Is it PAO-GAO competition or metabolic shift in EBPR system? Evidence from an experimental study
Ufuk G. Erdal, Zeynep K. Erdal, Glen T. Daigger and Clifford W. Randall

ABSTRACT
Reduced EBPR performance in full and bench-scale EBPR studies was linked to the proliferation of GAOs but often time with the lack of any evidence. In this study, a detailed enzymatic study was coupled with batch tests and electron microscopy results for a realistic explanation. The results eliminated the possibility of population shift from PAO to GAO or other non-PAO due to the short batch test period provided which would not allow a population shift and further justified with the electron microscopy results. The results indicate that glycogen serves not only as source of reducing power for PHA production but also serves as an alternative energy source when the poly-P pool of the PAOs is depleted. Slow generation of ATP via glycolytic pathway at 5°C cannot satisfy energy requirements of EBPR cells to complete several cell functions including acetate uptake and PHA storage. However, the glycolytic pathway is efficiently operable at warm temperatures (>20°C). The reduced performance of enhanced EBPR facilities operated at warm temperature may not be a result of GAO proliferation; instead it may be related the efficient use of the glycolytic pathway by PAOs which results in more glycogen storage and less P uptake, thereby reducing the EBPR performance.

Key words | biochemical pathways, EBPR, GAO, metabolic shift, PAO, phosphorus removal

INTRODUCTION
Wastewater treatment plants can achieve phosphorus removal as long as wastewater containing organic substrate in the form of short-chain volatile fatty acids (SCVFAs) is introduced to the anaerobic zone of the treatment plant, and the mixed liquor is alternated between anaerobic and aerobic conditions. The purpose of the alternation is to favor the growth of phosphorus accumulating organisms (PAOs). PAOs can store inorganic phosphorus as intracellular poly-phosphate (poly-P) to a much greater extent than is needed for growth metabolism, and can use the energy stored in the poly-P bonds to polymerize and store poly-hydroxyalkanoates (PHAs) from SCVFAs. The first observation of EBPR deterioration by population change was reported by Cech & Hartman (1993). Their microscopic examinations detected clusters of large coccoid Neisser-negative cells usually arranged in tetrads, that accumulated glycogen under aerobic conditions, but did not store polyphosphate in excess. They named them “G-bacteria” but they have since then been called “glycogen accumulating organisms” (GAOs). Both PAOs and GAOs are said to accumulate PHA anaerobically. The main difference between them is that PAOs use phosphate as the energy source under anaerobic conditions, while GAOs use only glycogen for energy under anaerobic conditions (Mino et al. 1998). Therefore, GAOs can take up organic material in the anaerobic stage without P release (Mino et al. 1998). PAOs are generally small and rod or oval shaped with large poly-P granules before anaerobic substrate uptake, whereas GAOs are thought to be large spherical organisms arranged in pairs or tetrads without poly-P inclusions. Neisser staining response is positive for PAOs, while it is negative for GAOs (Liu et al. 1997). Glucose addition was...
reported to suppress the PAOs by Cech & Hartman (1993). Matsuo (1994) reported inhibition of EBPR performance when a high anaerobic/aerobic contact time ratio was applied. Liu et al. (1997) postulated that a wastewater with a low P/C ratio (2/100) would stimulate the growth of GAOs because there is insufficient P to support a large PAO population. In Liu’s study, the P-content of sludge decreased down to 1.5% TSS during steady state operation at lower feed P/C values. Filipe et al. (2001) proposed that a pH value of 7.25 or below favors GAOs over PAOs and causes EBPR efficiency to decrease.

Often, the reduced EBPR performance in full and bench-scale EBPR studies is linked to proliferation of GAOs, with lack of any evidence. In addition, no study has been performed to show whether the reduced EBPR performance was a result of a major metabolic shift or the competition between poly-P and non-poly-P organisms, even though detailed micro-ecological studies have suggested that the taxonomic features of PAO and GAO are quite similar (Seviour & Blackall 1999). In this study, a detailed enzymatic study was coupled with batch tests and electron microscopy results for a realistic explanation.

METHODS AND MATERIAL

Two identical modified lab scale University of Cape Town (UCT) configuration EBPR systems containing two anaerobic (2 L each), two anoxic (2 L each) and three aerobic (3.5 L each) reactors in series and fed with synthetic wastewater, were operated at 20 and 5°C at steady state for more than two years. Solids retention time (SRT) of the systems was 20 and 10 days for 5 and 20°C, respectively. Synthetic feed was prepared daily to contain 450 mg/L acetate, and sufficient supplemental yeast extract (an average COD concentration of 500 mg/L) COD. Full characterization of feed water quality can be find elsewhere Erdal et al. (2002). The phosphate in the forms of potassium di-hydrogen phosphate and potassium mono-hydrogen phosphate was continuously dosed to the first anaerobic zone as shown in Figure 1.

