minutes), and decant (10 minutes). A programmable logic controller was used to control operations. Effluent was decanted each cycle and replaced with an equal volume of substrate to maintain an 18-hour hydraulic residence time. The solids residence time (SRT) was controlled at 10 days by manually wasting 200 mL of mixed liquor at the end of an aerobic period once a day. Anaerobic conditions were maintained by stopping aeration approximately 30 minutes before the settling period to allow dissolved oxygen (DO) depletion; DO measurements confirmed that anaerobic conditions prevailed. Air was introduced through stone diffusers to create aerobic conditions (DO $>$ 2 mg L^{-1}). Reactors were completely mixed with magnetic stir bars. Nitrification was inhibited principally through the regular addition of thiourea, occasionally augmented with 2-chloro-6(trichloromethyl) pyridine (Clesceri et al., 1998). All pumping was performed using peristaltic pumps (Watson Marlow Bredel, Wilmington, Massachusetts).

Analytical Techniques. Samples were collected to monitor pH, DO, soluble reactive phosphate (SRP), nitrate (NO_3), ammonia (NH_3), VFAs, mixed-liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), glycogen, PHA, glycerol, and methanol. For soluble constituents, samples were first centrifuged to remove biomass and then filtered through a 0.22-μm syringe filter (Millipore Corp., Billerica, Massachusetts) prior to testing. Total phosphorus was determined in accordance with Hach (Loveland, Colorado) method 8190 while SRP was determined in accordance with Hach method 8048 (both methods equivalent to Standard Methods 4500-PE [Clesceri, 1998]). Biomass phosphorus was recovered for analysis through digestion at 150 °C in nitric acid. Soluble NO_3 was determined in accordance with Hach method 10020, while soluble NH_3 testing followed Hach method 10031. A Spectronic 20 Genesys spectrophotometer (Thermo-Fisher Scientific Corp, Waltham, Massachusetts) was used to measure the absorbance of the reacted sample at a wavelength of 890 nm for TP/SRP, 410 nm for NO_3, and 655 nm for NH_3. Phosphate, NO_3, and NH_3 concentrations were determined using a standard curve ($R^2 >$ 0.99).

Table 2—Influent substrate carbon speciation (average, standard deviation; range also presented for the glycerol substrate).

	Substrate concentrations (mg/L)		
Organic carbon species	**Raw wastewater + fermenter liquor**	**Raw wastewater + crude glycerol**	**Raw wastewater**
Acetate	73.2 ± 34.9	31.1 ± 18.7	14.2 ± 17.7
Propionate	65.5 ± 53.4	13.0 ± 10.0	0
Butyrate	10.1 ± 7.9	1.1 ± 1.9	0
Valerate	1.0 ± 2.6	0.1 ± 0.3	0
Methanol	0	15–20	0
Glycerol	0	60–70	0

Mixed-liquor suspended solids and MLVSS were measured in accordance with Standard Methods 2540 D and 2540 E (Clesceri et al., 1998), respectively. Measurement of pH was accomplished with a Thermo-Fisher Scientific Accumet AP85 Waterproof pH/Conductivity Meter. Dissolved oxygen measurements were collected using a Hach HQ30d Meter with a LDO101 DO Probe.

Volatile fatty acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids) and methanol were quantified using a Hewlett-Packard 6890 series gas chromatograph (Agilent Technologies, Santa Clara, California) equipped with a flame-ionization detector and a Hewlett-Packard 7679 series injector. The system was interfaced with the Hewlett-Packard GC ChemStation software version A.06.01. The VFA separation was achieved using a capillary column (Heliflex AT-AquaWax-DA , 30 m × 0.25 mm ID, W. R. Grace & Co., Deerfield, Illinois), which was ramped from an initial 50 °C to 200 °C in three steps (following 2 minutes at 50 °C, ramp to 95 °C at 30 °C per minute then to 150 °C at 10 °C per minute and hold for 3 minutes; finally, ramp to 200 °C at 25 °C per minute; and hold for 12 minutes) with helium as the carrier gas (1.2 mL per minute). The split/splitless injector and detector were operated isothermally at 210 and 300 °C, respectively. Prior to analysis, samples were acidified to a pH of 2 using HCl; 0.5 μL of each sample was injected in 20:1 split mode. Volatile fatty acid and methanol concentrations were determined through retention time matching with known standards (Sigma-Aldrich Co., St. Louis, Missouri; Thermo-Fisher Scientific) and linear standard curves ($R^2 > 0.99$). Soluble glycerol concentrations were quantified colorimetrically (570 nm) using the EnzyChrom Glycerol Assay Kit from BioAssay Systems (Hayward, California). Pure glycerol standards were used to generate a standard curve ($R^2 > 0.96$).

Biomass PHA content was determined by gas chromatography/mass spectrometry (GC-MS) as described in Coats, Mockos, and Loge (2011). Total intracellular PHA content was determined on a percent dry weight cell basis (mass PHA [mass of biomass]$^{-1}$, w/w). Glycogen was determined as total glycogen with dried biomass samples according to Parrou and Francois (1997).

Quantitative Polymerase Chain Reaction. The quantitative polymerase chain reaction (qPCR) was used to quantify 16S rDNA from total bacteria, Accumulibacter (the model PAO), and glycogen accumulation organisms (GAOs) to provide an estimation of relative abundance. Total bacterial and Accumulibacter 16S rDNA were quantified with primer sets 341f/534r and 518f/846r, respectively (He et al., 2007). The GAOs were quantified using primer set GAOQ431f/GAOQ989r (designed to target *Candidatus* Competibacter phosphatis, which is a putative model GAO [Crocetti et al., 2002]) and the total bacteria primer set. In addition, a primer set targeting the GB lineage (specifically GB612f/GAOQ989r [Kong et al., 2002], coupled with the total bacteria primer set) was used to quantify *Gammaproteobacteria*. The GB lineage, also referred to as the *Competibacter* lineage, is proposed to capture the predominant GAOs within the class *Gammaproteobacteria* that would be present in EBPR WRRFs (Kong et al., 2006; Oehmen et al., 2007). DNA was extracted from each reactor according to the procedure outlined in Coats, Watkins, Brinkman, and Loge (2011), with qPCR settings per Winkler et al. (2011).

