Functionally Relevant Microorganisms to Enhanced Biological Phosphorus Removal Performance at Full-Scale Wastewater Treatment Plants in the United States

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ABSTRACT: The abundance and relevance of *Accumulibacter phosphatis* (presumed to be polyphosphate-accumulating organisms [PAOs]), *Competibacter phosphatis* (presumed to be glycogen-accumulating organisms [GAOs]), and tetrad-forming organisms (TFOs) to phosphorus removal performance at six full-scale enhanced biological phosphorus removal (EBPR) wastewater treatment plants were investigated. Coexistence of various levels of candidate PAOs and GAOs were found at these facilities. *Accumulibacter* were found to be 5 to 20% of the total bacterial population, and *Competibacter* were 0 to 20% of the total bacteria population. The TFO abundance varied from nondetectable to dominant. Anaerobic phosphorus (P) release to acetate uptake ratios (P_{rel}/HAc_{up}) obtained from bench tests were correlated positively with the abundance ratio of *Accumulibacter*/(*Competibacter* +TFOs) and negatively with the abundance of (*Competibacter* +TFOs) for all plants except one, suggesting the relevance of these candidate organisms to EBPR processes. However, effluent phosphorus concentration, amount of phosphorus removed, and process stability in an EBPR system were not directly related to high PAO abundance or mutually exclusive with a high GAO fraction. The plant that had the lowest average effluent phosphorus and highest stability rating had the lowest P_{rel}/HAc_{up} and the most TFOs. Evaluation of full-scale EBPR performance data indicated that low effluent phosphorus concentration and high process stability are positively correlated with the influent readily biodegradable chemical oxygen demand-to-phosphorus ratio. A system-level carbon-distribution-based conceptual model is proposed for capturing the dynamic competition between PAOs and GAOs and their effect on an EBPR process, and the results from this study seem to support the model hypothesis. *Water Environ. Res.,* **80,** 688 (2008).

KEYWORDS: enhanced biological phosphorus removal, glycogen-accumulating organisms, polyphosphate-accumulating organisms, tetrad-forming organisms, *Accumulibacter*, *Competibacter*.

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Introduction

Enhanced biological phosphorus removal (EBPR) has been applied worldwide in full-scale activated sludge facilities to achieve low effluent phosphorus (P) concentrations. However, process reliability varies among wastewater treatment plants (WWTPs), and many facilities suffer from periodic upsets (Gu et al., 2004, 2005; Neethling et al., 2005; Stephens et al., 2004).

Successful EBPR processes rely on the selection of polyphosphate-accumulating organisms (PAOs) via uptake of volatile fatty acids (VFAs) and phosphorus release in an anaerobic zone followed by phosphorus uptake into polyphosphate storage granules in a subsequent aerobic zone. Lack or deterioration of phosphorus removal performance was attributed to the proliferation of glycogen-accumulating organisms (GAOs) in laboratory-scale EBPR reactors (Cech and Hartman, 1990, 1993; Satoh et al., 1994) and also at full-scale facilities (Gu et al., 2005; Saunders et al., 2003). The GAOs are able to compete with PAOs for VFAs in the anaerobic zone without phosphorus release and with phosphorus uptake only for cell synthesis. Because VFAs are often the limiting substrate for EBPR processes, an abundance of GAOs could potentially affect phosphorus removal efficiency.

The candidate PAOs identified include *Accumulibacter phosphatis* in the *Rhodocyclaceae* group of the *Betaproteobacteria* (Crocetti et al., 2000; Hesselmann et al., 1999; Oehmen et al., 2007), *Actinobacteria* (Kong et al., 2005), and *Malikia spp.* (Spring et al., 2005). The candidate GAOs identified include *Amaricoccus* spp. and *Defluvicoccus vanus*-related organisms in the *Alphaproteobacteria*, *Betaproteobacteria* (*Quadracoccus* sp.), *Competibacter* in the *Gammaproteobacteria*, and possibly some *Actinobacteria* (*Micropruina glycogenica*, *Tetrasphaera* spp., and *Kineosphaera limosa*) (Crocetti et al., 2002; Kong, Beer, Seviour, Lindrea, and Rees, 2002; Kong et al., 2001, Kong, Ong, Ng, and Liu, 2002; Liu et al., 2001; Meyer et al., 2006; Seviour et al., 2000, Tsai and Liu, 2002; Wong et al., 2004). Candidate GAOs consist of several different morphologic types. Among them, characteristic cocci-arranged tetrads or sheets are commonly observed, and they are referred to as tetrad-forming organisms (TFOs) (Cech and Hartman, 1990, 1993). However, not all TFOs have the GAO phenotype. Investigation of TFOs in two laboratory-scale sequence batch reactors revealed diverse phylogenetic affiliation and physiological traits, in terms of intracellular polyphosphate and polyhydroxyalkanoates (PHA) accumulation (Tsai and Liu, 2002; Wong et al., 2004).

Fluorescence in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes allows the detection and quantification of candidate PAOs and GAOs in EBPR activated sludges.

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Rhodocyclus-group organisms were found to account for 26 and 73% of the total PAO populations in two plants surveyed by Zilles et al. (2002). Saunders et al. (2003) also found various levels of *Accumulibacter* in the plants they studied, suggesting the important role they may play in EBPR processes. However, a study of PAOs in full-scale EBPR systems in Australia by Beer et al. (2006) indicated that *Actinobacteria* comprised dominant populations containing polyphosphate in many plants instead of *Accumulibacter*.

This study investigated the abundance of candidate PAOs and GAOs, including *Accumulibacter, Competibacter,* and TFOs at six full-scale EBPR plants in the United States, for which, operation and performance data were collected over a 2-year period. The VFA uptake and phosphorus release and uptake characteristics were evaluated with mixed liquor from each plant in batch tests. Both light microscope and molecular techniques were used to examine the presence of candidate PAOs and GAOs in the plant mixed liquor samples. From these results, possible relationships between the population distribution of PAOs and GAOs and phosphorus removal efficiency and stability were evaluated.

Methods

This study evaluated the phosphorus removal performance, relative PAO/GAO populations, and mixed liquor characteristics of VFA uptake and phosphorus release under anaerobic conditions and phosphorus uptake under aerobic conditions for six full-scale EBPR WWTPs in the United States. Operation and performance data were collected and summarized for the 2001 through 2003 time period. Mixed liquor samples were obtained from each facility in August through September 2003, to evaluate the composition and characteristics of the VFA-utilizing populations. The performance data collected from the WWTPs include influent flow, temperature, solids retention time (SRT), and influent and effluent total and soluble phosphorus across the EBPR systems. In addition, intensive sampling was conducted for 2 to 3 months at plants to determine other wastewater characteristic and system performance data, such as metals, flocculated and filtered chemical oxygen demand (ffCOD), and VFA. The flocculation–filtration method by Mamais et al. (1993) was applied to both secondary influent and effluent samples to obtain their ffCOD concentrations, with their difference being the readily biodegradable COD (rbCOD) concentration. The secondary influent rbCOD to soluble orthophosphorus (rbCOD/P) ratios were then determined.

