Effect of Anaerobic HRT on Biological Phosphorus Removal and the Enrichment of Phosphorus Accumulating Organisms

Erik R. Coats[1]*, David L. Watkins[2], Cynthia K. Brinkman[3], Frank J. Loge[4]

ABSTRACT: The purpose of this research was to develop a better understanding of the dynamic effects of anaerobic hydraulic retention time (HRT) on both enhanced biological phosphorus removal (EBPR) performance and enrichment of phosphorus accumulating organisms (PAOs). The research was conducted using laboratory-scale sequencing batch reactors inoculated with mixed microbial consortia and fed real wastewater. Exposing microorganisms to extended anaerobic HRTs is not recommended for EBPR configured systems. In this research, however, longer anaerobic exposure did not negatively affect performance even if volatile fatty acids were depleted. Further, extended anaerobic HRTs may positively affect phosphorus removal through enhanced aerobic uptake. The EBPR consortia also appear to maintain reserve energetic capacity in the form of polyphosphate that can be used to survive and grow under variable operational and environmental conditions. Finally, the tested EBPR systems yield mixed microbial consortia enriched with PAOs (specifically Candidatus Accumulibacter phosphatis) at approximately 7.1 to 21.6% of the total population. *Water Environ. Res.*, **83,** 461 (2011).

KEYWORDS: enhanced biological phosphorus removal, volatile fatty acids, polyphosphate, qPCR, DGGE, polyphosphate accumulating organisms.

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Introduction

Enhanced biological phosphorus removal (EBPR) is a wastewater treatment process designed to create mixed microbial consortia capable of sequestering and storing excess quantities of soluble orthophosphate (as polyphosphate). Process success is dependent upon repetitively cycling the microorganisms (referred to as phosphate accumulating organisms, or PAOs) between anaerobic (no external electron acceptor) and aerobic (oxygen as the electron acceptor) environments. Under anaerobic conditions, organic acids (acetate, propionate, etc.) present in the influent wastewater are stored intracellularly by microbes as polyhydroxyalkanoates (PHA), with glycogen reserves providing necessary metabolic reducing equivalents for PHA synthesis (Arun et al., 1988; Hesselmann et al., 2000; Lemos et al., 2003; Satoh et al., 1992). Hydrolysis of polyphosphate reserves produce the energy required for anaerobic carbon update and PHA synthesis when it is cleaved from the cell (Mino et al., 1998). The hydrolyzed phosphate is released from the cell as a metal phosphate complex, primarily complexed with K^+ and Mg^{2+}, in symport with a hydrogen ion via the secondary phosphorus transport system (Mulkerrins et al., 2004; van Veen et al., 1994). This action also results in an increase in bulk solution phosphorus, K^+, and Mg^{2+} concentrations. In the subsequent aerobic environment, glycogen reserves are restored using PHA (Maurer et al., 1997; Pereira et al., 1996). Excess phosphorus removal from bulk solution occurs aerobically when the microorganisms—using PHA reserves also for energy production, growth, and cellular maintenance—sequester a greater amount of phosphorus than released anaerobically.

Success of the EBPR process hinges largely on providing sufficient anaerobic contact time so that the carbon and polyphosphate metabolisms are fully induced and volatile fatty acids (VFAs) are depleted. For full-scale wastewater treatment facilities (WWTFs), it typically is recommended that an anaerobic hydraulic residence time (HRT) of 0.25 to 1.0 hour is adequate to induce the target metabolisms (Barnard, 1984; Barnard et al., 1998; Grady Jr. et al., 1999). Based on the temporal variability in wastewater flow at full-scale facilities, however, maintaining a stable and constant anaerobic HRT is not realistic. The commonly perceived risk of extended anaerobic HRT is VFA depletion, which can induce a phenomenon referred to as ''secondary phosphorus release'' (Barnard et al., 1998). Specifically, when readily biodegradable organic carbon is depleted from bulk solution under anaerobic conditions, it has been observed that microbes can continue to anaerobically hydrolyze polyphosphate (to produce energy for maintenance and survival), with bulk solution phosphorus concentrations continuing to increase. With the resulting imbalance of phosphorus released to VFAs stored as PHA, insufficient energy (as PHA) is available to drive aerobic phosphorus removal (Barnard et al., 2006). This can lead to reduced overall phosphorus removal. In effect, secondary phosphorus release can cause EPBR upset.

Although research has shown that excessive anaerobic HRT can adversely affect EBPR, it is unclear how much is ''excessive''. Lopez et al. (2006) found that long anaerobic HRT (greater than 12 hours) affected intracellular carbon storage polymers and caused a reduction in the rate of aerobic phosphorus removal. The tested anaerobic HRTs, however, were long relative to that recommended for design. Similarly, Lu et al. (2007) studied extended anaerobic starvation (monitored daily up to 8 days) with

[1]* Department of Civil Engineering, University of Idaho, BEL 129, Moscow, ID 83844-1022; e-mail: ecoats@uidaho.edu.

[2] J-U-B Engineers, Inc., Boise, Idaho.

[3] Research scientist in the Department of Civil Engineering, University of Idaho, Moscow, Idaho.

[4] Department of Civil and Environmental Engineering, University of California Davis, Davis, California.

Table 1—Summary description of treatment reactor operational configuration. The reactor naming scheme was as follows: "F" represents fermenter-fed [i.e., 90% raw wastewater, 10% fermenter liquor (v/v)], the first number represents the bioreactor solids retention time (SRT), the second number is overall hydraulic retention time (HRT) in hours, and the last two numbers are anaerobic and aerobic cycle time, respectively, in hours.