Mean amplification efficiencies for the total bacterial and PAO primer sets were 90.0 ± 0.05% ($n = 85$) and 99.1 ± 0.07% ($n = 95$), respectively. Mean amplification efficiencies for GAO and GB lineage were 78.8 ± 0.1% (GAOs; $n = 79$) and 88.7 ± 0.11% (GB lineage; $n = 85$). The cycle threshold was set at a constant value (0.5) based on location within the log-linear region for determination of Cq values (cycle number at which the measured fluorescence exceeds the cycle threshold). Relative PAO and GAO abundance was estimated per Winkler et al. (2011). Gel electrophoresis of qPCR products confirmed the presence of a single band for all samples.

Polymerase Chain Reaction for DNA Amplification and Denaturing Gradient Gel Electrophoresis. Amplification of 16S rDNA fragments was carried out on genomic DNA using the PAO primer set described above. A GC clamp was added to the forward primers (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G). The PCR reaction was performed with 5 minutes of initial denaturation at 94 °C and 30 cycles of 94 °C for 15 seconds, 57 °C annealing (PAOs) for 15 seconds, 72 °C extension for 45 seconds, and a 72 °C final extension for 5 minutes. To confirm successful amplification, DNA was resolved by 1.5% agarose gel electrophoresis using 1X TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM 0.5 M EDTA [pH 8.0]) for 45 minutes at 6 V cm^{-1}. The gel was stained in 1X TAE containing 1X SYBR Gold (Invitrogen, Eugene, Oregon) for 30 minutes, visualized with a UV transilluminator, and photographed. The PCR products were separated on a 6% polyacylamide gel in a 30 to 70% denaturing gradient using the Bio-Rad DCode system (Bio-Rad Laboratories, Hercules, California). The operating conditions were 60V at 60 °C for 17 hours. After electrophoresis, the gel was stained in 1X TAE containing 1X SYBR Gold (Invitrogen, Eugene, Oregon) for 30 minutes, visualized with a UV transilluminator, and photographed.

Results and Discussion

The research presented and discussed herein is based on operations assessed over a period of 800 days. For the first 288

Table 3—Performance summary for the EBPR reactors ([1]high ratio due to very low influent phosphorus associated with the real raw wastewater used on that operational day). Data presented for reactors V2-EBPR and G2-EBPR represent results collected after the substrate switch (COD = chemical oxygen demand; SD = standard deviation).

Parameter	Units	Reactor	Minimum	Maximum	Average	SD
Influent phosphorus	mgP/L	R-EBPR	0.49	3.76	2.04	1.02
		V-EBPR	3.02	7.03	4.79	1.28
		G-EBPR	0.35	5.44	2.91	1.32
		V2-EBPR	0.35	3.57	1.96	1.17
		G2-EBPR	3.02	5.39	4.32	0.98
Effluent phosphorus	mgP/L	R-EBPR	0.40	3.29	1.90	0.94
		V-EBPR	0.00	0.62	0.22	0.20
		G-EBPR	0.01	0.38	0.10	0.10
		V2-EBPR	0.03	0.12	0.06	0.03
		G2-EBPR	0.03	0.28	0.10	0.10
Influent vfa:influent p	mgVFA (as COD)/mgP	R-EBPR	0.00	71.14[1]	7.53	18.73
		V-EBPR	21.79	70.78	44.14	20.53
		G-EBPR	6.04	201.20[1]	41.13	57.71
		V2-EBPR	24.66	201.20[1]	75.38	72.86
		G2-EBPR	21.79	70.78	49.52	20.51

days, the parent reactors (V-EBPR, G-EBPR, and R-EBPR) were assessed for bulk solution phosphorus cycling and overall phosphorus removal. Subsequent investigations focused on characterizing the process in more detail; evaluating microbial function and structure relative to current theory; assessing process stability; and assessing the relative impacts of CG on EBPR.

EBPR Performance—VFA vs. Crude Glycerol Augmentation. *Phosphorus Removal and Cycling*. Excellent phosphorus removal was achieved by the MMC-fed both crude glycerol (CG)- and VFA-augmented wastewater (Table 3; Figures 1a, 1b), including the respective MMC that had experienced a shift in substrate (V2-EBPR, G2-EBPR). Results reported in Table 3 represent data collected during comprehensive bioreactor performance investigations. Beyond these data sets, routine effluent phosphorus measurements over the duration of the study (data not shown) confirmed stable and consistent phosphorus removal, with effluent phosphorus consistent with that presented in Table 3. Influent phosphorus concentrations varied over the project study, as would be expected of raw wastewater; however, EBPR performance nonetheless remained stable.

Considering that both wastewaters contained VFAs (Table 2) and the influent VFA-to-phosphorus ratio exceeded that observed to achieve successful EBPR (Horgan et al., 2010), the observed phosphorus removal performance would be reasonable. However, contradicting EBPR theory, not all consortia exhibited the theoretically required anaerobic–aerobic phosphorus cycling. While the VFA-fed MMC exhibited the required anaerobic phosphorus release response (Fig. 1a), in contrast, the average anaerobic phosphorus release in the CG-augmented reactors was nearly an order of magnitude lower than observed in the VFA-fed reactors (Figure 1b). Moreover, the CG-fed MMC did not always realize anaerobic phosphorus release; over the entire operational period, on eight occasions (five for G-EBPR; two for V2-EBPR following a permanent switch to CG; one for V-EBPR following a temporary switch to CG) zero anaerobic phosphorus release was observed. Despite the theoretical metabolic inconsistencies, ultimately the CG-fed MMC achieved excellent phosphorus removal. Such efficient and effective phosphorus removal without strict compliance to EBPR theory was not an anticipated outcome of this study. Of note, whereas the raw wastewater fed MMC received a similar substrate (same raw wastewater as the CG-fed reactors) and also exhibited no substantive anaerobic phosphorus release, in contrast to the CG-fed reactors, the R-EBPR MMC achieved negligible phosphorus removal (Figure 1c).