Batch tests were conducted to evaluate anaerobic VFA uptake and phosphorus release rates and aerobic phosphorus uptake characteristics using mixed liquor samples from the aerobic basin of each facility that were shipped overnight on ice. Thee samples were purged with nitrogen for 15 to 60 minutes to reach the initial anaerobic condition before carbon (sodium acetate) addition. Sodium acetate (60 to 70 mg/L as acetate) was added to 3.5 to 4 L mixed liquor in a 5-L reactor mixed with a magnetic stir plate and stirring bar. During the initial anaerobic period (45 minutes), the reactor was sparged with nitrogen gas, and, in the following aerobic period (200 to 250 minutes), the reactor was sparged with air. Samples taken at the start of the experiment and then at 10- to 15-minute intervals were analyzed for soluble phosphorus and VFAs. Oxidation–reduction potential and pH were continuously monitored, but not controlled.

The same mixed liquor that was used for bench batch phosphorus release and uptake and release tests was subject to microscopic observation of chemical staining of cells with Neisser stain and 4′, 6-diamidino-2-phenylindole (DAPI) to monitor the presence of PAOs and TFOs. The TFOs were semiquantified by counting the number of fields out of 10 fields in which they were present. For DAPI stain, the activated sludge from the aerobic zone was fixed, stained, and observed using confocal laser-scanning microscopy (Zeiss, LSM 5 PASCAL, AxioSkop 2, Oberkochen, Germany) equipped with epifluorescence. An estimate of PAO abundance level number was given based on visual observation, with 1 = some found, 2 = easily found, and 3 = abundant. The same mixed liquor samples were also fixed and prepared on microslides (Paul Marienfeld, Lauda-Königshofen, Germany) for FISH analyses. For FISH, bacteria were probed with a EUBMIX probe (Daims et al., 1999); *Accumulibacter* were probed with PAO421, PAO651, and PAO846 together as so-called ''PAOMIX''(Crocetti et al., 2000); *Competibacter* were probed with GAOQ989 and GB-G2 (Crocetti et al., 2002, Kong, Beer, Seviour, Lindrea, and Rees, 2002); and *Alphaproteobacteria* were probed with ALF969. Details of the FISH and quantification protocol used are described in Saunders et al. (2003). Briefly, microscopic slides that were covered with sludge samples were subject to FISH hybridization and then visualized with a BioRad Radiance 2000 confocal laser scanning microscope (Hercules, California) using an Olympus 60× oil-immersion objective collecting 8-bit, 512 × 512 pixel images. The area containing Cy3-labeled specific probe (PAOMIX or GAOQ989) cells was quantified as a percentage of the area of Cy5-labeled bacterial probe (EUBMIX) within each image using Image Pro Plus 4 Software (Media Cybernetics, Bethesda, Maryland).

Dissolved phosphorus was analyzed by inductively coupled plasma via an atomic emission spectrophotometer (model 955, Jarrell Ash, Franklin, Massachusetts) following filtration through a 0.22-μm glass-fiber filter. The VFAs (acetic, propionic, isobutyric, butyric, and valeric acids) were analyzed by gas–liquid chromatography using a flame ionization detector and a 0.32-mm internal diameter × 30 m Hewlett Packard (Palo Alto, California) capillary-free-fatty-acid-phase column (Agilent Technologies; Wilmington, Delaware) with helium carrier gas at 2.7 mL/min on a Perkin Elmer auto-system gas chromatograph (Perkin Elmer, Waltham, Massachusetts). Samples were filtered through 0.22-μm glass-fiber filters and acidified to pH 2 with 5.25 N sulfuric acid, then stored at 4°C until analysis. The following oven temperature program was used: 80°C for 1.5 minutes, 20°C/min to 160°C for 1 minute, then 10°C/min to 220°C for 3 minutes. The detection limit was 3 to 5 mg/L for a 1-μL sample injection.

Results and Discussion

Full-Scale Enhanced Biological Phosphorus Removal Performance. Phosphorus removal performance and key operating parameters are summarized for the EBPR WWTPs in Table 1. The facilities represent a range of EBPR process configurations, including anaerobic–anoxic–aerobic (A2O), Virginia initiative plant (VIP), University of Cape Town (UCT), and modified UCT (MUCT). Their treatment capacities ranged from 18 900 to 113 600 m^3/d (5 to 30 mgd). The EBPR process influent readily biodegradable COD to soluble phosphorus ratio (rbCOD/P) ranged from 5 to 38 mg/mg. Secondary effluent total phosphorus (TP) concentration was less than 1.0 mg/L for all the WWTPs, except one that averaged 1.2 mg-P/L, indicating effective EBPR processes for all the facilities. The reliability of phosphorus removal in the EBPR facilities was assessed from a cumulative frequency of percent occurrence versus secondary clarifier effluent total phosphorus concentration. For an effluent total phosphorus concentration of

Table 1—Configurations, operating conditions, and phosphorus removal for the EBPR WWTPs.

		WWTP					
		A	B	C	D	E	F
Parameter	Units	(A2O)	(A2O)	(VIP)	(VIP)	(MUCT)	(UCT)
Average flow	m^3/d	83 300	75 700	113 600	75 700	18 900	18 900
	mgd	22	20	30	20	5	5
Temperature	°C	20	—	21	—	20	21
SRT	days	11	9.8	9.5	8.5	6.7	9.4
SI rbCOD/PO_4-P[a]	g/g	29	20	27	5	38	25
SI TP[b]	mg/L	6.1	6	5.3	14	6.5	8.8
SI PO_4-P	mg/L	3.4	3	4.1	12	3.4	5.6
SE TP	mg/L	0.51	0.82	0.64	1.2	0.15	0.9
SE PO_4-P	mg/L	0.26	0.54	0.55	NA	NA	0.75
CF of SE TP<0.5mg/L[c]	%	80	64	70	24	95	76
Notable features		Good phosphorus removal, add fermenter effluent to primary clarifier	Not very stable	Good phosphorus removal, with annual upset in summer	Unstable phosphorus removal	Good phosphorus removal, very stable process, adds sugar waste to primary clarifier	Good phosphorus removal

[a] SI = secondary influent readily biodegradable COD (rbCOD)-to-soluble-phosphorus ratio based on data collected for a period of 1 to 3 weeks when this study was conducted in 2003.
[b] SI TP = Secondary influent average total phosphorus during 2001 to 2003.
[c] Cumulative frequency that measured daily secondary effluent total phosphorus concentration less than 0.5 mg/L during the 2-year period from 2001 to 2003.

less than or equal to 0.50 mg/L, the cumulative frequency of occurrence ranged from 24 to 95%.