Reactor name	SRT (days)	HRT (hrs)	Anaerobic time (hrs)	Aerobic time (hrs)	Cycle time (hrs)	Reactor volume (L)
F.10.12.1.3	10	12	1	3	4	1.2
F.10.12.3.3	10	12	3	3	6	0.8
F.9.12.2.4	9	12	2	4	6	0.8

an enriched Accumulibacter PAO population, and confirmed that phosphorus release will continue once VFAs are depleted (i.e., secondary phosphorus release) (Barnard et al., 1998). Again no design or operational guidance was implemented, and the anaerobic HRTs were long. Regarding shorter anaerobic HRTs, Kuba et al. (1993) tested laboratory-scale EBPR reactors at anaerobic HRTs ranging from 1.75 to 3 hours, and observed no apparent process problems. Their research, however, was principally focused on investigating the effects of nitrate (in lieu of oxygen) on EBPR, and thus no conclusions were drawn regarding variable anaerobic HRT.

The purpose of this research was to develop a better understanding of the effects of variable anaerobic HRT on both EBPR performance and PAO enrichment. Central to this research was the use of real wastewater and mixed microbial consortia.

Materials and Methods

Wastewater Sources and Characteristics. Raw wastewater (screened and degritted) was obtained approximately biweekly from the Moscow, Idaho, EBPR WWTF. The Moscow facility treats approximately 7,570 to 15,140 m^3 of wastewater per day (2 to 4 mgd) in an A^2/O-oxidation ditch process with no primary solids fermentation (Tchobanoglous et al., 2003). Fermenter liquor was produced in a laboratory bench-top fermenter fed thickened primary solids from the Pullman, Washington, WWTF. Pullman operates a conventional modified Ludzack-Ettinger (MLE) process with a high rate, completely mixed, mesophilic anaerobic digester. All wastewater was stored at 4 °C until used (Tchobanoglous et al., 2003).

The fermenter liquor-raw wastewater mixture (10:90%, v/v) organic acid concentration varied from 140 to 180 mg (as total organic acid)/L^{-1}, with acetic acid being the predominant form (60 to 65%). Other forms of organic acids detected (order of highest to lowest concentration) were propionate (25 to 30%), butyrate (8 to 10%), and valerate (< 3%). Comparatively, the raw wastewater organic acid concentrations were dominated by acetate and propionate, with minimal butyrate and valerate; total VFA concentrations were 44 to 55 mg (as total organic acid)/L^{-1}. The 90:10 mixture ammonia-nitrogen and phosphorus concentration ranged from 25 to 45 mg NH_3-N/L^{-1} and 20 to 25 mg P/L^{-1}, and the raw wastewater exhibited concentrations of 20 to 25 mg NH_3-N L^{-1} and 4 to 6 mg P/L^{-1}, respectively. No nitrate was detected in either waste stream.

Treatment Bioreactor Operating Conditions. Three independent sequencing batch reactors (SBRs), each seeded with a mixed microbial consortium obtained from the Moscow, Idaho, EBPR WWTF aerobic basin, were operated in cyclical anaerobic-aerobic conditions at room temperature (approximately 21 °C). Table 1 summarizes the operational parameters for each reactor. The SBR naming scheme was as follows: "F" represents "fermenter-liquor"-fed (substrate was 90% raw wastewater, 10% fermenter liquor, v/v); the first number represents the solids residence time (SRT; in days); the second number is HRT (in hours); and the last two numbers are anaerobic and aerobic cycle time (hours), respectively. Each operational cycle included the following periods: feed, anaerobic, aerobic, settle, and decant. The settling period (10 minutes) in all reactors was followed by a two-minute decant period and an eight-minute feed period. With each cycle, 400 mL of treated wastewater was decanted and replaced with a commensurate volume of wastewater to maintain the target HRT. The SRT was controlled by wasting daily an appropriate volume of mixed liquor at the end of the aerobic period before removal of any effluent for HRT control (e.g., to maintain a 10-day SRT, 10% of the reactor volume was removed, so that 10% of the biomass was removed). Each reactor remained fully mixed when biomass was removed for SRT control to ensure that a consistent quantity of biomass was removed daily.

Anaerobic conditions were established by diffusing nitrogen gas into each SBR for the first eight minutes of the anaerobic period to achieve a dissolved oxygen concentration < 0.2 mg/L^{-1}. Aerobic conditions were maintained by diffusing air through the reactor to maintain a dissolved oxygen level greater than 2 mg/L^{-1}. Gas diffusion was accomplished with ceramic fine-bubble diffusers. Reactors were completely mixed except during the settle and decant periods; mixing was accomplished with magnetic stirrers. Digital timers were used to control mixing, gas, and pumping, and all pumping was performed using peristaltic pumps (Watson Marlow Bredel, Wilmington, Massachusetts). Nitrification was inhibited in all reactors by the addition of thiourea (influent wastewater contained 10 mg thiourea per liter).

Fermenter liquor was produced in a 12-L completely mixed primary solids fermenter, operated as a SBR with an SRT and HRT of four days. The daily decant was centrifuged at approximately 10,000 rpm, with the supernatant (i.e., fermenter liquor) recovered and stored at 4 °C.

Analytical Techniques. Soluble reactive phosphate was determined in accordance with Hach method 8048 (equivalent to Standard Methods 4500-PE). All samples were centrifuged to remove biomass and then filtered through a 0.22-μm syringe filter (Millipore Corp, Billerica, Massachusetts) before testing. A Spectronic® 20 Genesys™ spectrophotometer (Thermo Scientific Corp, Waltham, Massachusetts) was used to measure absorbance at 890 nm. Phosphate concentrations were determined using a standard curve ($R^2 > 0.99$). Total suspended solids (TSS) and volatile suspended solids (VSS) were measured in accordance with Standard Methods 2540 D and 2540 E (American Public

Health Association et al., 1998). The pH was measured using a Thermo Scientific Corp. Accumet AP85 Waterproof pH/Conductivity Meter (Waltham, Massachusetts). Dissolved oxygen measurements were collected using a Hach (Loveland, Colorado) HQ30d meter with LDO101 dissolved oxygen probe.

