

# MOLECULAR MICROBIAL ECOLOGY OF ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL IN AERATED-ANOXIC ORBAL™ PROCESSES

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## ABSTRACT

The traditional process for enhanced biological phosphorus removal (EBPR) in wastewater treatment involves an anaerobic zone followed by an aerobic zone. Although there is no strict anaerobic zone in aerated-anoxic Orbal™ processes, phosphorus removal in excess of that required for cell growth does occur. The microbial ecology of polyphosphate accumulating organisms (PAO) in two full-scale Orbal™ wastewater treatment plants was investigated using flow cytometry to physically separate PAO from non-PAO and fluorescent in situ hybridization (FISH) to identify the main bacterial groups in the PAO subpopulation. Although organisms related to *Rhodocyclus* were present in these full-scale Orbal™ processes, they were not enriched in the PAO subpopulation, suggesting that the operational characteristics of aerated-anoxic processes select for a PAO community significantly different from that observed in traditional EBPR processes.

## KEYWORDS

Aerated-anoxic, EBPR, Orbal™, phosphorus removal, polyphosphate, *Rhodocyclus*

## INTRODUCTION

The design and operation of enhanced biological phosphorus removal (EBPR) processes is based on the cycling of activated sludge through anaerobic and aerobic stages, fermentation of organic matter with production of volatile fatty acids, and separation of anaerobic and anoxic environments (Grady *et al.*, 1999; Rittmann & McCarty, 2001). These key characteristics are based on empirical observations of biological nutrient removal processes and on the biochemical requirements of polyphosphate-accumulating organisms (PAO) selectively enriched in EBPR environments (Grady *et al.*, 1999; Hesselmann *et al.*, 2000; Mino *et al.*, 1998). Additional operational criteria, such as solids retention time, influent organic matter to phosphorus ratio, pH, and temperature, which are also based on empirical observations, appear to be strongly linked to the microbiology of the process (Filipe *et al.*, 2001; Filipe *et al.*, 2001; Grady *et al.*, 1999).

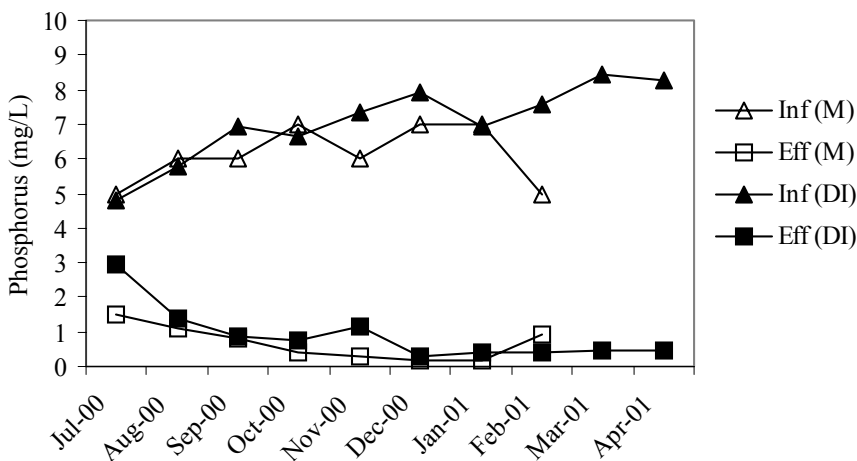
Although EBPR processes are reasonably well understood conceptually and operationally, the microbiology of EBPR has proven more difficult to analyze and the identity of the relevant PAO in full-scale processes remains elusive. This gap between engineering advances and