With the atypical phosphorus cycling response associated with the CG-fed MMC, it was suggested that phosphorus removal by the CG-fed MMC was perhaps chemically associated and not biological. However, if chemical precipitation was the driver, phosphorus removal would have been observed both anaerobically and aerobically, yet phosphorus removal only was observed aerobically and consistent with EBPR theory. However, beyond this observation, investigations were conducted on both the G-EBPR and V-EBPR MMC to evaluate the fate of phosphorus between the anaerobic and aerobic cycles. On two separate occasions, three samples were collected from each reactor (capturing both anaerobic and aerobic phosphorus effects): one at the end of cycle (just prior to commencing a new cycle), one at the end of the anaerobic period, and one after 45 minutes aerobic for the subsequent cycle. Total and soluble reactive phosphorus analyses were conducted on unfiltered/filtered supernatant (i.e., phosphorus in bulk solution, $P_{bulk\ solution}$) and on unfiltered/filtered samples recovered from washed (vigorously vortexed then centrifuged) biomass with a 0.9% NaCl solution to remove phosphorus potentially bound to the biomass (i.e., biomass-bound phosphorus, $P_{on\ biomass}$). Additionally, the washed biomass was digested for phosphorus quantification ($P_{in\ biomass}$). For both MMC a negligible amount of phosphorus was observed bound to the cell wall (<0.072%, mg P:mg biomass [$P_{on\ biomass}$]), all of the phosphorus released anaerobically was SRP, and the biomass phosphorus content ($P_{in\ biomass}$) decreased anaerobically and then increased aerobically (as would be expected of EBPR). The phosphorus mass balance closed (i.e., $P_{in\ biomass} + P_{bulk\ solution} + P_{on\ biomass}$) for the three sample times. Collectively, these results confirmed that phosphorus removal was not chemically driven.

For the samples collected (above), biomass phosphorus content (volatile suspended solids [VSS] basis) for the G-EBPR

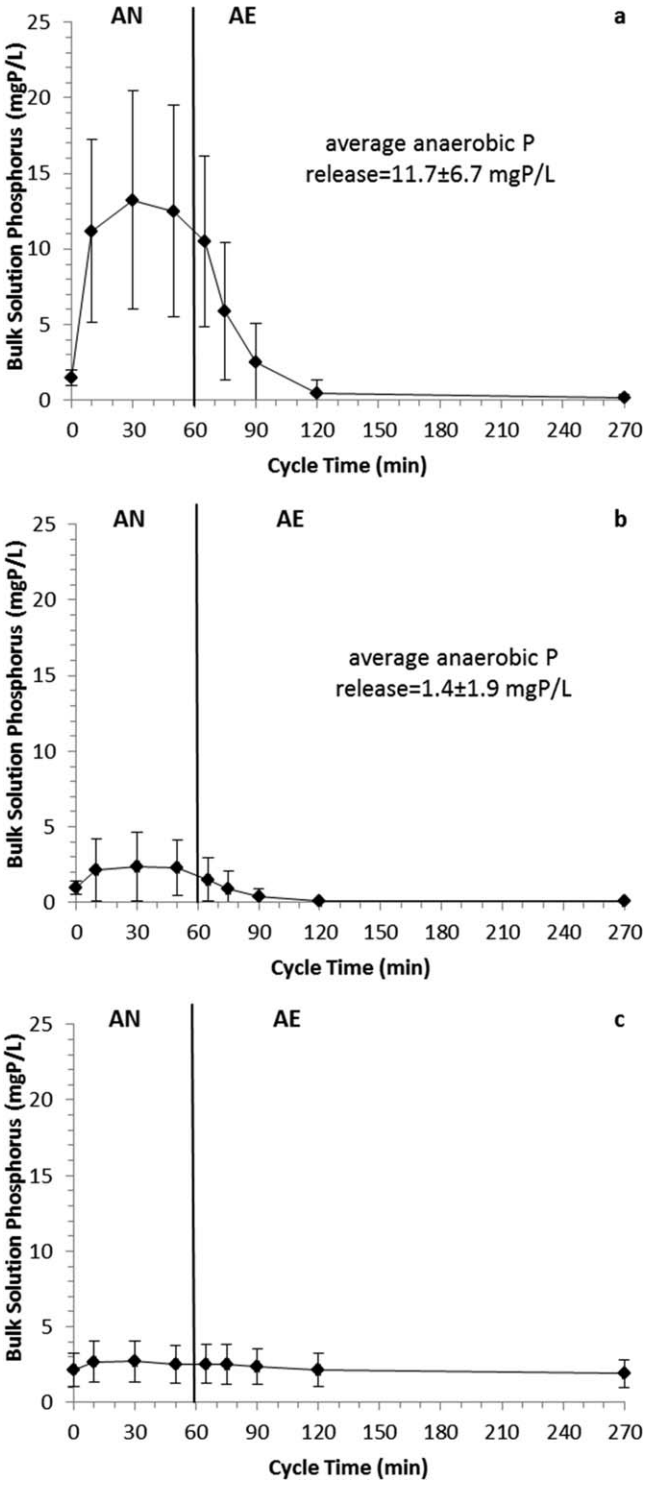

Figure 1—Bulk solution phosphorus cycling in the three study reactors (average and standard deviation ($n = 15$ [V-EBPR (1a)], $n = 15$ [G-EBPR (1b)], $n = 10$ [R-EBPR (1c)]). Results represent data collected during comprehensive performance assessments.

MMC ranged from 1.3 to 1.9%, while the V-EBPR MMC exhibited phosphorus content ranging from 0.9 to 3.0%. More generally, based on the data presented in Tables 1 and 3, the estimated average VSS phosphorus content in G-EBPR and V-EBPR MMC was 2.2 and 2.8%, respectively. Growth of ordinary heterotrophic organisms (OHOs) was estimated to account for 20 to 37% of the observed phosphorus removal (typical for OHOs for basal metabolic demands [Tchobanoglous et al., 2014]) in the G-EBPR and V-EBPR reactors, on average; thus, the majority of the removed phosphorus would be stored as polyP by the PAOs. While estimated biomass phosphorus content was less than cited for PAO-enriched consortia (e.g., 3.0 to 6.0% mg phosphorus per mg VSS [Seviour and Nielsen, 2010]), it must be noted that most EBPR research is based on the enriched PAO culture being fed significantly larger quantities of phosphorus than present in typical wastewater (as used in this study). Higher intracellular concentrations of biomass phosphorus would be a consequence of the much larger mass of phosphorus fed.

Carbon Utilization. As noted, the G-EBPR MMC was provided an atypical (relative to EBPR theory) organic carbon source (Table 2). Specifically, the CG contained relatively large quantities of glycerol (a primary byproduct of biodiesel synthesis) and some residual methanol (which is used to catalyze biodiesel production). Some VFAs were also present in the mixed substrate, derived from the raw wastewater. Despite receiving an atypical EBPR carbon substrate, ultimately the G-EBPR MMC consumed all available soluble carbon during the short anaerobic period (Figure 2a; methanol was completely consumed but due to low concentrations is not shown). As would be expected, the V-EBPR MMC consumed all the influent VFAs anaerobically (Figure 2b). Although the V2-EBPR and G2-EBPR carbon usage patterns are not shown, the response was consistent with the "parent" MMC. Regarding fate of the influent carbon, all MMC synthesized PHA anaerobically (Figure 2; Table 4) in accordance with EBPR theory; the VFA-fed MMC synthesized more PHA (both in total and on a MLVSS basis; Tables 4 and 5). All consortia also used glycogen consistent with EBPR theory (Figure 2).