Organic acid (e.g., acetic, butyric, propionic) concentrations were measured using a Hewlett-Packard (Palo Alto, California) 6890 Series gas chromatograph with a flame-ionization detector (FID). The temperature of the column (Grace Davison Discovery Sciences, Deerfield, Illinois; Alltech® Heliflex® AT™ Wax Column, length 30 m, internal diameter 0.32 mm) was held constant at 150 °C; the injector was maintained at 250 °C; and the detector was operated at 250 °C. Helium was used as the carrier gas at a flow rate of 1.2 mL/min^{-1}. Before injection, samples were acidified to a pH of approximately 2. Then 0.5 μL of sample was injected in 20:1 split mode for analysis. The respective sample organic acid concentrations were confirmed with known standards and quantified using a standard curve ($R^2 > 0.99$).

Samples for total soluble minerals analysis were first filtered through a 0.22 μm filter and then analyzed by acid digestion followed by inductively coupled plasma spectrometry, in accordance with U.S. Environmental Protection Agency Method 200.7. Analyses were performed by the Holm Research Center, Analytical Sciences Laboratory, University of Idaho, Moscow, Idaho.

Polymerase Chain Reaction for Deoxyribonucleic Acid Amplification. Deoxyribonucleic acid (DNA) from each reactor was extracted using an UltraClean™ soil DNA extraction kit (MoBio Laboratories, Inc., Solana Beach, California). Samples were stored at −20 °C until used. Amplification of 16S ribosomal ribonucleic acid (rRNA) fragments was carried out on genomic DNA using bacterium-specific 341f (CCT ACG GGA GGC AGC AG) and 907r (CCG TCA ATT CMT TTG AGT TT) primers. A GC clamp was added to the forward primers (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G). The primers were selected because they have been shown to cover a highly conserved region within the domain bacteria (Baker et al., 2003). The polymerase chain reaction (PCR) was performed with five minutes of initial denaturation at 94 °C and 30 cycles at 94 °C for one minute, 55 °C annealing for one minute, 72 °C extension for two minutes, and a 72 °C final extension for five 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 ethylenediaminetetraacetic acid, 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 and destained with deionized water for 10 minutes, visualized with a UV transilluminator, and photographed using Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, New York).

Denaturing Gradient Gel Electrophoresis Analysis. The PCR products were separated on a 6% polyacylamide gel in a 30 to 80% denaturing gradient using the Bio-Rad DCode system (Bio-Rad Laboratories, Inc., Hercules, California). The operating conditions were 55 V 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 and destained with deionized water for 10 minutes, visualized with a UV transilluminator, and photographed using Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA).

Quantitative Real-Time Polymerase Chain Reaction. Quantitative real-time polymerase chain reaction (qPCR) was applied to 16S rDNA extracted from each EBPR reactor to estimate the relative abundance of the *Candidatus* Accumulibacter *phosphatis* lineage in the total bacterial community. The qPCR was conducted on a StepOne Plus™ Real-Time PCR System (Applied Biosystems, Foster City, California) using SYBR® Green PCR master mix (Applied Biosystems, Foster City, California) with a total reaction volume of 25 μl. Primer set 341f/534r was used to quantify total bacterial 16S rDNA genes, and primer set 518f and PAO-846r was used to quantify total *Candidatus* Accumulibacter *phosphatis* 16S rDNA genes (He et al., 2007; Yoshida et al., 2005). The former is a universal bacterial primer; the latter primer is specific to *Candidatus* Accumulibacter *phosphatis* (He et al., 2007). The operational conditions for qPCR were: 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C, one minute at 60°C, and one minute at 72°C. Data collection was performed during each amplicon extension phase. The qPCR calibration curves were produced by serial dilution of extracted genomic DNA for each sample within each assay. Quantification was performed using StepOne Software (v2.0). Amplification efficiency of PCR was estimated from the slope of the standard curve by the formula $10^{-1/\text{slope}} - 1$. Standard deviations were calculated from the average for the triplicate runs.

Results and Discussion

The results presented and discussed herein represent performance for each independent EBPR reactor monitored multiple times over approximately one week after each had reached steady-state operation. Reactor steady-state conditions were assumed following an operational period equal to three SRTs, and by monitoring reactor mixed liquor suspended solids (MLSS) concentrations.

Performance at Steady-State Anaerobic Hydraulic Retention Time. Three bench-scale SBRs were independently operated in a cyclical anaerobic-aerobic pattern to enrich for microbial consortia that would achieve biological phosphorus removal. Each consortium was fed 10% fermenter liquor and 90% raw wastewater (v/v) and operated at a 12-hour total HRT. The principle variable across the three SBRs was the length of the anaerobic period. The consortium in reactor F.10.12.1.3 was exposed to a one-hour anaerobic period each cycle; the consortia in reactors F.9.12.2.4 and F.10.12.3.3 were exposed to two- and three-hour anaerobic periods each cycle, respectively. Table 1 summarizes the reactor operational configurations. As shown (Figures 1A–1C), under these operational conditions each microbial consortium exhibited typical EBPR behavior, with phosphorus release commencing immediately and rapidly when anaerobic conditions were imposed, followed by rapid phosphorus uptake aerobically. Observed anaerobic phosphorus release rates (Table 2) varied substantially across the three reactors, with the highest and lowest rates differing by more than a factor of two. However, these release rates were comparable to that observed in full-scale EBPR facilities (Gu et al., 2008). Regarding aerobic phosphorus uptake, complete removal in the reactor operated at a one hour anaerobic HRT required more than 30 minutes of aerobic exposure. The microorganisms exposed to longer anaerobic HRTs achieved complete phosphorus removal within (and likely less than) 30 minutes aerobically. Not only did phosphorus removal occur in a shorter time, it would appear that aerobic kinetics were