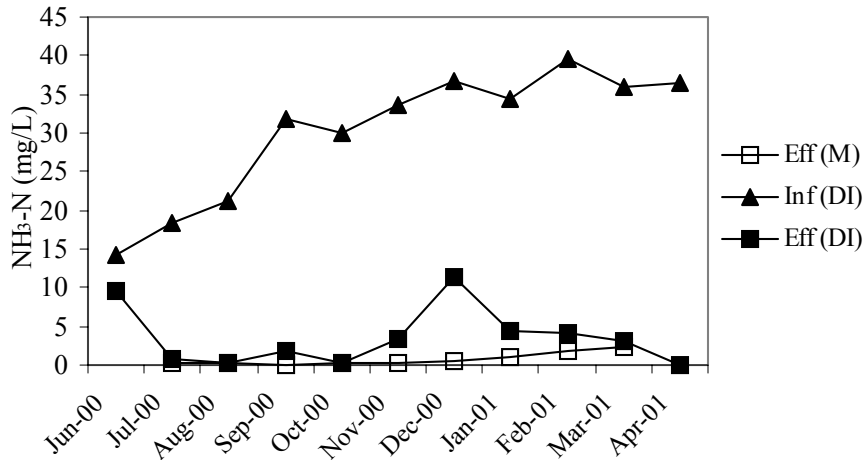
microbiological knowledge is partly due to the long-standing assumption that *Acinetobacter* sp. were the dominant PAO in EBPR processes, since these organisms were repeatedly isolated from EBPR treatment plants. It was not until the application of in situ hybridization and other molecular-based methods that it became evident that *Acinetobacter* sp. were not the numerically dominant PAO in EBPR (Bond *et al.*, 1995; Cloete & Steyn, 1987; Wagner *et al.*, 1994).

To date, there are no reports of pure cultures or fully characterized cocultures capable of performing the anaerobic/aerobic biochemical operations conducive to biological phosphorus removal. Although highly enriched cultures of a *Rhodocyclus*-related organism are typically obtained in lab-scale reactors fed with acetate as the only carbon source, attempts to isolate this organism have failed (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999; Liu *et al.*, 2001). Evidence that *Rhodocyclus* relatives are relevant in full-scale EBPR is starting to emerge (Zilles *et al.*, 2001). While the identification of this industrially relevant PAO is significant, the inability to study this organism in pure culture has limited progress on understanding its biochemistry. The contributions of other, as yet unidentified organisms also appear to be significant in full-scale EBPR processes, further complicating the analyses (Zilles *et al.*, 2001).

The practical consequence of these limitations is that the engineering conceptualization of EBPR processes continues to be based on empirical observations and models that indicate the requirement of separate anaerobic, anoxic, and aerobic stages for stable biological nutrient removal. This approach leaves out alternative designs that, although not in complete agreement with the anaerobic/anoxic/aerobic paradigm, can be successfully operated for biological phosphorus removal. For instance, aerated-anoxic Orbal™ processes are oxidation ditch type reactors (Applegate *et al.*, 1980) that do not have a strictly anaerobic stage but can adequately achieve effluent phosphorus levels below 1 mg P/L through biological means (Figure 1).

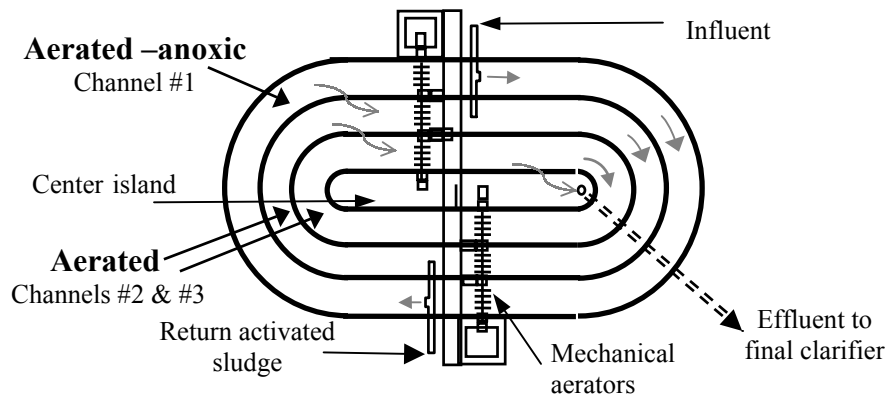
**Figure 1. Monthly averages of influent and effluent total phosphorus and ammonia-nitrogen at Dane-Iowa (DI) and Marshall (M) wastewater treatment plants.**