Evaluating the anaerobic carbon stoichiometry, the CG-fed MMC consistently used significantly more glycogen than the VFA-fed MMC, presumably for PHA synthesis (Table 5 for G-EBPR, V2-EBPR). Comparatively, the VFA-fed MMC used more VFAs for PHA synthesis (V-EBPR, G2-EBPR). Glycerol can also be metabolized anaerobically to acetyl-CoA (Gupta et al., 2009), a precursor to PHA synthesis; since all glycerol was consumed anaerobically, it would appear that the CG-fed MMC also used this substrate in the synthesis of PHA. Comparing anaerobic carbon utilization metrics with that theoretically predicted for PAOs and GAOs (as summarized by Oehmen et al. [2010]), the VFA:PHA ratio for the VFA-fed MMC (Table 5) was more consistent with a blended PAO/GAO-enriched consortium (theoretical ratio of 1.22 to 2.33); conversely, the theoretical PAO/GAO metrics do not align with the responses of the CG-fed MMC (reinforcing the presumed utilization of glycerol in PHA production). Considering the glycogen:VFA ratio, data for the VFA-fed MMC compared to Oehmen et al. (2010) suggest a more PAO-enriched consortium, while again the CG-fed MMC data do not align with either PAOs or GAOs (again likely due to the utilization of glycerol).

Effects of Substrate on Process Resiliency and Stability. Considering the positive results using CG to drive EBPR, subsequent investigations assessed the relative resiliency and stability of the respective EBPR reactors when subjected to a switch in substrate.

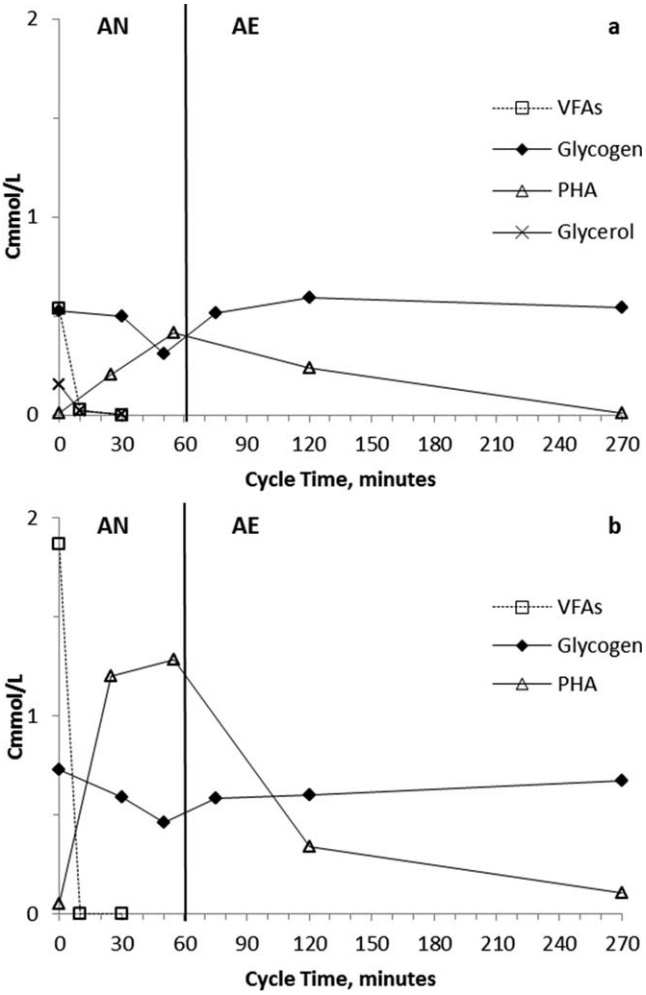

Figure 2—Average bulk solution (VFAs, glycerol) and intracellular biomass carbon (PHA, glycogen) for reactors G-EBPR (a) and V-EBPR (b) over an operational cycle. Results represent data collected during comprehensive performance assessment of the respective reactors ($n = 9$).

A Permanent Change in Substrate. The purpose of this aspect of the study was to evaluate how the MMC that were stabilized on either VFA- or CG-augmented wastewater would respond and perform phosphorus removal when the substrate source was permanently switched. "Child" reactors V2-EBPR and G2-EBPR were established using inocula from their respective parent MMC and initially operated receiving the normal substrate (i.e., V2-EBPR receiving the VFA-rich substrate and vice versa) for a time period longer than three SRTs. Performance of the respective MMC was assessed before (i.e., baseline) and more extensively after the substrate switch. As shown, the V2-EBPR MMC exhibited no ill effects associated with the substrate switch to CG (Figure 3). Because the CG-augmented wastewater was not the prescribed EBPR substrate and also considering that the G-EBPR MMC had not exhibited prototypical EBPR behavior (i.e., inconsistent anaerobic phosphorus release), it was expected that the VFA-enriched V2-EBPR MMC would require some length of time to ultimately stabilize and achieve stable phosphorus removal using the CG substrate. However, functionally the V2-EBPR MMC maintained process stability despite the changed substrate. In stark contrast, the G2-EBPR MMC, which was now receiving VFA-rich wastewater, did not respond resiliently. As shown (Figure 3), for the first 12 days following the substrate switch, the MMC continued to perform excellent phosphorus removal. Thereafter, however, the G2-EBPR MMC entered a process upset period that lasted for 39 days; it was not until day 51 that process stability was regained. It has been suggested that following a significant process disturbance, bioreactor stability may not be regained for a period of time equal to three SRTs (Grady Jr. et al., 2011); interestingly, the length of time required for G2-EBPR to regain stability was 39 days, or slightly longer than three operational SRTs. Once reactor G2-EBPR reached process stability, excellent phosphorus removal occurred for the duration of the study operational period (Table 3).

To understand how the substrate switch potentially affected the respective microbial populations, DNA was extracted from biomass collected from all bioreactors on operational days 385 and 457 (i.e., bracketing the transition period depicted in Figure 3). The DNA was PCR amplified with 16S rDNA PAO primers and separated through denaturing gradient gel electrophoresis

Table 4—EBPR bioreactor organic carbon utilization data (SD = standard deviation).