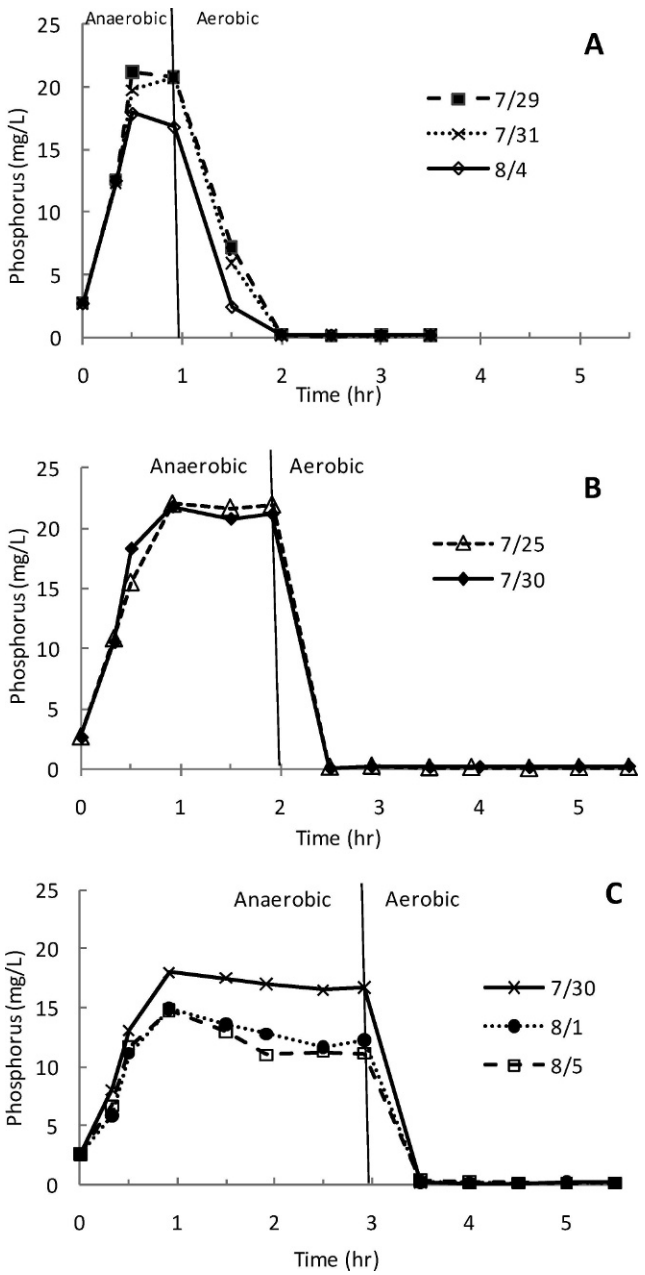

Figure 1—Bulk solution phosphorus profiles for each of the three bench-scale wastewater treatment reactors tested in this study. Shown are the bulk solution phosphorus concentrations over multiple operational cycles for each reactor (1A: F.10.12.1.3; 1B: F.9.12.2.4; 1C: F.10.12.3.3).

enhanced in the SBRs operated at longer anaerobic HRTs (Fig. 1A versus Figs. 1B and 1C). Nevertheless, although the reactors were operated at different anaerobic HRTs, effluent phosphorus concentrations were consistently low and comparable in magnitude (Table 2). With an influent VFA-to-influent phosphorus ratio exceeding 25, this observed high-quality EBPR performance would be expected (Horgan et al., 2010). The results further correlate well with those of Lopez-Vazquez et al. (2008), who propose optimal ranges of VFA species (e.g., acetate-to-propionate) to select for PAOs. Consistent with their observations, the operating conditions in all bioreactors were such that PAOs should broadly predominate.

In each reactor, although VFAs were consistently depleted within one hour anaerobically, no secondary phosphorus release was observed. This observation is in contrast with that observed by some investigators (Barnard et al., 1998; Stephens et al., 1998; Wouters-Wasiak et al., 1996); although it was consistent with others (Kuba et al., 1993). One potential explanation for this seemingly contradictory phenomenon could be the occurrence of surface reaeration during the anaerobic period. Both Plósz et al. (2003) and Koch et al. (2000) observed such a phenomenon in preanoxic denitrification (both at a laboratory level and in full-scale treatment facilities). Upon closer inspection of the results in Figure 1, a slight decrease in bulk solution phosphorus anaerobically can, in fact, be observed [in particular for the SBR operated at a three-hour anaerobic HRT (Figure 1C)]. This phenomenon also has been observed (although not discussed) by others in EBPR systems (Hesselmann et al., 2000; Neethling et al., 2005). Although such an occurrence—if, in fact, surface reaeration was occurring—would be considered undesirable in EBPR, overall process performance was not impaired.

Additional evidence of typical EBPR activity occurring was documented by monitoring bulk solution mineral concentrations during the anaerobic period. As noted, in EBPR systems hydrolyzed phosphate is released anaerobically from the cell as a metal phosphate complex (principally K^+ and Mg^{2+}) (Mulkerrins et al., 2004). Thus, bulk solution mineral concentrations should stabilize once anaerobic phosphorus release ceases. In fact, this phenomenon was observed (Figure 2). Significant amounts of Mg^{2+} and K^+ were released into bulk solution; this ended when phosphorus concentrations stabilized. Further, on a stoichiometric basis, the ratios of K^+:P (0.22 mol:mol) and Mg^{2+}:P (0.32 mol:mol) were consistent with current theory (Mulkerrins et al., 2004). The dominance of Mg^{2+} in phosphate transport was similarly observed by van Veen et al. (1994).