Acetate Uptake and Phosphorus Release and Phosphorus Uptake in Batch Tests. The results of anaerobic acetate uptake and phosphorus release rates, aerobic phosphorus uptake rates and specific net phosphorus uptake, and release per unit of biomass in the batch tests are summarized in Table 2. Also shown in Table 2 are the anaerobic-phosphorus-release-to-acetate-uptake-rate ratio (P_{up}/HAc_{rel}), aerobic-phosphorus-uptake-to-anaerobic-phosphorus-release-rate ratio (P_{up}/P_{rel}), and the range of pH recorded during the tests. Phosphorus release and uptake rates varied greatly among the WWTPs, ranging from 5.6 to 31.9 mg-P/gVSS · h and from 2.4 to 9.7 mgP/gVSS · h, respectively. The aerobic phosphorus uptake rates were found to be lower than anaerobic phosphorus release rates for all plants studied, and the ratio of phosphorus uptake rate to phosphorus release rate ranged from 0.24 to 0.69. The specific net phosphorus release and net phosphorus uptake ranged from 0.005 to 0.024 g-P/gVSS and from 0.004 to 0.015 g-P/gVSS, respectively. Three plant samples showed higher net phosphorus release than phosphorus uptake. Sludge transportation and storage could result in secondary phosphorus release (as evidenced by the relatively high initial soluble phosphorus concentrations in the tests) that was not associated with intracellular carbon energy synthesis. The disturbance of the balance between the intracellular energy and carbon storage could lead to reduced phosphorus uptake in the following cycle (Stephens et al., 1998; Wang and Park, 2001), as

Table 2—Summary of results from batch phosphorus release and uptake tests.

Plant	P_{rel}/HAC_{up} mol/C-mol	Anaerobic phosphorus release rate mg-P/gVSS · h	Aerobic phosphorus uptake rate mg-P/gVSS · h	Acetate uptake rate mg/gVSS · h	P_{up}/P_{rel}	Net phosphorus uptake g-P/gVSS	Net phosphorus release g-P/gVSS	pH range during the test
A	0.39	16.5	9.7	42.5	0.59	0.0088	0.0083	7.4 to 8.2
B	0.5	13.7	3.3	18.6	0.24	0.0075	0.0097	7.2 to 7.8
C1*	0.38	12.4	8.5	27	0.69	0.01	0.0096	7.2 to 8.2
C2*	0.31	13.6	3.8	40.5	0.28	0.0066	0.0083	7.1 to 8.3
D	0.29	10.8	4.3	28.5	0.40	0.0077	0.0077	7.9 to 8.2
E	0.27	5.6	2.4	16.1	0.43	0.0041	0.0051	7.7 to 7.9
F	0.75	31.9	7.9	31	0.25	0.015	0.0243	7.2 to 8.0

* Two separate samples taken at plant C at two different times within 3 months, one taken during good performance, another taken during performance deterioration.

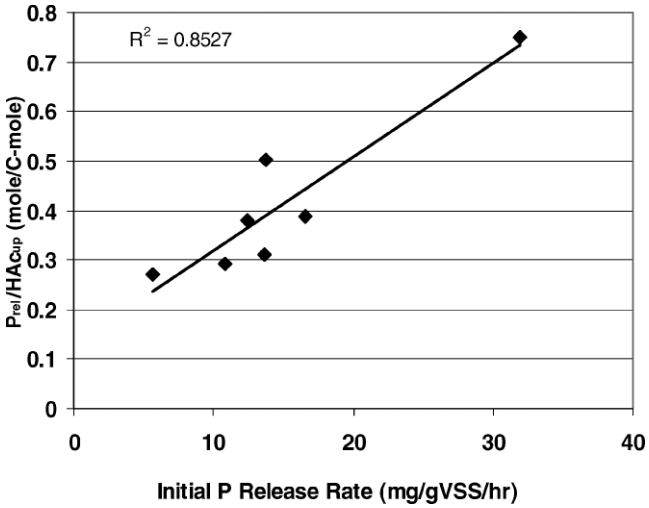


Figure 1—Relationship between P_{rel}/HAc_{up} ratio and initial phosphorus release rate in the batch tests.

observed in this study. Initial acetate uptake rates varied from 16.1 to 42.5 mgHAc/gVSS·h. The P_{rel}/HAc_{up} obtained in this study ranged from 0.27 to 0.75 mole/C-mole, which is in the range of values reported in the literature (0.01 to 0.93 moleP/C-mole acetate) (Bond et al., 1998; Saunders et al., 2003; Schuler and Jenkins, 2003; Wentzel et al., 1988). Based on current understanding of the biochemical pathways of the phosphorus-removal process, the theoretical P_{rel}/HAc_{up} ratio is proposed to be 0.5 to 0.7 mol/C-mol for PAOs (see summary in Schuler and Jenkins, 2003). Values that are lower than theoretical predictions indicate that there are GAOs present in the sludge because they compete for VFA utilization without contributing to phosphorus release.

In previous reports, the P_{rel}/HAc_{up} ratio was correlated with both the initial phosphorus release and the initial acetate uptake rates (Saunders et al., 2003; Schuler and Jenkins, 2003). Although we found a linear correlation between the P_{rel}/HAc_{up} ratios and initial phosphorus release rates (Figure 1), there was no clear correlation between P_{rel}/HAc_{up} ratios and initial acetate uptake rates (data not shown). The latter correlation is likely expected for systems where the increase in P_{rel}/HAc_{up} and in initial acetate uptake rates was caused by an increase in PAO abundance, such as the laboratory-scale reactors (Liu et al., 1997; Schuler and Jenkins, 2003). In those systems, the influent rbCOD/P ratios were varied by keeping the influent rbCOD concentration constant and changing the influent phosphorus concentration. An increase in influent phosphorus led to higher PAO abundance, which then led to both higher acetate uptake rate and a higher P_{rel}/HAc_{up} ratio. However, for full-scale systems that have varying influent rbCOD and phosphorus levels, increases in P_{rel}/HAc_{up} and initial acetate uptake rates do not always co-occur, because the P_{rel}/HAc_{up} ratio is dictated by the relative PAO-to-GAO abundance level. For example, elevation of GAO abundance would lead to higher acetate uptake rates and a lower P_{rel}/HAc_{up} ratio, if PAO abundance remains the same. The unclear correlation between P_{rel}/HAc_{up} ratios and initial acetate uptake rates implies that the plants surveyed in this study had various combinations of PAOs and GAOs, as confirmed by the microscopic observation of candidate PAOs and GAOs (Table 2). Further evaluation also revealed positive correlation between the anaerobic P_{rel}/HAc_{up} ratio and the specific anaerobic net phosphorus release and between the P_{rel}/HAc_{up} ratio and the specific aerobic net phosphorus uptake, as shown in Figures 2 and 3. These results imply that the systems that had a higher relative PAO-to-GAO abundance level had a higher fraction of PAOs in the biomass also (Table 3).

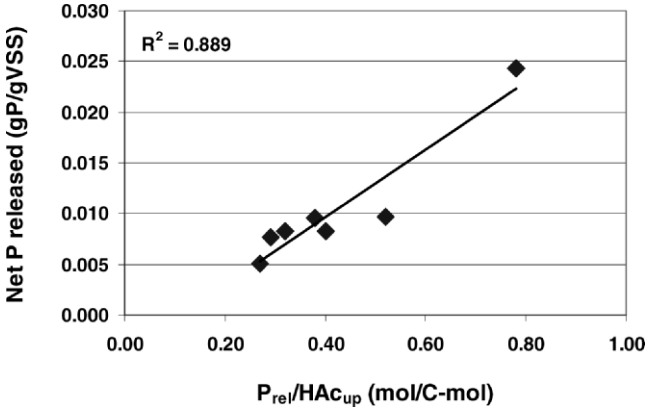


Figure 2 —Relationship between anaerobic net phosphorus release per unit biomass and P_{rel}/HAc_{up} ratio in the batch tests.