Parameter	Units	Reactor	Average	SD
Anaerobic VFA uptake	mgVFA (as COD)/L	V-EBPR	67.80	36.62
		G-EBPR	20.67	6.96
		V2-EBPR	24.08	5.21
		G2-EBPR	61.21	20.32
	Cmmol/L	V-EBPR	1.86	1.09
		G-EBPR	0.53	0.22
		V2-EBPR	0.69	0.16
		G2-EBPR	1.72	0.58
Anaerobic glycogen consumption	Cmmol	V-EBPR	1.13	0.38
		G-EBPR	1.00	0.34
		V2-EBPR	2.05	0.59
		G2-EBPR	1.47	0.78
Anaerobic PHA synthesis	Cmmol	V-EBPR	3.43	2.79
		G-EBPR	1.62	1.65
		V2-EBPR	1.56	1.14
		G2-EBPR	1.96	0.65

Table 5—Summary of anaerobic EBPR stoichiometric parameters (SD = standard deviation).

Parameter	Units	Reactor	Average	SD
Anaerobic phosphorus release (P(rel))	mg/L	VFA-fed MMC	10.10	6.92
		CG-fed MMC	3.27	4.47
Anaerobic P(rel): VFA(uptake) (P:C ratio)	Pmol/Cmol (total VFAs)	VFA-fed MMC	0.24	0.20
		CG-fed MMC	0.17	0.19
	Pmol/Cmol (HAc basis)	VFA-fed MMC	0.53	0.37
		CG-fed MMC	0.32	0.41
Anaerobic VFA:PHA	Cmmol/Cmmol	VFA-fed MMC	1.54	0.61
		CG-fed MMC	0.69	0.34
Anaerobic glycogen:PHA	Cmmol/Cmmol	VFA-fed MMC	0.63	0.39
		CG-fed MMC	2.16	2.77
Anaerobic glycerol:PHA	Cmmol/Cmmol	VFA-fed MMC	–	–
		CG-fed MMC	0.24	0.20
Anaerobic PHA:MLVSS	Cmmol/gVSS	VFA-fed MMC	0.89	0.93
		CG-fed MMC	0.67	0.84

(Figure 4) to develop a fingerprint of the respective populations. Each denaturing gradient gel electrophoresis (DGGE) band, or "signal", within a lane represents a unique amplified DNA sequence present in the sample. Although the individual bands alone do not provide phylogenetic information about the populations in the reactors, comparatively this molecular technique can be used to broadly assess the potential similarity between the different substrate-fed reactors and to identify potentially important community members (de Araujo and Schneider, 2008). Pertinent observations from this molecular analysis are as follows.

- Both "child" reactors were established from and operated consistent with their "parents", suggesting that the respective fingerprints should have been similar if not identical. In that regard, the microbial fingerprints for V-EBPR and V2-EBPR on day 385 (lanes 3 and 5, respectively) indicate that the two MMC were, in fact, nearly identical before the substrate switch. However, the same cannot be said of the G-EBPR and G2-EBPR consortia (lanes 9 and 7, respectively). The two fingerprints do exhibit similarity; however, there are signals present in lane 7 that are not present in lane 9. The same quantity of DNA was added to all PCR-DGGE reactions; further, the MLSS on day 385 for reactors G-EBPR and G2-EBPR was similar. The difference in PAO fingerprints between the two reactors suggests that, while G2-EBPR was inoculated from G-EBPR, ultimately population differences developed over the 18-day period following inoculation.
- Considering V2-EBPR before and after the substrate switch, comparing lanes 5 and 6, it would appear that the MMC diversity increased with the switch to the CG substrate

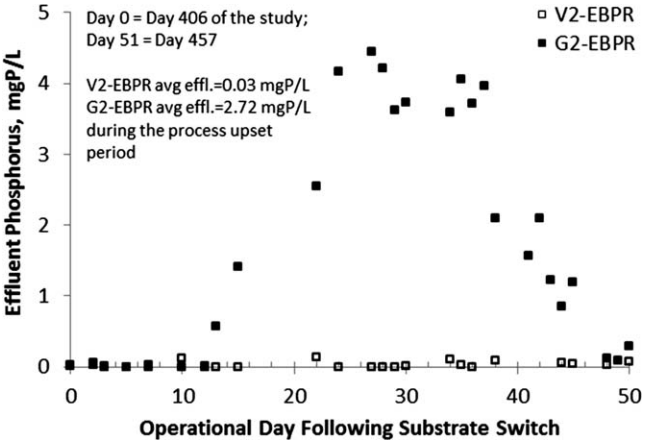

Figure 3—Effluent phosphorus for V2-EBPR and G2-EBPR immediately following the switch in substrate and for the subsequent 51 operational days until process stability (as assessed by effluent phosphorus) was achieved for both reactors. Note that V2-EBPR maintained stability throughout, while G2-EBPR initially maintained stability but experienced a process upset for approximately 39 days until regaining stability.

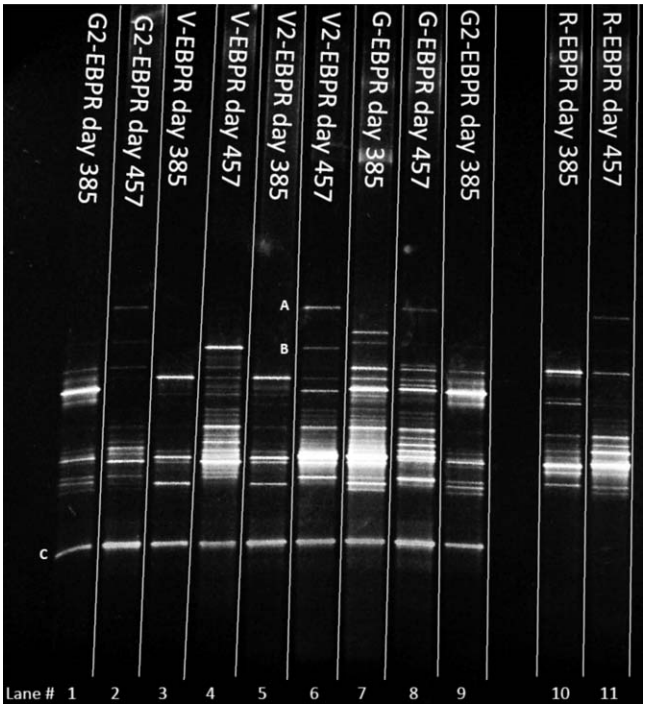

Figure 4—Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA (PAO primer set) extracted from biomass for all reactors on operational days 385 and 457.