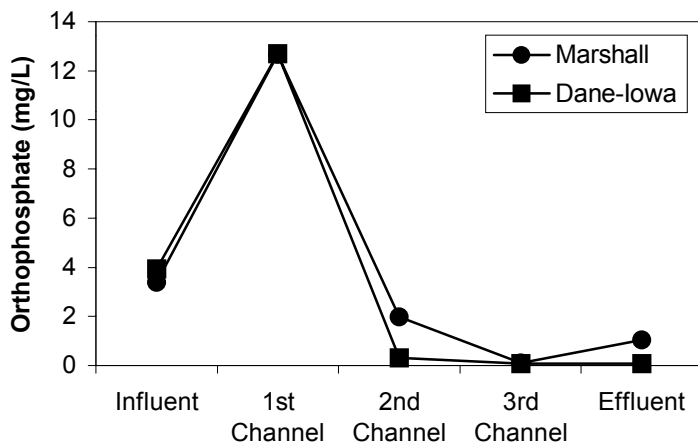


Orbal™ treatment processes are optimized for nitrogen removal (Daigger & Littleton, 2000; Park *et al.*, 2001) and operate under the concept that simultaneous nitrification and denitrification can be enhanced in an anoxic stage by providing aeration at a level below the oxygen uptake rate so that the oxygen concentration remains near zero (Smith, 1996). The concentric closed-loop plant design (Figure 2) provides for an initial anoxic stage that typically represents ~ 50% of the total reactor volume, another characteristic that is different from traditional biological nutrient removal reactors (Grady *et al.*, 1999). Under these conditions, phosphorus release is observed in the aerated-anoxic stage, while excess phosphorus uptake occurs in the aerobic stage (Figure 3), indicating the presence of organisms performing the EBPR biochemical cycle.

**Figure 2. Schematic of aerated-anoxic Orbal™ processes.**



**Figure 3. Phosphorus release and uptake in Marshall and Dane-Iowa wastewater treatment plants.**



Whether the organisms responsible for biological phosphorus removal in Orbal™ wastewater treatment plants (WWTPs) are the same as those present in traditional EBPR processes or whether the mode of operation of Orbal™ processes selects for different PAO is unknown. Thus, our research was aimed at determining which PAO were important at two full-scale Orbal™ treatment plants. Based on recent reports correlating the presence of organisms related to *Rhodocyclops* sp. with phosphorus removal, we used fluorescent in situ hybridization (FISH) and cloning to address whether these organisms were present in full-scale Orbal™ processes. Furthermore, to investigate which organisms were accumulating polyphosphate in the full-scale processes, we used flow cytometry to physically separate PAO from non-PAO in activated sludge samples followed by FISH to characterize the PAO-enriched subpopulation (Hung *et al.*, 2001; Noguera *et al.*, 2000).

## METHODOLOGY

### Full-scale Orbal™ processes

The two full-scale wastewater treatment plants examined in this study were oxidation ditch reactors using the aerated-anoxic Orbal™ process as described in Figure 2 and Table 1. In the outer, aerated-anoxic channel dissolved oxygen (DO) was kept low by supplying oxygen at a rate less than the utilization rate. In this channel, simultaneous nitrification/denitrification occurs (Daigger and Littleton, 2000; Park *et al.*, 2001). Phosphorus and nitrogen removal for both plants are shown in Figure 1. Influent ammonia-nitrogen data was not available for the Marshall WWTP. While the cause of the ammonia nitrogen spike at Dane-Iowa starting in November, 2000 has not been fully determined, it should be noted that the plant began operating in June, 2000, and prior to the upset, batch septage dumps were pumped directly to the aeration basin. Nitrification improved after that practice was discontinued in favor of more uniform metering of the septage to the aeration basin.