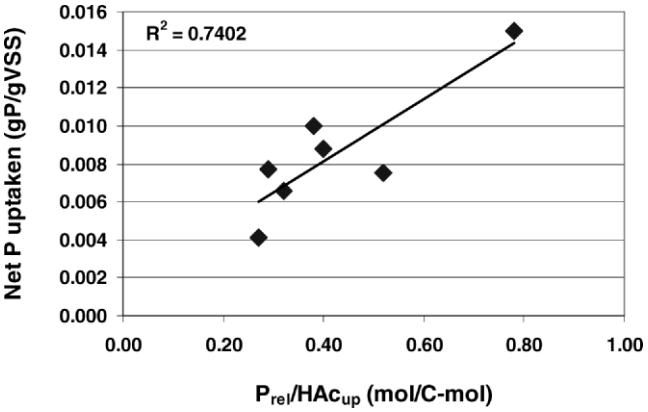


Figure 3—Relationship between aerobic net phosphorus uptake per unit biomass and P_{rel}/HAc_{up} ratio in the batch tests.

Factors that may affect phosphorus and acetate uptake rates include temperature, SRT, and pH. The temperature was kept constant for all experiments at 22 to 25°C and therefore was not expected to affect the acetate uptake rates significantly (Whang and Park, 2001). Even though SRT was considered as a factor influencing the selective enrichment of PAOs/GAOs and affecting the acetate uptake rate (Whang and Park, 2001; Zilles et al., 2002), association of SRT with acetate uptake rates and with the P_{rel}/HAc_{up} ratio was not evident in this study (data not shown). This was probably because the SRT values at the WWTPs studied (6.7 to 11 days) were beyond the range that could affect the competition of PAOs and GAOs (3 to 5 days; Whang and Park, 2001). The pH was found to be a key factor that affected the metabolic rates and competition between PAOs and GAOs (Filipe et al., 2001). The acetate uptake rate by GAOs was shown to increase with pH up to 6.5, being the greatest at pH 7, and starting to decrease at pH 7.5, whereas, for PAOs, the acetate uptake rate was shown to increase with pH up to 6.5, remain nearly constant from pH 7.0 to 8.5, and start to decrease at pH 8.5 (Filipe et al., 2001; Schuler and Jenkins,

Table 3—Phosphorus-release-to-acetate-uptake ratio and quantification of PAOs and GAOs.

Plant	*Accumulibacter*/(*Competibacter* + TFO) ratio	P_{rel}/HAC_{up} (P-mol/C-mol)	Abundance				
			Accumulibacter % of Bacteria[a]	*Competibacter* % of Bacteria[a]	Abundance of PAOs[b]	Abundance of TFO[c]	*Alphaproteobacteria* % of Bacteria[a]
F	5	0.75	5 ± 5	<1	3	1	<1
B	0.7	0.5	5 ± 5	5 ± 5	3	2	<1
A	3.8	0.39	15 ± 5	<1	2	4	5 ± 5
C (7/03/03)	1.9	0.38	15 ± 5	5 ± 5	2	3	15 ± 5
C (9/03/03)	1.5	0.31	15 ± 5	5 ± 5	2	5	15 ± 5
C (9/12/03)	1.2	—	15 ± 5	5 ± 5	2	8	15 ± 5
D	0.7	0.29	10 ± 5	15 ± 5	1	0	5 ± 5
E	0.6	0.27	5 ± 5	<1	1	8	5 ± 5

[a] Abundance of *Accumulibacter, Competibacter*, and *Alphaproteobacteria* are from FISH results.
[b] Abundance of organisms containing polyphosphate were estimated from microscopic observation of Nessier and DAPI stain; 1 = some found, 2 = easily found, and 3 = abundant.
[c] TFO results are from microscopic observation of Neisser stain. The value of this abundance level is the number of microscopic fields (1000× magnification) in which the TFOs were found out of every 10 fields observed, averaged from two separate slides and at least 20 fields monitored.

2002). Therefore, the correlation patterns of anaerobic phosphorus release rate and acetate uptake rate with pH varies depending on relative PAO and GAO levels in the system. In our experiments, the pH was between 7.2 and 8.3 (Table 2). The extent of differences in pH values among plant samples could contribute to, but could not account for, the great variation of acetate uptake and phosphorus release and uptake rates observed (Table 2).

Observation of Candidate Polyphosphate- and Glycogen-Accumulating Organisms. Both candidate PAOs and GAOs were found to coexist at various levels in all the WWTPs (Table 3). *Accumulibacter* were found to be 5 to 20% of the total bacterial population, and *Competibacter* were 0 to 20% of the total bacteria population. The abundance of TFOs varied from nondetectable to dominant.

Accumulibacter were found at all plants with various process configurations and influent wastewater characteristics, suggesting that they could be important to EBPR. However, no correlation was observed between the number of cells containing polyphosphorus (according to Neisser and DAPI staining) and *Accumulibacter* abundance. This could indicate that *Accumulibacter* are not the only PAOs present in full-scale EBPR systems, and the diversity of PAO populations is larger than our current understanding (Beer et al., 2006; Oehmen et al., 2007; Saunders et al., 2003; Wong et al., 2005; Zilles et al., 2002). However, because we did not confirm that all cells containing polyphosphorus actually participated in the well-known EBPR transformations of anaerobic phosphorus release (in a stoichiometric relationship with acetate uptake) and aerobic phosphorus uptake, it is difficult to be emphatic about the latter conclusion. It is well-known that polyphosphorus is a common component in many cells, where it has myriad functions (McInerney et al., 2006).

Higher enrichments of *Rhodocyclus*-related organisms were proposed to be associated with the UCT process compared with the A/O process (Zilles et al., 2002). This association was not really supported in our study. Plants E and F, which were operated in modified UCT and UCT mode, did not contain a higher abundance of *Accumilibacter* than those of plants A and B, which were run in A2O mode. This suggests that there are factors other than process configuration that affect *Accumulibacter* abundance. It is also possible that the difference, if any, is finer than any of the investigative methods could detect in this study.

Competibacter, a candidate GAO in the *Gammaproteobacteria* lineage, was found in 3 of the 6 plants investigated. The TFOs were found at various levels in all plants except one. The TFOs were frequently found in laboratory systems that exhibited no or deteriorated EBPR activities (Crocetti et al., 2002; Kong et al., 2001; Liu et al., 1997; Tsai and Liu, 2002). Although TFOs apparently often possess the GAO phenotype, a recent study revealed that the physiological traits of TFOs are quite diverse, in terms of their ability to accumulate carbon and/or polyphosphate (Tsai and Liu, 2002). The presence of both *Competibacter* and TFOs were observed in full-scale EBPR plants by Wong et al. (2005). Interestingly, only *Competibacter* (but no TFOs) were found at plant D. The reason the *Competibacter* is selected at this plant is not completely clear. One noticeable feature of plant D, however, is that it had a much lower rbCOD/P ratio (5 mg/mg) compared with the other plants, as a result of its high influent phosphorus (14 mg/L). The rbCOD/P ratio seemed to affect the selection of different GAO populations, as shown by Crocetti et al. (2002). In their study, two sequence batch reactors that were fed with different influent rbCOD/P ratios had different microbial community and potential GAOs identified. Whether the rbCOD/P ratio had an affect on the selection of different GAO populations needs further investigation.