Although longer anaerobic HRTs seemed to have no apparent adverse effect on EBPR process performance, the results do suggest that the microbes managed phosphorus reserves differently as HRT increased. Specifically, one biochemical response by the consortia can be quantified through the microorganisms' use of phosphorus and VFAs within the anaerobic period. The success of EBPR is theoretically and centrally predicated on significant anaerobic phosphorus release associated with VFA uptake and storage. In fact, the phosphorus released-to-VFA uptake ratio has been proposed as a measure of successful process performance (Filipe et al., 2001; Smolders et al., 1994). For each EBPR SBR analyzed, the ratios were assessed for phosphorus released anaerobically (on a mmol P basis) to the VFA uptake (on a mmol carbon basis) (commonly referred to as the P:C) and the P:C:VSS (Table 2). The P:C (based on total VFAs) was effectively the same for the one- and two-hour anaerobic HRTs, but decreased by approximately 33% at the three-hour anaerobic HRT. In particular, comparing reactors F.9.12.2.4 and F.10.12.3.3 (same total cycle length and thus same mass of carbon fed per cycle), significantly less phosphorus was released per microbe for the consortia experiencing a longer anaerobic HRT. By normalizing the ratios to VSS (P:C:VSS) or, conversely, only considering acetate (the model VFA in EBPR), similar conclusions can be drawn. These results would suggest that the F.10.12.3.3

Table 2—Summary of parent bioreactor average volatile suspended solids (VSS), average phosphorus release, and average effluent phosphorus concentrations (see also Figure 1).

Reactor	Average biomass (mg VSS/L^{-1})	Average phosphorus release					Avg. Effluent P (mg P/ L^{-1})
		mgP× gVSS^{-1}	mgP× (gVSS^{-}h) $^{-1}$	mmolP× (mmolC*gVSS) $^{-1}$	mmolP× mmolC^{-1}	mmolP× mmolHAc^{-1}	
F.10.12.1.3	2 793	6.2	12.4	0.11	0.30	0.44	0.15
F.9.12.2.4	2 630	7.3	8.0	0.12	0.31	0.59	0.18
F.10.12.3.3	2 547	5.3	5.8	0.08	0.20	0.33	0.15

consortium anaerobically processed VFAs in a more energetically efficient manner. Contrasting these results with the theoretical model of Filipe et al. (2001), the P:C (HAc basis) should have ranged from 0.68 to 0.70 (for the anaerobic pH values of 7.7 to 7.8 in this study), which are significantly higher than actually observed.

In summary, these results suggest that 1) mixed microbial consortia in real wastewater environments are potentially more energetically efficient than would be predicted using synthetic wastewaters or 2) operating with real wastewater enriches for a different consortia, or both. The former conclusion is supported by Gu et al. (2008), who observed P:C (HAc basis) in full-scale EBPR facilities comparable to that presented herein. Observations from Beer et al. (2006) support the latter conclusion.

Performance Stability. Under constant anaerobic HRT conditions, the respective consortia maintained efficient EBPR performance. Influent wastewater volumes entering a full-scale WWTF will vary over time (both within a day and over a year), however, and thus a given microbial consortium will experience variable anaerobic HRTs. To assess the potential effect on the microorganisms, biomass from reactor F.9.12.2.4 was subjected to different anaerobic HRTs—one and three hours—than imposed during steady-state operations. The microorganisms were supplied with the same wastewater quantity, on a volumetric basis, as the parent treatment reactor. As shown (Figure 3), the consortium performed comparable to that observed in its parent SBR. The observed maximum phosphorus release was both similar to that of the treatment reactor (Figs. 1B versus 3) and between the two batch tests. Effluent concentrations were less than 0.12 mg P/L^{-1}. It would appear, however, that as observed in the steady-state treatment reactors, longer anaerobic exposure induces more rapid aerobic phosphorus removal.

Combined with variable HRT, microorganisms in full-scale WWTFs also can experience variable VFA loading. As noted, anaerobic phosphorus release metabolisms are theoretically and intrinsically linked to anaerobic VFA metabolisms (Mino et al., 1998). In this regard, Filipe et al. (2001) suggested that "shock" VFA loading could impair EBPR performance and yield higher effluent phosphorus concentrations. Results from this investigation suggest otherwise. Using microorganisms from reactor F.9.12.2.4, when the consortium was exposed to an approximately 320% increase in VFAs, excess energy [in the form of hydrolyzed polyphosphate reserves, as indicated by a nearly 70% increase in bulk solution phosphorus (Figs. 4A versus 4B)] was available to achieve complete VFA uptake. Note that VFAs were fully depleted in approximately 30 minutes in both batch reactors tested, even though both were inoculated with the same quantity of microorganisms. Although the consortium generated signifi-

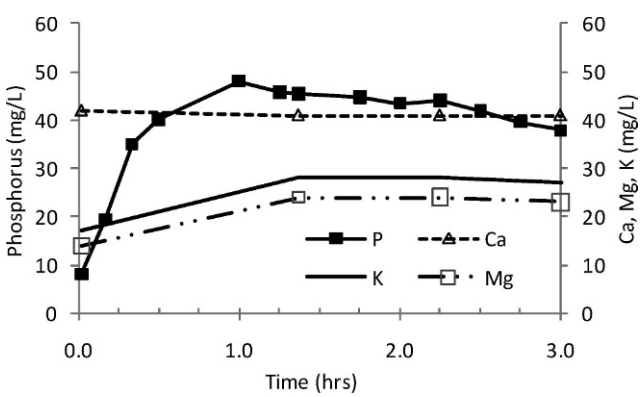

Figure 2—Soluble phosphorus (P), calcium (Ca), potassium (K), and magnesium (Mg) concentrations under anaerobic conditions in a batch reactor. As shown, the microbial consortium released P anaerobically (consistent with enhanced biological phosphorus removal theory, EBPR). Concentrations of Mg and K increased concurrent with anaerobic P release (also consistent with EBPR theory). Calcium concentrations were constant over the test period. The microbial seed was derived from reactor F.9.12.2.4, and fed 90% raw wastewater and 10% fermenter liquor. All data was obtained following the addition of substrate.