(which may, in part, also explain the observed process stability following the change in substrate [Figure 3]). Not only were numerous signals more intense on day 457 (suggesting the MMC evolved to yield a higher concentration of those particular species in V2-EBPR), two new "signals" can be observed (A and B). Signal A also appears in two other lanes from MMC on day 457 (lane 2, G2-EBPR, fed VFA-rich substrate; and lane 8, G-EBPR day 457, fed CG) but not in the V-EBPR MMC fed VFA-rich substrate (lane 4). Signal B appears prominently in the V-EBPR MMC on day 457 but is essentially non-existent otherwise.

- Examining G2-EBPR (lanes 1 and 2 for days 385 and 457), in contrast to the V2-EBPR reactors, it would appear that the switch in substrate (to VFA-rich substrate) decreased the microbial diversity in this reactor. The apparent shift in population and reduced diversity could explain the process instability observed with the G2-EBPR reactor following the permanent substrate switch (Figure 3).
- The respective microbial fingerprints for the child reactors following the substrate switch did not mirror that of their substrate counterparts prior to the substrate switch. Specifically, the V2-EBPR MMC (day 457, lane 6, fed CG) was quite dissimilar from that of G2-EBPR (day 385, lane 1, fed CG), while the G2-EBPR MMC (day 457, lane 2, fed VFAs) also exhibited dissimilarity to V2-EBPR (day 385, lane 5, fed VFAs). Although it would appear that the respective MMC did adapt to the change in substrate, such adaption does not suggest that the dominant microbial populations were exclusively substrate-dependent.
- In considering all lanes for MMC fed CG (1, 6, 7, 8), it would appear that the CG substrate yielded a more diverse MMC than did the VFA-rich wastewater.
- Comparing the fingerprints between the respective EBPR consortia (lanes 1 through 9) and the poorly performing raw wastewater reactors (R-EBPR, lanes 10, 11), given some similarities it could be suggested that perhaps there were some PAOs present in the R-EBPR consortia (see also separate discussion later in this article) but that the substrate was insufficient to induce the necessary metabolisms for successful phosphorus removal. However, there was at least one dominant signal unique to the successful EBPR MMC that was not present in the R-EBPR reactors (signal C).

One-time Switch in Substrate. As supported by a vast body of research on EBPR, sufficient organic carbon (typically VFAs, but as demonstrated herein not exclusively) is required to enrich for the proper microbial consortium capable of necessary EBPR metabolisms to achieve very low effluent phosphorus. However, full-scale EBPR WRRFs can experience a dilution of the influent substrate, for example through process failure associated with a primary solids fermenter or through excess infiltration/inflow entering the sanitary sewer collection system. Under such conditions, EBPR process failure could occur. Operating under a federally regulated permit, such failures can lead to expensive permit violations. Many EBPR facilities thus employ process redundancy, often in the form of chemical phosphorus removal, to ensure permit compliance.

In lieu of installing expensive infrastructure to ensure stable phosphorus removal performance, considering the results

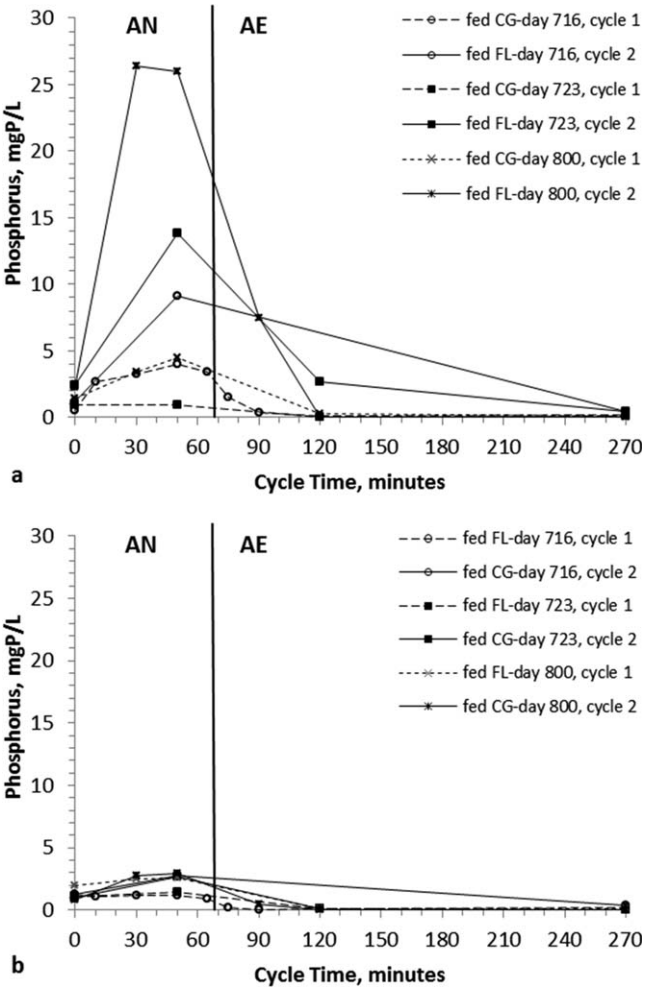

Figure 5—Phosphorus cycling data for reactors V-EBPR (a) and G-EBPR (b) when subject to single event change in substrate (V-EBPR received crude glycerol [CG] for a single cycle followed by regular substrate the subsequent cycle; vice versa for G-EBPR) on days 716, 723, and 800 of the investigation period.

presented herein, it could be more economical to maintain a supply of CG on site to augment the influent wastewater on an as-needed basis. The premise of this suggestion is that a VFA-sustained EBPR process can be quite resilient, as discussed above; temporarily augmenting the process with CG could thus help maintain permit compliance. To assess the potential effects of short-term shifts in substrate on EBPR performance, for one operational cycle on three different days the substrate fed to reactors V-EBPR and G-EBPR was switched (i.e., V-EBPR received CG and vice versa). As shown, the respective MMC performed excellent phosphorus removal regardless of receiving a different substrate (Figure 5); moreover, no process upset was observed immediately thereafter nor for the days that followed (data not shown). Nominal anaerobic phosphorus release occurred when the V-EBPR MMC was fed CG wastewater (Figure 5a), but phosphorus cycling returned to the norm once the MMC were fed the VFA-rich substrate. In contrast, the G-EBPR exhibited little to no anaerobic phosphorus release regardless of the substrate (Figure 5b). Regarding carbon utilization, both MMC used all substrate provided anaerobically

during the substrate switch, and also cycled PHA and glycogen consistent with theory.