The presence of *Alphaproteobacteria* was investigated because a number of TFOs reported belong to *Alphaproteobacteria* (Beer et al., 2002; Meyer et al., 2006; Seviour et al., 2000; Wong et al., 2004). The abundance of *Alphaproteobacteria* was not able to be correlated with the level of total TFOs. However, this finding is expected, considering the diversity of bacteria in this subdivision and the likely diversity of TFOs.

Correlation of P_{rel}/HAc_{up} Ratio with *Accumulibacter*, *Competibacter*, and Tetrad-Forming Organism Abundance. The P_{rel}/HAc_{up} ratio has previously been used as an indicator of PAO abundance in relation to GAO abundance, in both laboratory-scale sequencing batch reactors (SBR) and full-scale processes (Liu et al., 1997; Saunders et al., 2003; Schuler and Jenkins, 2003). It was proposed that P_{rel}/HAc_{up} ratios higher than 0.5 indicate PAO dominance, values less than 0.25 indicate GAO dominance, and values in between indicate mixed populations. In reality, the P_{rel}/HAc_{up} is an indication of organism activity, which

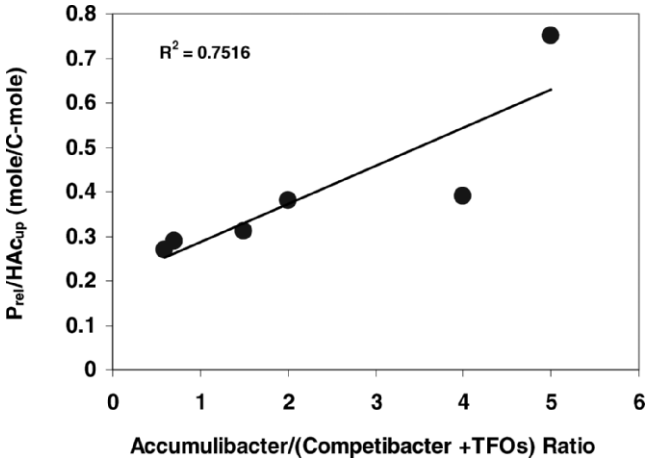


Figure 4—Relationship between P_{rel}/HAc_{up} ratio and abundance ratio of *Accumulibacter* over the sum of *Competibacter* and TFOs.

may not necessarily be proportional to organism abundance. In any case, results in the present study are consistent with the previous findings. Five of the six plants surveyed had P_{rel}/HAc_{up} ratios in the range 0.25 to 0.5 P-mole/C-mole and were found to have mixed *Accumulibacter* (presumed to the abundant PAOs), *Competibacter* (presumed to be the abundant GAOs), and TFO populations (Table 3). Excluding plant B, there seem to be a positive correlation between P_{rel}/HAc_{up} ratio and the *Accumulibacter*/(*Competibacter* + TFO) ratio (Figure 4). Plant F, which had the highest P_{rel}/HAc_{up} ratio of 0.75, had the highest *Accumulibacter*/(*Competibacter* + TFO) ratio. Plants D and E, which showed the lowest P_{rel}/HAc_{up} ratios, had the lowest *Accumulibacter*/(*Competibacter* + TFOs) ratios in their systems.

No consistent correlation between P_{rel}/HAc_{up} ratio and *Accumulibacter* abundance was observed for all the plants studied (Table 3). Plants B and F had higher P_{rel}/HAc_{up} ratios and did not contain a higher abundance of *Accumulibacter*, indicating the existence of other PAOs.

Effect of Glycogen-Accumulating Organisms on Carbon-Source-Utilization Efficiency in Enhanced Biological Phosphorus Removal. The P_{rel}/HAc_{up} is an indicator of carbon-utilization efficiency, because it shows the amount of phosphorus that can be removed per unit amount of carbon utilization. The proliferation of GAOs reduce the P_{rel}/HAc_{up} ratio as a result of their contribution to acetate uptake, but not to the phosphorus release. A general reverse correlation between the P_{rel}/HAc_{up} ratio and the overall abundance of *Competibacter* and TFOs (presuming they were the important GAOs in the systems) was observed (Figure 5). This implies that *Competibacter* and TFOs together might represent the dominant GAO population in these full-scale plants. The negative correlation between the P_{rel}/HAc_{up} ratio and TFO abundance was observed at all except one plant (plant D) (Figure 5b). Plant D had a low P_{rel}/HAc_{up} ratio, did not contain TFOs, and had the highest abundance of *Competibacter*. Despite the diverse phylogenetic affiliations and physiological traits of the TFOs (Tsai and Liu, 2002), the correlation of P_{rel}/HAc_{up} ratio with TFO abundance might suggest that TFOs represent the dominant GAOs in most full-scale plants in the United States.

Because molecular-technique-based analyses are typically not accessible for most WWTPs, simple microscopic observations of TFOs could potentially help operators monitor possible GAO proliferation. Of course, extreme caution must be taken for exceptions, such as plant D, which did not have any TFOs, but had a high abundance of *Competibacter*. Phosphorus release and uptake tests should be conducted with microbial assessments to monitor the status of the EBPR process.

Competition Between Polyphosphate- and Glycogen-Accumulating Organisms and Its Effect on Enhanced Biological Phosphorus Removal Performance and Stability. Good phosphorus removal with effluent <1 mg/L was achieved with coexistence and variation of GAO and PAO populations in the

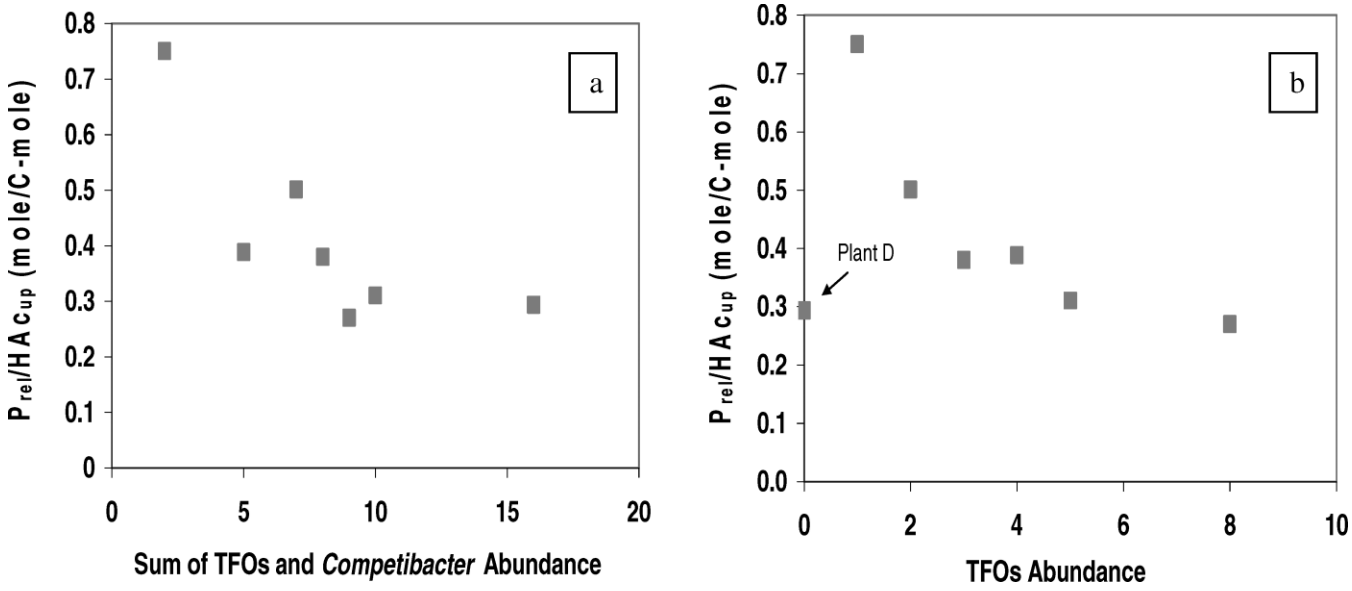


Figure 5—(a) Correlation between P_{rel}/HAc_{up} ratio and sum of *Competibacter* and TFO abundance level, and (b) correlation between P_{rel}/HAc_{up} ratio and TFO abundance level.