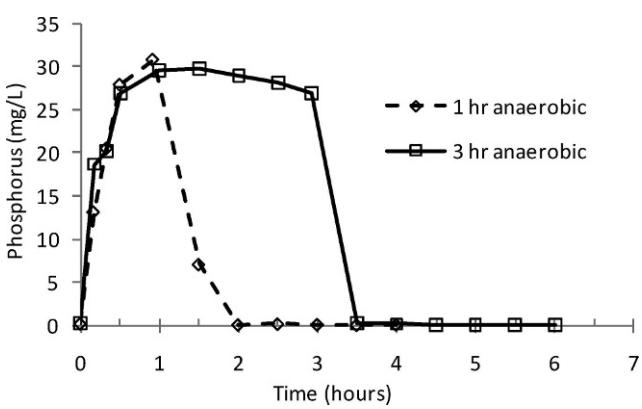

Figure 3—Anaerobic-aerobic bulk solution phosphorus cycling in a single-run batch reactor using biomass obtained from treatment reactor F.9.12.2.4. The microorganisms, which had been conditioned to a two-hour anaerobic hydraulic retention time (HRT), were subjected to either one- or three-hour anaerobic HRTs, followed by a three-hour aerobic period. Shown are bulk solution phosphorus concentrations for the two batch tests.

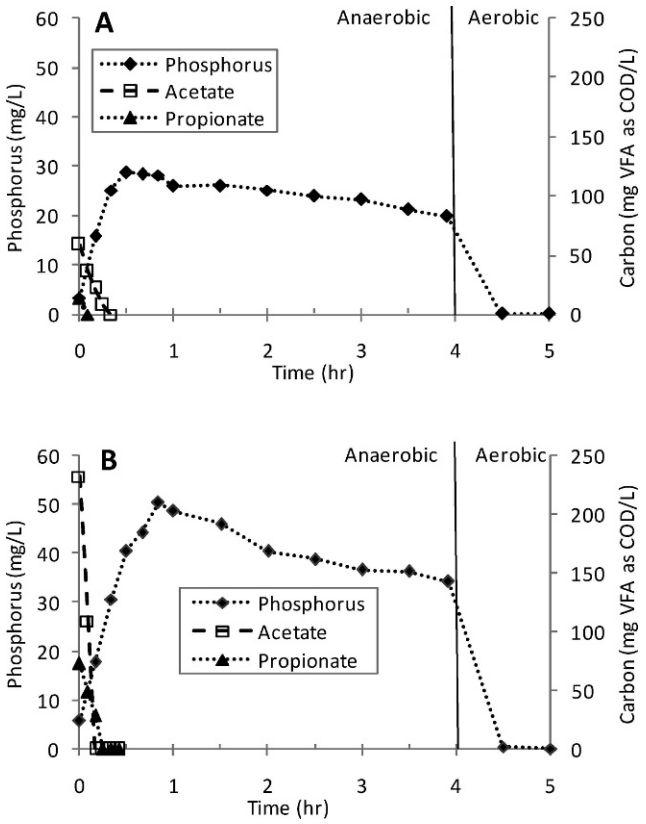

Figure 4—Bulk solution phosphorus and volatile fatty acid (VFA; reported on a chemical oxygen demand, COD, basis) profiles for two single-run batch reactors, each inoculated with biomass from treatment reactor F.9.12.2.4 and fed substrate at the following raw wastewater-to-fermenter liquor ratios (volumetric basis): (A) 90:10 and (B) 70:30.

cantly higher bulk solution phosphorus concentrations anaerobically when provided more VFAs, overall phosphorus removal was nevertheless comparable. The P:C was 0.37 Pmmol:Cmmol for the 90:10 batch test, decreasing to 0.16 for the 70:30 batch test; comparatively, the parent reactor exhibited a P:C of 0.31. Thus, although the same microbial consortium expended more total energy metabolizing VFAs, on a unit-carbon basis less energy was required. These results suggest that EBPR consortia are quite capable of adapting to dynamic operational and substrate conditions to maintain stable performance.

Effect of Anaerobic Hydraulic Retention Time on the Mixed Microbial Consortia. It is assumed that by operating a WWTF in the prescriptive EBPR manner, the mixed microbial consortium will become enriched for the requisite PAO population (Oehmen et al., 2007; Tchobanoglous et al., 2003). This presumption is further supported by both Onuki et al. (2002) and Ren et al. (2007) who, after applying PCR/denaturing gradient gel elecrophoresis (DGGE) on EBPR reactors, concluded that simply exposing microbes to repeated anaerobic/aerobic cycling ultimately eliminated microbes not capable of BPR. In this regard, and according to Oehmen et al. (2007) and Lopez-Vazquez et al. (2007), the P:C exhibited by the microbial consortia in our reactors (Table 2) would suggest that the three SBRs were dominated by PAOs. Debate remains as to both the uniqueness and the relative distribution of PAOs within the total microbial population (He et al., 2007; He et al., 2008; Liu et al., 2001; Zhou et al., 2008). Research has further suggested that assessment of PAO populations in EBPR systems would be best achieved using reactors that simulate full-scale operations with real wastewater (Beer et al., 2006).