**Table 1. Operational Conditions**

	Dane-Iowa WWTP	Marshall WWTP
Process	aerated-anoxic Orbal™	aerated-anoxic Orbal™
Location	Mazomanie, WI	Marshall, WI
MGD (m <sup>3</sup> /d)	0.58 (2,190)	0.57 (2,150)
Aerated-anoxic hydraulic residence time	10.9 hours	9.2 hours
Aerated-anoxic DO	0.03 mg/L	0.1 mg/L
Aerobic hydraulic residence time	11.4 hours	9 hours
Aerobic DO	0.76-4.88 mg/L	0.5-4 mg/L
Solids retention time	20 days	15 days

### Fluorescent in situ hybridization and microscopic analysis

Microscopic analysis was performed as described here and reported previously (Zilles *et al.*, 2001). Grab samples of activated sludge were collected from the inner channel (aerobic stage) and stored on ice during transport to the laboratory. Settled mixed liquor samples were mechanically disrupted by repetitive purging through a 26G needle at least 30 times. Samples were then fixed in 3% paraformaldehyde-PBS (final concentration) for 30 minutes at room temperature. Following fixation, cells were collected by filtration through a 0.2 micron pore size polycarbonate filter and transferred to gelatin-coated slides (Amann *et al.*, 1990). Polyphosphate staining was for 1 hr with 1 microgram/mL DAPI/dH<sub>2</sub>O (Hung *et al.*, 2001; Zilles *et al.*, 2001). Oligonucleotide probes, hybridization conditions, and related references are listed in Table 2. Unlabeled BET42a and GAM42a were used as competitors for each other as previously described (Wallner *et al.*, 1995). Modifications were made to PAO462 and PAO846 to include sequences related to *Rhodocyclus* identified in Nine Springs WWTP (modified probe sequences: PAO462b, 5'-CCGTCATCTRCWCAGGGTATTAAC and PAO846b, 5'-GTTAGCTACGGYACTAAAAGG). The combination of four probes used to target *Rhodocyclus* have no mismatches with the organisms identified by Hesselmann *et al.* (1999) and Crocetti *et al.* (2000). Depending on the hybridization stringency, these probes will also bind to other organisms in the *Rhodocyclus tenuis* subgroup and may include organisms in the closely related *Dechlorimonas agitata* subgroup. Cells were visualized on a Zeiss Axioplan 2 epifluorescent microscope (Carl Zeiss, Thornwood, NY). Typical microscopic analysis consisted of manually counting the cells in at least ten digital images per well from duplicate wells, resulting in total cell counts of 500-1500 per sample. The 95% confidence intervals on cell counts were typically  $\pm 5-10\%$ .

**Table 2. Oligonucleotide probes and hybridization conditions**

Probe*	Targeted organisms	% Formamide
EUB338 <sup>1</sup>	<i>Bacteria</i>	20
ALF1b <sup>2</sup>	<i>alpha-Proteobacteria</i>	20
BET42a <sup>2</sup>	<i>beta-Proteobacteria</i>	35
GAM42a <sup>2</sup>	<i>gamma-Proteobacteria</i>	35
HGC <sup>3</sup>	Gram positive, high G+C content	25
CF <sup>3</sup>	Flexibacter-Cytophaga-Bacteroides	35
RHC439 <sup>4</sup> , PAO462b <sup>5,6</sup> , PAO651 <sup>5</sup> , PAO846b <sup>5,6</sup>	<i>Rhodocyclus</i>	35

\* References: <sup>1</sup>Amann *et al.*, 1996, <sup>2</sup>Manz *et al.*, 1992, <sup>3</sup>Wagner *et al.*, 1994, <sup>4</sup>Hesselmann *et al.*, 1999, <sup>5</sup>Crocetti *et al.*, 2000, <sup>6</sup>Zilles, Kim, and Noguera, unpublished results