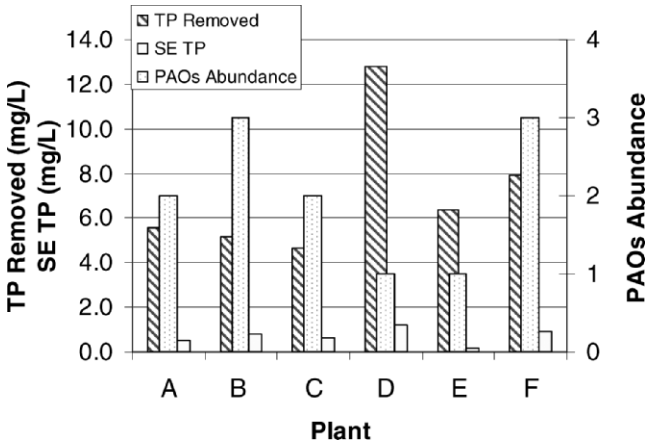


Figure 6—Relationship between phosphorus removal performance and PAO abundance at full-scale EBPR plants.

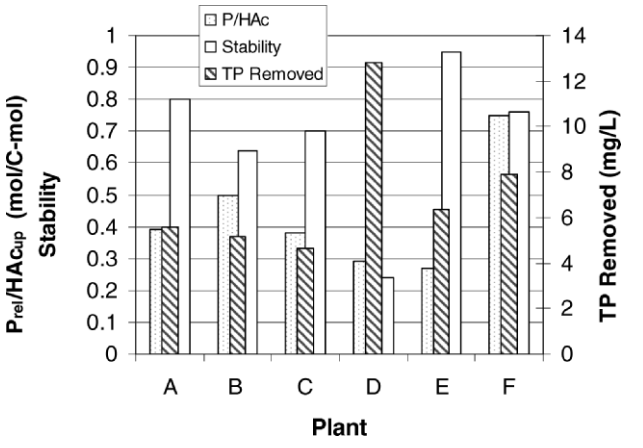


Figure 7—Phosphorus removal performance and relationship to P_{rel}/HAc_{up} ratio at full-scale EBPR plants. Stability is the cumulative frequency of percent occurrence that secondary effluent total phosphorus<0.5 mg/L during the study.

plants studied. Effluent phosphorus concentration, process stability, and amount of phosphorus removed in the EBPR process were not directly related to PAO abundance level and the P_{rel}/HAc_{up} ratio (Figures 6 and 7). Although the presence of GAOs affected carbon-utilization efficiency for phosphorus removal, it did not seem to directly affect the performance of a stable EBPR process. The following hypothesis is proposed to explain why an EBPR process can performance well and maintain its stability, even with various abundance levels of PAOs and GAOs in the system, as shown in this study. Despite the complexity and uncertainties with metabolic pathways that the PAOs and GAOs use, at the system level, the bottom line that governs the competition outcome is their ability to compete for carbon sources for growth. From this point-of-view, the competition can be described using a rather simplified carbon-flow-based conceptual model, as shown in Figure 8. The key aspects are as follows:

(1) The relative anaerobic VFA uptake rates (kinetics) of PAOs versus the GAOs will determine what percentage of the influent carbon the PAOs and GAOs will obtain. As carbon (volatile fatty acids or rbCOD) flows (Q_c) into the anaerobic phase of an EBPR system, PAOs (biomass as X_{PAO})and GAOs (biomass as X_{GAO}) will compete for carbon uptake and storage. The VFA uptake rates (q_c^{PAO} and q_c^{GAO}) differ for PAOs and GAOs, and they are affected by many parameters, including pH, temperature, substrate type, and SRT (Filipe et al., 2001; Schuler and Jenkins, 2002; Whang and Park, 2001; Whang et al., 2002; Zilles et al., 2002). As a result, before entering the following aerobic phase, the carbon is already distributed between PAOs and GAOs in the form of intracellular carbon polymers (PHA).

(2) Then, in the aerobic phase, the intracellular carbon polymers (such as PHA or glycogen, noted as $C_{PHA}^{PAO}, C_{PHA}^{GAO}$) will be used for cell synthesis, for both PAOs and GAOs. The amount of phosphorus that can be removed depends on the net biomass production of PAOs, which depends on the amount of carbon that is stored and available for growth and yield values (Y_{PAO} and Y_{GAO}). The net amount of carbon available for growth is the total amount, C (i.e., in PHAs), minus the need for maintenance and for restoring intracellular polymers (glycogen or polyphosphorus) used in the anaerobic phase, as follows:

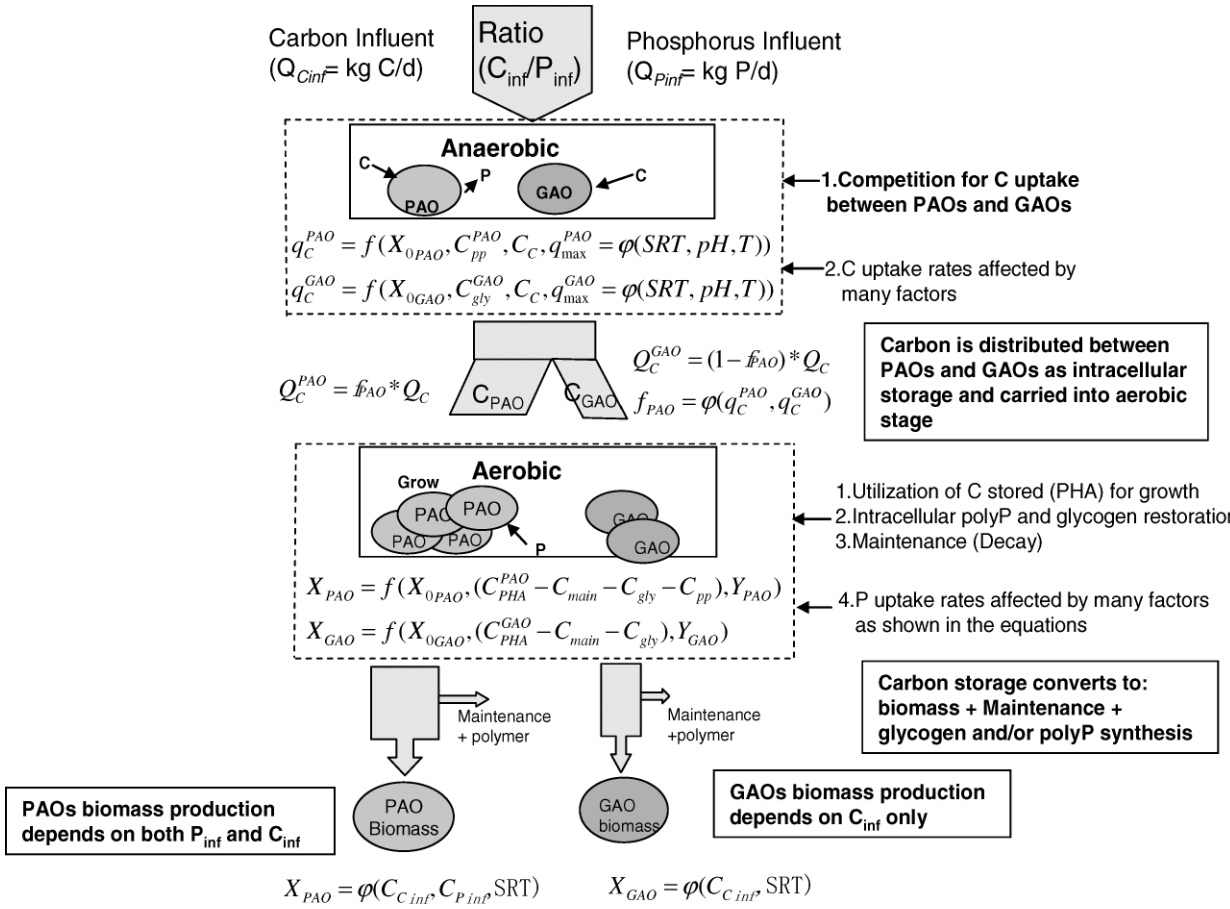
$$(C_{PHA}^{PAO} - C_{main} - C_{gly} - C_{pp}) \text{ for PAOs} \quad (1)$$

$$(C_{PHA}^{GAO} - C_{main} - C_{gly}) \text{ for GAOs} \quad (2)$$

The environmental and operational factors (pH, temperature, and SRT) in the aerobic phase can affect the PHA utilization rate and the energy need for maintenance, and, therefore, they will affect the biomass production of PAOs and GAOs.

(3) The maximum PAO abundance in a system depends on the influent phosphorus concentration and the carbon source available. There is a stoichiometric requirement of carbon for a given amount phosphorus to be removed. The minimum rbCOD/P ratio for complete phosphorus removal was found to be 8 to 10 g/g in an acetate-fed SBR EBPR by Schuler and Jenkins (2003). Under the condition that gives competitive advantage to PAOs, the PAOs will take up sufficient VFA in the anaerobic phase to drive subsequent aerobic phosphorus uptake for complete phosphorus removal. If the influent carbon is more than what is required for complete phosphorus removal, the spare carbon can be sequestered by GAOs and allow GAOs to coexist with PAOs, without negatively affecting the EBPR performance, as shown in all the plants studied. Based on this understanding, the PAO fraction in the sludge biomass in a system should be proportional to the influent phosphorus concentration, provided that there is sufficient influent carbon. Examination of the relationship between the influent phosphorus concentrations and net phosphorus release measured in the phosphorus uptake and release batch test (which is an indicator of PAO abundance or activity level) showed positive correlation for all plants except D, as shown in Figure 9. Similar correlation was also demonstrated by Schuler and Jenkins (2003). These results support the proposed hypothesis. The exception of plant D was likely the result of the insufficient influent rbCOD/P ratio (5 g/g) at plant D, which limited the PAO proliferation.

(4) On the other hand, if the process conditions change, such that they become favorable for GAOs for VFA uptake, GAOs will continue to proliferate and eventually outcompete PAOs and cause a system failure. This is because the growth of GAOs will



Scenario I: PAOs have favorable anaerobic C uptake, PAO biomass production is proportional to influent P provided that there is sufficient influent C; excessive C is converted to GAOs biomass; P removal can be stable even in presence of GAOs;
Scenario II: GAOs have favorable anaerobic C uptake, GAO biomass production only depends on influent C, GAOs will continue to proliferate until they outcompete PAOs; P removal can deteriorate due to insufficient PAOs activities.

Figure 8—Carbon-based conceptual model for PAO and GAO competition. Some symbols and equations are adopted from Whang and Park (2001).

only depend on the carbon available, unlike PAOs, whose proliferation depends not only on carbon available, but also the influent phosphorus level.

Correlation of rbCOD/P Ratio with Enhanced Biological Phosphorus Removal Performance. For EBPR WWTPs, the effluent phosphorus concentration and phosphorus removal stability are of the greatest concern. A positive correlation between phosphorus removal performance stability and influent rbCOD/P ratio was observed in this study (Figure 10), which was also shown in other investigations (Liu et al., 1997; Schuler and Jenkins, 2003). An influent rbCOD/P ratio greater than 15 to 20 was recommended to achieve effluent phosphorus <1 mg/L and for more stable performance (Randall et al., 1992; Tetreault et al., 1986). Phosphorus removal performance for WWTPs A and E were expected to be affected by the addition of carbon to the anaerobic zone, with plant A receiving primary sludge fermentation supernatant and plant E receiving sugar waste. The WWTPs E and A had the best performance reliability and also had higher influent rbCOD/P ratios.

A higher influent rbCOD/P ratio correlates with stable phosphorus removal performance, but, at the same time, leads to higher GAO populations and therefore lower carbon utilization efficiency. A rbCOD/P ratio of <13 led to PAO dominance; a rbCOD/P >50 led to GAO dominance; and a rbCOD/P ratio between these values led to coexistence of both PAOs and GAOs (Ahn, Lee, and Kwon, 2006; Liu et al., 1997; Schuler and Jenkins, 2003). The plants surveyed in the current study had carbon-to-phosphorus ratios ranging between 5 and 38, therefore predicting that our evaluated plants contained a combination of PAO and GAO populations, which is consistent with our observation.

For most municipal WWTPs, the influent carbon-to-phosphorus ratios range from 5 to 40, and this is where coexistence of both PAOs and GAOs is expected. As previously discussed, an EBPR process in which both PAOs and GAOs coexist can maintain good and stable performance, as long as the conditions are favorable for PAOs to preferably uptake sufficient carbon to removal all the influent phosphorus, only leaving the spare carbon for GAOs to sequester. The positive correlation of process stability with the increasing influent rbCOD/P ratio, and by extension with the elevating GAOs abundance in the system, suggests the possibility that GAOs may actually serve as a buffer to attenuate the effect of fluctuation of influent carbon loading on the PAOs, because PAOs seem to be sensitive to process disturbances (Ahn, Park, and Kim,

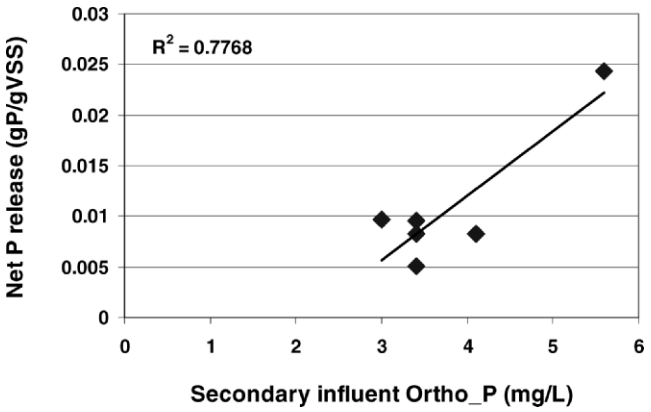


Figure 9— Correlation between secondary influent ortho-phosphorus concentration at the plants and the net phosphorus release measured in phosphorus release and uptake batch tests.