To investigate the relative microbial diversity in the three EBPR reactor configurations tested in this research, DNA was extracted from each bioreactor during steady-state operations. The DNA was PCR amplified with 16S rDNA primers, then separated through DGGE (Figure 5). Each DGGE band within a lane represents a unique amplified DNA sequence present in the sample. Although the individual bands alone do not provide any phylogenetic information about the populations in the reactors, this molecular technique can be used to broadly assess the potential similarity between treatment reactors and to identify important mixed-microbial consortia community members (de Araujo et al., 2008).

Several relevant observations can be drawn from the DGGE results. First, considering the broadly conserved eubacterial 16S rDNA data, little if any difference can be seen between the standard PCR and touchdown PCR results. These results confirm that minimal nonspecific priming occurred (touchdown PCR is applied to minimize mispriming), and thus the results are an accurate representation of the microbial population. Comparing the three reactors, the bacterial populations appear similar. This was expected because the three EBPR reactors performed comparably (Table 2). Although the populations were similar, there were some subtle differences. For the one-hour anaerobic HRT (F.10.12.1.3), bands "a" and "c" are unique, and band "b" also can be observed in reactor F.9.12.2.4. These microbial species seemingly disappear at the three-hour anaerobic HRT (F.10.12.3.3). At the two-hour anaerobic HRT, band "d" appears to be more predominant than observed in the other two reactors (it is even questionable if this band is present in F.10.12.3.3). Finally, for reactor F.10.12.3.3, two new bands appear, albeit faintly (e, f). Collectively, these results suggest that certain bacterial species were not necessary for EBPR success and that bioreactor operational condition (in this case, anaerobic HRT) can vary and enrich for certain microbial diversity without impairing EBPR performance. Similar observations were drawn by Liu et al. (2000) and Gu et al. (2008).

In addition to investigating the broad eubacterial population, extracted DNA was amplified using 16S rDNA primers specific to *Candidatus* Accumulibacter *phosphatis* (He et al., 2007). As shown in Figure 5 (lanes 3, 6, and 9), two bands are predominantly common to all three reactors (j, k). Considering that this primer set was theoretically specific to a single PAO species that contains only two rRNA (rrn) gene copies, this result was expected (Garcia Martin et al., 2006). Additional bands can be observed in the reactors operated at longer anaerobic HRTs, however, which suggests that either the primers amplified additional *Candidatus* Accumulibacter species or that the primers were not necessarily specific to the species. The spatial differences between bands that were presumed to be *Candidatus* Accumulibacter *phosphatis* (j, k) and the additional bands that appear in the longer anaerobic HRT reactors (g, h, i) was quite significant, which means that the G+C content in the two groups of amplicons was significantly different (the denaturing gradient used in this study was 20 to 70%).