### PAO separation by flow cytometry

Activated sludge samples were fixed and dispersed as described above and stained with DAPI (final concentration 5 microgram/mL) overnight in the dark and on ice. Flow cytometry was performed with a FACStar Plus dual laser flow cytometer (Becton Dickinson, San Jose, CA). DAPI was excited with a multi-line UV generated from a krypton laser at 340 nm (Coherent, Palo Alto, CA) and emission signals were collected through a 450/25 band-pass filter and a 575/25 band-pass filter. The cells with a high 575/450 ratio were collected as previously described (Hung, *et al.*, 2001; Noguera, *et al.*, 2000; Zilles, *et al.*, 2001) for subsequent microscopic or molecular analysis.

### Extraction and purification of genomic DNA

Grab samples of activated sludge were collected from the inner channel. Samples were transported on ice, 1 mL of settled mixed liquor was concentrated by centrifugation at 10,000 x g for 3 min, and resuspended in 1 mL (final volume) in 10 mM tris (pH 8.0), 1 mM EDTA buffer. Lysozyme was added (final concentration of 2 mg/mL) and the mix was incubated at 37 °C for 1 hr. Proteinase K and sodium dodecyl sulfate were added to a final concentration of 60 microgram/mL and 0.5% wt./vol. respectively, and the solution was incubated for 30 min at 70°C. Protein in the lysed cell suspensions was extracted in phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated by adding 0.2 volumes of 3 M sodium acetate, pH 5.2, and 2 volumes of cold ethanol followed by incubation at -70 °C for 1 hr. Nucleic acids were recovered by centrifugation at 14,000 x g for 30 min and washed twice in 70% ethanol. The dried pellet was resuspended in sterile deionized water.

## Amplification, cloning, and sequencing 16S rRNA encoding genes

16S rDNA fragments were obtained by PCR amplification using RHC439 (5'-TCGGCGGGGAAGAAATNG-3' (Hesselmann *et al.*, 1999) and reverse universal primer 1492r (5'-GGYTACCTTGTTACGACTT-3') (Lane, 1991)). Each reaction tube contained 50 microliters of 1 x PCR buffer B (Promega, Madison, WI), 2.5 U Taq polymerase (Promega), 0.4 microM of each primer, 0.2 mM each of deoxynucleotide triphosphates, 2.5 mM MgCl<sub>2</sub>, 100 micrograms/mL BSA, and ~100 ng genomic DNA. Taq polymerase was added after a 5 min hot start at 80°C, followed by an initial denaturation at 94°C and 30 cycles of denaturation (90 sec at 94°C), annealing (60 sec at 65°C), extension (180 sec at 72°C) and a final extension at 72°C for 7 min. The 1.1 kilobase fragment was verified by gel electrophoresis and purified using a Qiaex II gel extraction kit (Qiagen, Valencia, CA). Purified fragments were ligated to p-GEM-T vector (Promega) and transformed into *Escherichia coli* str. JM109 competent cells. Plasmids were extracted using Wizard<sup>®</sup> Plus miniprep DNA purification system (Promega) and inserts detected by gel electrophoresis. Plasmid inserts were cycle sequenced with Big Dye (Applied Biosystems, Foster City, CA) sequencing kit using T7 and SP6 primers and approximately 1 microgram plasmid template. Products were purified using Autoseq<sup>™</sup>G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ) then separated and analyzed on an automated DNA sequencer (ABI models 377XL, Applied Biosystems, Foster City, CA).

## RESULTS

### Presence of PAO in aerated-anoxic Orbal<sup>™</sup> processes

With chemical data supporting biological phosphorus removal in the aerated-anoxic processes (Figure 3), activated sludge samples from the Dane-Iowa and Marshall WWTPs were analyzed for the presence of PAO. PAO were identified and quantified using DAPI staining for polyphosphate (Hung *et al.*, 2001; Kawaharasaki *et al.*, 1999; Streichan *et al.*, 1990; Zilles *et al.*, 2001). As shown in Table 3, both plants contained significant numbers of PAO. The quantities of PAO observed in activated sludge from these aerated-anoxic plants is consistent with levels observed in traditional full-scale EBPR processes (i.e. 14-36% in modified UCT process (Zilles *et al.*, 2001)) and thus supports the existence of biological phosphorus removal.