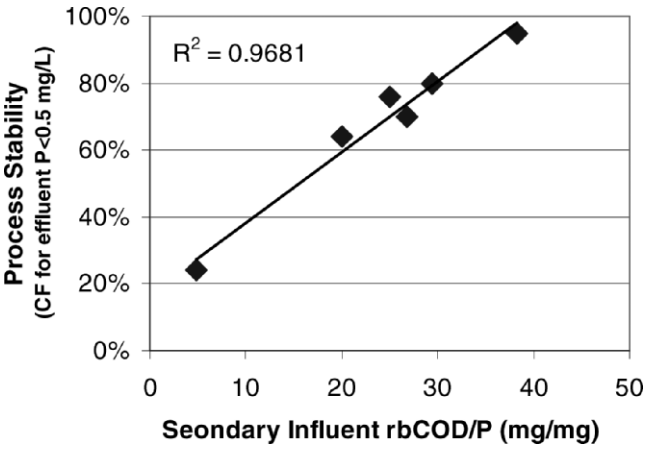


Figure 10—Positive correlation between influent rbCOD/P ratio and EBPR stability at full-scale EBPR plants.

2006). However, changes in conditions of a stable EBPR system that becomes more favorable for the GAOs could potentially result in proliferation of GAOs and outcompete PAOs, eventually leading to deterioration of phosphorus removal. Therefore, understanding and controlling the operation conditions favorable for anaerobic VFA uptake and aerobic growth of PAOs is necessary to maintain the system stability. Competition between PAOs and GAOs can be influenced by many factors, including rbCOD/P ratio, substrate type, SRT, anaerobic hydraulic retention time, temperature, pH, dissolved oxygen, and feeding strategy (and probably more). It is worth mentioning that facilities that have higher rbCOD/P ratios, such as plants B and E, may achieve higher stability, but might also be more prone to upsets, as a result of GAO proliferation, because a higher carbon-to-phosphorus ratio means more GAOs in the system. Periodic summer upsets experienced at plant C are an example of phosphorus removal deterioration that could be related to GAO proliferation, possibly induced by higher temperatures in the summer, which favor development of GAOs. This was evidenced by three consecutive analyses of *Accumulibacter* and TFOs at plant C. An increase in GAOs (TFOs) was observed when samples taken in July and September 2003 were compared (Tables 2 and 3). The increase of TFOs correlated with a decrease in P_{rel}/HAc_{up} ratio and an increase in effluent total phosphorus. The average effluent total phosphorus at plant C increased from <0.3 mg/L during March to July to 1.0 to 2.0 mg/L during August to September 2003 (Figure 11).

Conclusions

The following conclusions can be drawn from this study:

- Quantitative observation of candidate PAOs and GAOs, in combination with batch phosphorus release and uptake tests, and full-scale plant performance evaluation are necessary to reveal the effects of these two populations on EBPR performance and stability. The candidate PAOs and GAOs studied, including *Accumulibacter phosphatis, Competibacter phosphatis*, and TFOs, were relevant to EBPR processes in the United States, although the results also suggested that the diversity of PAOs and GAOs are larger than our current understanding.
- The effluent phosphorus concentration, amount of phosphorus removed, and process stability in an EBPR system are not directly related to high PAO abundance or mutually exclusive with a high GAO fraction. Effective phosphorus removal is achieved with coexistence and a great variation of combined PAO and GAO populations in the six full-scale EBPR plants in the United States. The presence of GAOs does not necessarily negatively affect EBPR performance, provided that the process condition favors the PAOs to uptake sufficient carbon for complete removal of influent phosphorus, leaving only the spare carbon to be sequestered by GAOs. Understanding and controlling the factors that affect VFA uptake rate of PAOs versus GAOs, including pH, temperature, SRT, and carbon substrate type, is critical.
- Stability of EBPR processes was shown to be correlated with higher influent rbCOD/P ratios, even though a higher rbCOD/P ratio often selected for a higher GAO level also. The EBPR process that has higher influent carbon (rbCOD), either by nature or from additional supplement, is expected to have more stable phosphorus removal. It should be noted, however, that facilities that have higher rbCOD/P ratios, although they may achieve higher stability, might also be more prone to upsets if conditions change to be favorable for GAOs, as a result of the relatively higher GAO abundance in the system.
- More research should be directed toward the study of maintaining EBPR stability with a combined PAO and GAO microbial community at rbCOD/P ratios common in WWTPs. Experimental design of laboratory studies on EBPR processes and mechanism should consider the system feed rbCOD/P ratios and effect on the PAO and potential PAO/GAO populations that may be encouraged. Such studies should include population characterization of GAOs and PAOs in interpreting results on phosphorus removal mechanisms, kinetics, and simulation model developments.

Credits

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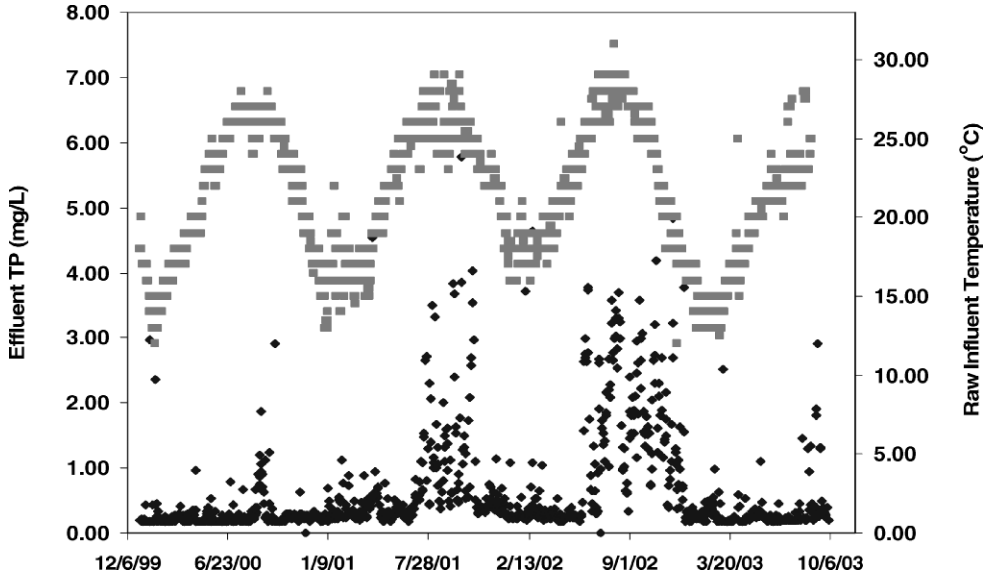

Figure 11—Effluent total phosphorus concentration and influent temperature at plant C. Phosphorus removal upsets were experienced during summer.

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