Use of Pure Glycerol. Recognizing that crude glycerol also contains methanol and other impurities associated with biodiesel production, and further considering the uncharacteristic EBPR observations discussed herein, some limited testing with pure glycerol (in lieu of CG) was performed to confirm that the MMC used glycerol for EBPR. On three occasions, the CG-fed MMC was supplied pure glycerol (once for a single operational cycle for both G-GBPR and V2-EBPR, and once for two cycles in series for G-EBPR). In all instances, the MMC exhibited the same phosphorus removal behavior as when supplied CG (data not shown).

Crude Glycerol Effects on Anaerobic EBPR Metabolisms. As discussed herein, the use of CG to achieve EBPR yielded excellent results. However, also as discussed, the theoretical anaerobic EBPR metabolisms were not consistently induced. Interrogation of the anaerobic process stoichiometry and energetics revealed some insights on these unexpected metabolic responses.

Phosphorus:Carbon Ratio. Anaerobic phosphorus release is intrinsically linked to polyP hydrolysis to generate energy (adenosine tri-phosphate [ATP]) for the uptake/conversion of VFAs to PHA. Consistent anaerobic phosphorus release observed in an EBPR system indicates the induction of a cascade of EBPR metabolisms that ultimately produce reclaimed water containing very low concentrations of soluble orthophosphate. Considering the theoretical importance of phosphorus release in EBPR success, a metric based on phosphorus release would be potentially useful in process monitoring and troubleshooting. To that end, Smolders et al. (1994) and later Filipe et al. (2001) proposed an empirical metric (known as the phosphorus:carbon [P:C] ratio) to encapsulate the relationship. The P:C ratio is calculated as the mass of phosphorus released divided by the mass of VFAs removed from bulk solution (mole basis). In studying EBPR, it can be valuable to quantify the P:C ratio and compare with empirical estimates.

As detailed above, indeed the MMC fed VFA-augmented raw wastewater exhibited prototypical anaerobic phosphorus release (Figure 2a; Table 5). Moreover, the P:C ratio was generally consistent with that predicted by EBPR theory (Table 5); according to the theoretical model of Filipe et al. (2001), the P:C (HAc basis) ratio should have ranged from 0.66 to 0.78 (for the anaerobic pH values of 7.6 to 8.3 observed). However, the observed P:C ratio decreased with increasing pH, which is opposite that suggested by Filipe. Although the CG-fed consortia were also provided with some VFAs (Table 2), as described the MMC released little to no phosphorus anaerobically (Figure 2b; Table 5). The average resultant P:C ratios were 0.32 (HAc basis) and 0.17 (total VFA basis). Comparatively, the Filipe model would suggest a range of 0.68 to 0.76 (HAc basis). Contrasted with the VFA-fed MMC, there was no apparent link between pH and the P:C ratio for the MMC fed CG. Ultimately this P:C analysis suggests that the CG-fed MMC were less reliant on polyP hydrolysis to generate energy in support of the anaerobic EBPR metabolisms. A similar response was observed by Zhou et al. (2008) on a PAO-enriched MMC.

Anaerobic Energetics. Considering the implications of low P:C ratio by the CG-fed MMC and further considering that the MMC at times exhibited no anaerobic phosphorus release, it was of interest to interrogate how the bacteria generated sufficient ATP to drive required EBPR anaerobic metabolisms (VFA uptake/catabolism). The P:C ratio only indirectly considers polyP energetics; a quantitative assessment of anaerobic energetics avoids such a limitation. In other words, by quantifying ATP generated based on known metabolisms and actual measurements made in this study, we can better describe what may have occurred in the CG-enriched MMC performing EBPR.

Adenosine tri-phosphate requirements for VFA uptake were estimated using an equation developed by Filipe et al. (2001): α (molATP molVFA^{-1}) = 0.16*pH$_{out}$-0.7985. For the CG-fed consortia, α varied from 0.35 to 0.51, and the α for the VFA-fed consortia ranged from 0.42 to 0.51. Whereas Filipe's equation was developed for an acetate only substrate, Zhang et al. (2008) concluded that the value α was not significantly influenced by VFA species, in part based on work by Oehmen et al. (2005). The estimated α values were higher than the 0.12 and 0.11 suggested by Filipe et al. (2001) and Oehmen et al. (2005), respectively, but similar to those of Schuler and Jenkins (2003) for cultures exhibiting a phosphorus accumulating metabolism. Regarding VFA uptake, estimates of required ATP (molATP molVFA^{-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).

To quantify anaerobic energy production, ATP synthesis from polyP hydrolysis was estimated to be generated on a 1 molATP to 1 molP(rel) ratio (Smolders et al., 1994). Beyond polyP hydrolysis, ATP is also generated anaerobically through substrate level phosphorylation associated with glucose (from glycogen) catabolism (Madigan and Martinko, 2006); a value of 0.5 molATP per Cmol was used, assuming the Embden-Myerhof pathway was utilized (which is consistent with Schuler and Jenkins [2003]). Similarly, the catabolism of glycerol will generate ATP through substrate level phosphorylation at 1 molATP per Cmol (Gupta et al., 2009). Applying these established metabolisms for quantifying ATP demand and production to data collected in our investigations, an ATP balance was developed for the respective reactors (Table 6). As shown, the estimated anaerobic ATP production for the CG-fed MMC nearly met demand; less VFAs fed to the MMC resulted in reduced ATP demand, and glycogen and glycerol catabolism provided the majority of the ATP required. Conversely, the ATP demand for the VFA-fed MMC was significantly greater than could be estimated for production. Saunders et al. (2007) proposed a model wherein PAOs can potentially produce ATP anaerobically through the ATP synthase mechanism associated with polyP hydrolysis and cellular phosphorus efflux; including this additional source of anaerobic ATP reduces but does not eliminate the negative balance for the VFA-fed MMC. In summary, based on these energetic assessments, it would appear that (i) MMC performing EBPR can use glycerol to generate ATP (both directly, and indirectly through increased glycogen storage) and thereby reduce the demand for polyP hydrolysis; and (ii) theoretical EBPR anaerobic energetic models insufficiently predict ATP production, ATP demand, or a combination of production and demand. Perhaps most interestingly this energetic assessment suggests that polyP hydrolysis is not necessarily a prerequisite for successful EBPR, if other energy substrates are available. However, additional molecular-level investigations are necessary to more fully understand these observations.

Table 6—Anaerobic bioenergetic assessment for the EBPR reactors.