**Table 3. Molecular characterization of activated sludge samples**

	Dane-Iowa WWTP	Marshall WWTP
Polyphosphate (%)	25	22
<i>Rhodocyclus</i> (%)	15	10

## **Presence of *Rhodocyclus* sp. in full-scale aerated-anoxic Orbal™ processes**

Since recent reports have indicated the involvement of organisms related to *Rhodocyclus* sp. in phosphorus removal (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999; Liu *et al.*, 2001; Zilles *et al.*, 2001), we searched for these organisms in the full-scale aerated-anoxic processes. Activated sludge samples from both Dane-Iowa and Marshall WWTPs were analyzed by FISH, using probes designed by Hesselmann *et al.* (1999) and Crocetti *et al.* (2000) to target the 16S rRNA of the organisms related to *Rhodocyclus* identified in their laboratory-scale reactors (Table 2). It is important to note that the four *Rhodocyclus* probes were used simultaneously to increase fluorescent signal. Although the probes have slightly different specificities, the only organism identified to date that is complementary to all four probes is the organism identified by Hesselmann *et al.* (1999) and Crocetti *et al.* (2000). Other organisms with zero or one mismatch to one or more probes fell either in the *Rhodocyclus tenuis* subgroup or in the *Dechlorimonas agitata* subgroup, suggesting that any false positives will come from closely related organisms. Organisms hybridizing with these *Rhodocyclus* probes represented a significant percentage of total microorganisms in both aerated-anoxic plants (Table 3), although their relative abundance was lower than the 20% of total organisms observed in a full-scale modified UCT process (Zilles *et al.*, 2001).

Clones recovered from Dane-Iowa activated sludge contained sequences greater than 99% identical to those reported from the laboratory reactors of Hesselmann *et al.* (1999) and Crocetti *et al.* (2000). Other recovered clones indicated the presence of a population targeted by RHC439 but not circumscribed by PAO462, PAO651, or PAO846, and having less than 95% similarity with any other known sequence.

## **Involvement of *Rhodocyclus* sp. in phosphorus removal**

To assess polyphosphate accumulation in organisms related to *Rhodocyclus* and begin addressing the involvement of other organisms, PAO were separated from other microorganisms in activated sludge from the Dane-Iowa WWTP by DAPI staining and flow cytometry (Hung *et al.*, 2001). This procedure was chosen over simultaneous staining and visualization of DAPI-stained FISH samples (Kawaharasaki *et al.*, 1999) as we have found that the number of PAO that can be visualized and quantified is significantly reduced in hybridized samples (Hung *et al.*, 2001; Zilles *et al.*, 2001). The flow cytometric sorting resulted in samples that contained 60-80% polyphosphate-positive cells, as compared to 25% in unsorted activated sludge. A comparison of the unsorted activated sludge samples with the sorted, PAO-enriched samples using FISH (Table 4) indicated that the physical concentration of PAO did not result in a significant increase in organisms related to *Rhodocyclus*, suggesting that these organisms were not the dominant PAO in the Dane-Iowa WWTP. Analysis of the sorted sample by FISH with group specific oligonucleotide probes did not reveal a dominant group or a significant change from unsorted activated sludge, suggesting either a broad diversity of PAO or PAO that were not detected by these probes (Table 4).