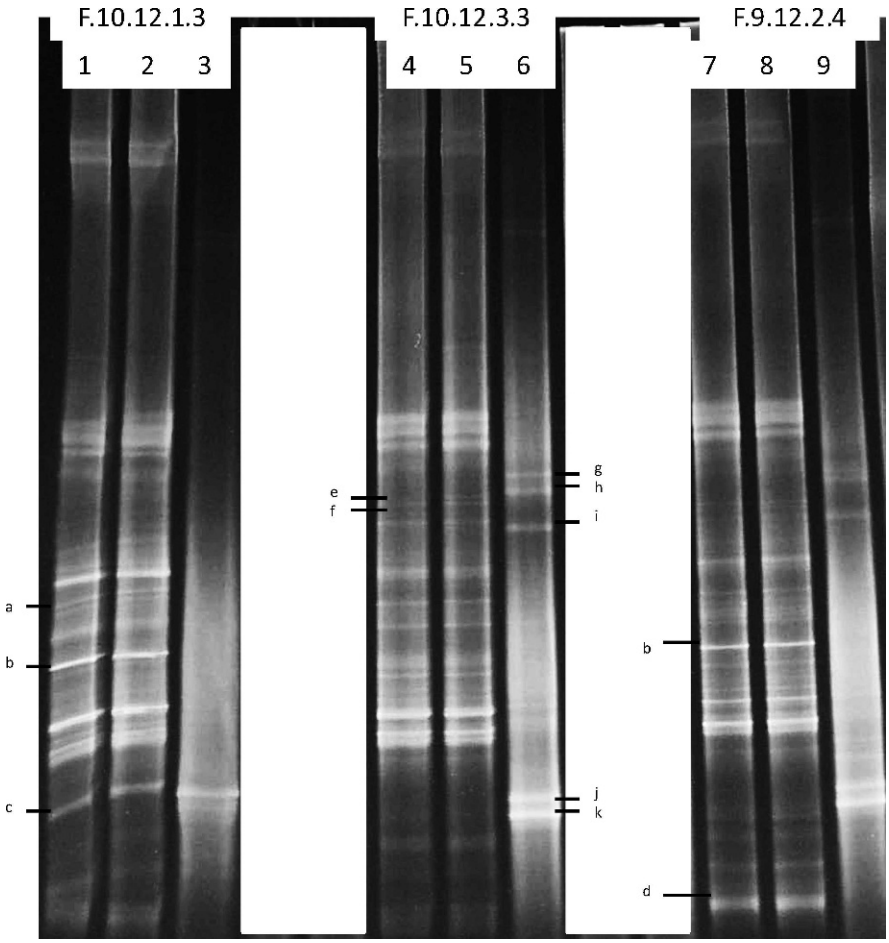

Figure 5—Denaturing gradient gel elecrophoresis (DGGE) with genomic deoxyribonucleic acid (DNA extracted and amplified from each reactor. Lane designations are as follows: (1) F.10.12.1.3–16S eubacterial primers; (2) F.10.12.1.3–16S eubacterial primers, touchdown polymerase chain reaction (PCR); (3) F.10.12.1.3–phosphorus-accumulating organisms (PAO) 16S primers; (4) F.10.12.3.3–16S eubacterial primers; (5) F.10.12.3.3–16S eubacterial primers, touchdown PCR; (6) F.10.12.3.3–PAO 16S primers; (7) F.9.12.2.4–16S eubacterial primers; (8) F.9.12.2.4–16S eubacterial primers, touchdown PCR; (9) F.9.12.2.4–PAO 16S primers. Naming scheme: "F" designation represents that the reactor was fed 90% raw wastewater + 10% primary solids fermenter liquor (v/v); the first number represents solids retention time (SRT); the second number hydraulic retention time (HRT); the third number is the length of the anaerobic period; and the fourth number is the length of the aerobic period.

Effect of Anaerobic Hydraulic Retention Time on Phosphorus-Accumulating Organisms. To further characterize the mixed microbial consortia in the three EBPR reactors, qPCR was used on the extracted genomic DNA. This molecular tool can be used to compare the relative quantity of microorganisms within and between samples. Quantification is based on the Cq parameter (quantification cycle), which represents the threshold concentration of amplified DNA that can be detected fluorescently by the qPCR instrument; the higher the Cq value, the less initial template material in the sample (Bustin et al., 2009). The qPCR process was used on the extracted DNA from each reactor using both eubacterial and the *Candidatus* Accumulibacter *phosphatis* primer sets.

The quality of qPCR data is assessed through both primer specificity and amplification efficiency. High specificity, as determined by the PCR melting curve, was confirmed by the observation of a single melting peak for both primer sets. For the eubacterial primers, a single peak was observed at approximately 82 °C; for the Accumulibacter primers, a single peak occurred at approximately 84 °C. Amplification efficiency, which should range from 90 to 110%, was established by generating calibration curves based on a series of dilutions of the extracted genomic DNA for each set of primers (He et al., 2007). For distinct amplicons to be compared quantitatively, the genes of interest must be amplified with comparable efficiencies (Bustin et al., 2009). Results from the qPCR calibration curves, established by applying a linear regression to the amplified dilution series, are summarized in Table 3. All calibration curves exhibited a linear range. Finally, negative controls for the eubacterial primer set exhibited a minimum Cq of 30; the Accumulibacter primer set negative control Cq was 39.

The relative quantity of *Candidatus* Accumulibacter *phosphatis* in each reactor was determined according to the ΔΔCq method as described by Livak and Schmittgen (2001). Quantitation was determined based on normalizing *Candidatus* Accumulibacter *phosphatis* data to the eubacterial 16S rDNA. In addition, it was assumed that bacteria in activated sludge contain an average of 4.1

Table 3—Summary of quantitative real-time polymerase chain reaction (qPCR) results, including information on the calibration curve and results on the relative quantity of *Candidatus* Accumulibacter *phosphatis* in the three tested enhanced biological phosphorus removal (EBPR) reactors.

		qPCR calibration curve ($n = 1$)				
Reactor	Target gene	Number of data points	Efficiency (%)	Slope	R^2	Relative quantity
F.10.12.1.3	Eubacterial 16S rDNA	5	96.1	−3.42	0.995	–
	Candidatus Accumulibacter *phosphatis*	4	96.0	−3.44	0.983	21.6%±3.4% ($n = 2$)
F.9.12.2.4	Eubacterial 16S rDNA	5	100.8	−3.3	0.994	–
	Candidatus Accumulibacter *phosphatis*	6	103.0	−3.3	0.981	7.1% ±0.9% ($n = 3$)
F.10.12.3.3	Eubacterial 16S rDNA	5	97.3	−3.39	0.997	–
	Candidatus Accumulibacter *phosphatis*	4	94.5	−3.46	0.998	16.8% ±2.1% ($n = 2$)

copies of the 16S rDNA gene; *Candidatus* Accumulibacter *phosphatis* is estimated to contain two copies of the target 16S gene (He et al., 2007; Kaetzke et al., 2005). As shown (Table 3), the mixed microbial consortia in reactor F.10.12.1.3 would appear to be more enriched for *Candidatus* Accumulibacter *phosphatis*; the consortia in reactor F.9.12.2.4 was least enriched. The estimated fraction of *Candidatus* Accumulibacter *phosphatis* present in the respective consortia ranged from 7.1 to 21.6%, which is consistent with that observed by He et al. (2007). Contrasting the qPCR results with the DGGE data (Figure 5), it is of note that the reactor with the fewest bands (F.10.12.1.3) was appeared to be the most enriched for *Candidatus* Accumulibacter *phosphatis*. These results reinforce that PCR/DGGE data can only be used to generally characterize a given microbial population, and that such data cannot be interpreted quantitatively.

Conclusions

The purpose of the research presented and discussed herein was to develop a better understanding on the dynamic effects of anaerobic HRT on both EBPR performance and on enrichment of PAOs. Key conclusions from this research are as follows.

- Although exposing microorganisms to extended anaerobic HRTs has been cautioned against for EBPR configured systems, the results suggest that longer anaerobic periods will not negatively affect performance.
- Extended anaerobic HRTs may positively affect phosphorus removal through enhanced aerobic uptake.
- EBPR consortia appear to maintain reserve energetic capacity in the form of polyphosphate that can be used to survive and grow under variable operational and environmental conditions.
- Anaerobic HRTs of 1 to 3 hours will result in the enrichment of PAOs (specifically *Candidatus* Accumulibacter *phosphatis*) at approximately 7.1 to 21.6% of the total population, and will further ensure successful biological phosphorus removal.

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