	ATP required (mmol)			ATP produced (mmol)				
Reactor	VFA uptake /transport	VFA activation to CoA	Total	PolyP	Glycogen	Glycerol	Total	%ATP acct'd for
CG-fed MMC ($n = 10$)	0.50	0.46	0.97	0.07	0.56	0.26	0.88	91%
VFA-fed MMC ($n = 9$)	1.78	1.43	3.22	0.64	0.48	0.00	1.11	35%

Polyphosphate Accumulating Organism and Glycogen Accumulation Organism Quantification. As a final assessment of the contrasting EBPR configurations, the respective MMC were assessed using qPCR to estimate the relative fraction of PAOs and GAOs present. *Accumulibacter* Type I (PAOI) and *Accumulibacter* Type II (PAOII) were investigated; both PAO types would be amplified with the primer set employed in this study. Recognizing the potential for GAOs in EBPR and the putative concern that such microorganisms can impair the EBPR process, GAOs were also quantified.

Considering the observed performance success of the EBPR reactors studied, as detailed herein, certainly the respective MMC were sufficiently enriched with PAOs. However, as summarized in Table 7, the actual fraction of PAOs in the bacterial consortium was relatively low. The VFA-fed MMC exhibited a PAO fraction ranging from 0.03 to 2.22% while the CG-fed MMC showed a PAO fraction ranging from 0.11 to 4.3%. Comparatively, these PAO fractions were less than observed in the authors' previous research (Coats, Watkins, Brinkman, and Loge 2011; Winkler et al., 2011). Nevertheless, the MMC was sufficiently enriched with PAOs to achieve the desired end-goal of excess phosphorus removal. Although the R-EBPR MMC did not perform phosphorus removal, the MMC did (at times) contain a very small fraction of PAOs; this observation suggests that the genetic potential for excess phosphorus removal existed within the R-EBPR MMC; however, the substrate was insufficient to initiate the necessary EBPR metabolisms.

In contrast to the PAOs, the GAO population was not detected until later in the study period (Table 7). Moreover, it would appear that the emergence of GAOs may have somewhat impaired the EBPR process, as the effluent phosphorus concentration increased on days 644 and 716 when GAOs were present. Indeed, for the V-EBPR reactor, effluent phosphorus was highest corresponding to the largest fraction of GAOs (day 716). Considering the CG-fed MMC, while GAOs appeared to be a dominant fraction in the G-EBPR reactor on day 716, excellent phosphorus removal still occurred as indicated by the effluent phosphorus concentration. In fact, the GAO fraction was very large (65.3%) yet effluent phosphorus was markedly better than observed in the V-EBPR reactor. Interrogation of the operating data did not provide any clues as to why the GAO fraction became more prominent in the later days of the study, particularly in the CG-fed reactor. Nevertheless, while qPCR data is not available beyond day 716, routine measurements of effluent phosphorus confirmed that EBPR success was maintained.

Conclusions

The objective of the research presented and discussed herein was to assess EBPR performance when a MMC was fed an atypical organic carbon substrate (CG). Investigations of EBPR reactors operated over the 800-day period yielded interesting observations as follows.

- Results suggest that CG is an organic carbon supplement that can be used to accomplish and/or help maintain EBPR

Table 7—Relative fraction of PAOs and GAOs within the respective bacterial community as estimated by qPCR for the DNA extracted on the operational days shown (nd = none detected).

	Operational day	Reactor				
		R-EBPR	V-EBPR	G-EBPR	V2-EBPR	G2-EBPR
PAOs	385	0.13±0.03	0.68±0.04 (0.0)	0.29±0.01 (0.01)	0.12±0.01 (0.01)	0.39±0.04 (0.0)
(effluent P, mg L^{-1})	457	0.18±0.01	0.26±0.03 (0.03)	0.64±0.05 (0.11)	0.33±0.03 (0.05)	0.85±0.02 (0.05)
	485	—	0.14±0.01 (0.06)	2.45±0.02 (0.03)	0.24±0.01 (0.04)	0.47±0.02 (0.05)
	520	—	2.22±0.20 (0.06)	4.30±0.68 (0.02)	0.62±0.04 (0.03)	2.96±0.07 (0.03)
	644	—	0.04±0.004 (0.25)	0.14±0.06 (0.12)	0.71±0.05 (0.12)	0.06±0.004 (0.28)
	716	0.01±0.002	0.03±0.004 (0.44)	0.11±0.005 (0.12)	—	—
GAOs	385			nd		
	457			nd		
	485			nd		
	520			nd		
	644	nd	0.06±0.01	4.65±0.81	1.17±0.12	0.45±0.02
	716	nd	4.90±0.95	65.3±7.1	—	—
GB lineage	385			nd		
	457			nd		
	485			nd		
	520			nd		
	644	nd	0.07±0.01	6.96±1.3	0.87±0.04	0.33±0.01
	716	nd	3.41±0.30	6.74±0.63	—	—

stability. Extrapolating from our results, a 37,854 m^3 per day (10 mgd) WRRF treating medium- to high-strength domestic wastewater (Tchobanoglous et al., 2003) would require 7.6 m^3 (2000 gallons) per day of CG, a volume that could be generated from a 7570 m^3 (2 MG) per year biodiesel plant (which is a small biodiesel production facility). Thus, even operating an EBPR system exclusively on CG is feasible.

- Research suggests that, in contradiction to current EBPR theory, polyP hydrolysis (to generate ATP) may not to be a necessary metabolic response. Rather, this research suggests that EBPR MMC will first leverage carbon substrate to generate the required ATP for VFA uptake and catabolism; as needed, the MMC will use its polyP reserves, but only if the substrate-driven energy production (e.g., glycerol) is insufficient.
- Although anaerobic phosphorus release did not appear intrinsically linked to successful removal of excess phosphorus aerobically, all EBPR MMC did cycle PHA and glycogen (consistent with theory) regardless of the substrate provided. Such a feast/famine carbon storage polymer response is associated with microbial stress, a metabolism implicated with excess phosphorus accumulation (Al-Najjar et al., 2011).
- Successful EBPR can be achieved even with a small fraction of PAOs present in the MMC. Moreover, EBPR can function properly when the consortia contain a relatively large fraction of GAOs.
- Although EBPR can be achieved using crude glycerol, the MMC enriched on VFAs was nonetheless more resilient. However, consortia acclimated to either carbon form did exhibit the potential to accommodate short-term shifts in substrate while maintaining excellent phosphorus removal.

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