**Table 4. Distribution of organisms in PAO-sorted sample from Dane-Iowa WWTP**

Group	Activated Sludge (% of total)		Sorted Sludge (% of total)	
	Dane-Iowa	Nine Springs*	Dane-Iowa	Nine Springs*
<i>Eubacteria</i>	83	78	50	77
<i>alpha-Proteobacteria</i>	4	10	7	5
<i>beta-Proteobacteria</i>	12	25	5	53
<i>gamma-Proteobacteria</i>	7	3	10	5
Gram positive	15	9	7	6
Flexibacter-Cytophaga- Bacteroides	4	nd	5	nd
<i>Rhodocyclus</i>	8	20	11	33

\*Results from Nine Springs WWTP are from Zilles *et al.* (2001). The Nine Springs WWTP operates as a modified UCT EBPR process. nd, not determined. The 95% confidence intervals on cell counts were typically  $\pm$  5-10%.

## DISCUSSION

The aerated-anoxic Orbal™ WWTPs do not follow the traditional design recommendations of an EBPR process because of the absence of a strictly anaerobic zone (Daigger and Littleton, 2000). However, these plants can be designed to achieve biological phosphorus removal by EBPR, as demonstrated by the observed low effluent phosphate concentrations (Figure 1) and the elevated orthophosphate concentrations in the aerated-anoxic channel of the Marshall and Dane-Iowa WWTPs (Figure 3). To promote environmental conditions consistent with EBPR processes, oxygen supply in the aerated-anoxic channel is maintained near zero by minimizing the number of surface aerators and the number of disks per surface aerator. Furthermore, because simultaneous nitrification and denitrification occur in the aerated-anoxic stage (Park *et al.*, 2001), nitrate accumulation at this stage is not significant, and thus, phosphate release in the aerated-anoxic channel is not affected by nitrate. Nevertheless, elevated phosphate concentrations in the aerated-anoxic channel are not always observed in Orbal™ WWTPs, and according to Daigger and Littleton (2000), EBPR in the close-loop configuration of Orbal™ treatment plants has been difficult to mathematically model using the IAWQ Activated Sludge Model 2. Daigger and Littleton (2000) hypothesized that EBPR in Orbal™ processes is possible due to the creation of anaerobic zones in the center of microbial flocs within the aerated-anoxic channel. This hypothesis is in agreement with the conceptual basis for simultaneous nitrification and denitrification in aerated-anoxic reactors, in which an anoxic zone would exist in the center of the flocs, where oxygen does not penetrate. However, the establishment of an additional anaerobic zone inside the flocs, where nitrate and oxygen are excluded but readily biodegradable organic matter is present, might be difficult to attain (Daigger and Littleton, 2000).

An alternative explanation for the observed EBPR activity in the Dane-Iowa and Marshall WWTPs is that strictly anaerobic conditions are not necessary for the selection of microorganisms that take advantage of cyclic phosphate uptake and release. Since pure cultures of organisms performing the EBPR biochemical cycle are not currently available, the physiological mechanisms that regulate the shift between phosphate uptake and release are not

completely understood, and therefore, there is uncertainty on the dissolved oxygen concentrations necessary to induce phosphorus release. For instance regulation could be similar to the shift from oxygen to nitrate as the electron acceptor in denitrifying organisms. Although for practical purposes, oxygen addition to an anoxic reactor is discouraged (Muyima *et al.*, 1997), denitrification can occur at DO concentrations well above zero (Rittmann and McCarty, 2001).

The recent implication of organisms related to *Rhodocyclus* sp. as the dominant PAO in well-controlled, acetate-fed, bench-scale EBPR reactors (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999), prompted us to investigate whether the same organisms were present at the Dane-Iowa and Marshall WWTPs and whether they could account for some of the biological phosphorus removal observed in these plants. Using the oligonucleotide probes developed by Hesselmann *et al.* (1999) and Crocetti *et al.* (2000), FISH analyses revealed that between 10% and 15% of the bacterial cells were *Rhodocyclus*-related organisms. Cloning confirmed that organisms greater than 99% identical to those identified by Hesselmann *et al.* (1999) and Crocetti *et al.* (2000) were present in the Dane-Iowa WWTP. However, additional evidence was required to directly correlate these organisms to EBPR. Since simultaneous visualization of polyphosphate granules and identification of PAO by FISH is not always possible (Zilles *et al.*, 2001), we favored the physical separation of PAO from non-PAO using flow cytometry (Hung *et al.*, 2001; Noguera *et al.*, 2000) and the subsequent characterization of the PAO-enriched subpopulation by FISH (Table 4). When this analysis was performed on activated sludge from the Dane-Iowa WWTP, *Rhodocyclus*-related cells were not concentrated, even though the sorted subpopulation contained three times more PAO than the unsorted activated sludge, suggesting that *Rhodocyclus*-related bacteria were not the significant PAO in the sample. This result is opposite to earlier observations with activated sludge from a full-scale modified UCT process, in which the concentration of *Rhodocyclus*-related cells increased when PAO were separated from non-PAO by flow cytometry (Zilles *et al.*, 2001). Further evidence that *Rhodocyclus*-related bacteria are not significant PAO in the Dane-Iowa WWTP can be seen in the relative concentration of *beta-Proteobacteria* in the sorted and unsorted samples (Table 4), since *Rhodocyclus* sp. belong to this phylogenetic group. While in the UCT process evaluated by Zilles *et al.* (2001), *beta-Proteobacteria* were the dominant group in the PAO-enriched sorted samples, this bacterial group was poorly represented in the sorted samples from the aerated-anoxic WWTP. Indeed, the physical enrichment of PAO by flow cytometry did not reveal correlations between PAO and any bacterial group defined by the group-specific oligonucleotide probes used, suggesting that in the Dane-Iowa WWTP the ability to accumulate polyphosphate is shared by a large diversity of bacteria.

This work confirmed that the activated sludge at the Dane-Iowa WWTP contained the same *Rhodocyclus*-related organisms responsible for EBPR in lab scale bench reactors operated with acetate as the sole carbon and energy source. However, given the low number of *Rhodocyclus*-related organisms in the sorted, PAO-enriched samples, it is unclear whether these *Rhodocyclus*-related organisms are capable of performing the EBPR metabolism in the environmental conditions provided by aerated-anoxic processes. In particular, the absence of strictly anaerobic conditions and the competition with denitrifiers for easily degradable organic material in the aerated-anoxic stage may exert unusual selective pressures that result in the development of polyphosphate accumulating microbial populations that are different from those found in

traditional EBPR processes. Although the molecular analyses described here provided an indication that *Rhodocyclus*-related organisms were not the most significant PAO in the Dane-Iowa WWTP, they did not yet reveal the identity of other PAO that may be more relevant in aerated-anoxic Orbal™ processes.

Finally, the existence of stable EBPR processes that do not meet the traditional design criteria of anaerobic/aerobic cycles and separation of anaerobic from anoxic zones suggests the potential for a broader range of EBPR processes. Identification of possible process variations requires thinking outside the current paradigm for EBPR, a task that would be greatly facilitated by having a better understanding of the microbial ecology and biochemistry of polyphosphate accumulation in WWTPs. The molecular tools used in this work made it possible to undertake a detailed comparison of the microbial community of full-scale traditional EBPR processes and aerated-anoxic processes performing EBPR. However, a thorough understanding of the key physiological mechanisms that regulate the EBPR metabolism, and therefore, of the real limits of non-traditional EBPR can only be assessed when pure cultures or well-defined mixed cultures of organisms that perform the cyclic uptake and release of phosphate are obtained.

## CONCLUSION

The phosphorus release and uptake within the treatment plant and the presence of polyphosphate-positive cells in the aerated-anoxic Orbal™ process confirm that the environmental conditions in this type of process can successfully select for a population of organisms performing the biochemical cycle characteristic of EBPR processes. However, the polyphosphate-accumulating community in the Dane-Iowa WWTP was not dominated by organisms related to *Rhodocyclus*, in contrast to results in traditional bench-scale and full-scale EBPR processes. This result suggests that the operational characteristics of Orbal™ processes may promote the growth of unique PAO.

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