Samples from the anaerobic, anoxic and aerobic stages of the pilot systems were visualized using a transmission electron microscope. Sample preparation, staining and sectioning of the microscopy samples were according to Erdal et al. (2002a,b). The enzyme assays were performed on all key enzymes involved in EMP Pathway, PP Pathway, ED Pathway, Glyoxylate Shunt, TCA Cycle, Branched TCA Cycle, Reverse TCA Cycle and Succinate-Propionate Pathway.

EBPR mixed liquor with enriched poly-P pools and depleted poly-P pools was exposed to anaerobic/aerobic cycling to determine PHA and glycogen storage and consumption patterns. The following procedure was followed. In each condition, a series of anaerobic batch tests utilizing 4L of activated sludge from the last anoxic reactor of the UCT system were performed in a DO free environment. At the beginning of each anaerobic test, unlimited acetate (600 mg/L) and micronutrients were added to fully ensure P release and PHA formation. At the end of the anaerobic test, the sludge was split into two flasks. The activated sludge in each flask was settled and the supernatant was poured and washed with distilled water. The successive settling and washing of biomass was repeated three times. Prior to starting the aerobic batch test, 200 mg/L P, 60 mg/L ammonia nitrogen and all necessary micronutrients were added to the first flask, whereas no P was added to the second flask. However, the same quantity of micronutrients and ammonia-N were added to Flask 2 prior to the aerobic batch test. Following 6 hrs of aerobic period, the biomass in each flask has oxidized the PHA formed during the anaerobic period. At the end of anaerobic test, the biomass in the first flask accumulated a large amount of poly-P utilizing enriched poly-P pool, whereas the biomass in the second flask accumulated only glycogen because of it’s depleted poly-P pool. Mixed liquor in flask 1 and 2 refers to enriched and depleted poly-P pool, respectively. The procedure to enrich and deplete poly-P pools of the EBPR mixed liquor is illustrated in Figure 2.

RESULTS

Steady State EBPR Performances at 20 and 5°C

The steady state performance data indicated that P removal was much greater at 5°C than 20°C. The transmission electron microscopy results have identified more diverse
populations at 20°C than those observed at 5°C. The disappearance of nitrification and reduced competition of substrate in the non-oxic zones resulted in an increased population of PAOs and thus greater EBPR efficiency at 5°C. Carbon mass balance calculations showed that glycogen utilization in the anaerobic zones and concurrent replenishment in the aerobic zones were significant at each temperature but greater at 20°C than at 5°C (Figure 3).

The average PHV content of the sludge was 18 and 4% at 20 and 5°C, respectively. These findings may support existence of GAOs which store PHA mainly in the forms of PHV and store glycogen preferably. There was, however, no indication of “GAOs” at either temperature (i.e., no distinctly coccoid cells in a tetrad or similar arrangement were observed in 20 and 5°C samples) when electron microscopy results were compared (Not shown in this manuscript).
Enzyme studies

The enzyme assays performed at both temperatures indicated that glycogen metabolism was present, and the reducing equivalents for PHA synthesis were shown to be obtained through the EMP pathway. The enzyme study showed that the branched TCA pathway was operative during anaerobic metabolism at 20°C to balance out the excess NADH produced during glycolysis. Among the investigated enzymes, phospho-fructo-kinase (PFK) enzyme, a key enzyme for the EMP pathway, was the most temperature dependent enzyme. More than 60% reduction in specific enzyme activity of PFK under anaerobic (0.194 vs. 0.017 U/mg) and aerobic (0.173 vs. 0.534 U/mg) conditions was observed at 20 and 5°C, respectively. Low temperatures were shown to slow down glycogen metabolism significantly, thereby providing a competitive advantage for poly-P metabolism.

Batch tests

As expected, EBPR mixed liquor with an enriched poly-P pool utilized acetate (460 mg/L) and stored PHA during anaerobic batch tests. The EBPR mixed liquor with a depleted poly-P pool also utilized acetate (330 mg/L) while consuming glycogen and subsequently storing PHA at 20°C (Figure 4). Figure 4 clearly showed that EBPR mixed liquor with an enriched PAO population can use an alternative energy source (glycogen) in the absence of poly-P pool.

It is apparent that glycogen is not only serving as the reducing power for PHA formation but also serves as an energy source for the EBPR consortium. The extensive use of glycogen by the enriched PAO sludge cannot be explained through a population shift from PAO to GAO because the batch tests were completed within 12 hours, and a significant population shift cannot take place within such a short period of time. The extensive use of glycogen by the PAOs is a result of a metabolic shift which occurred when the PAO’s preferred energy source (poly-P) was depleted.

The same test was repeated at 5°C. EBPR mixed liquor with enriched poly-P utilized acetate (450 mg/L) and glycogen at 5°C but with much slower reaction rates as expected (Figure 5).
However, EBPR mixed liquor with depleted poly-P utilized very small amount of acetate (50 mg/L) and glycogen (40 mg/L) during anaerobic batch tests performed at 5°C. As enzyme studies suggested, slow generation of ATP via glycolytic pathway at 5°C was not able to satisfy the energy requirements of the EBPR consortia to complete several cell functions including acetate uptake and PHA storage. Again, poor acetate uptake with poly-P depleted PAO sludge was not related to population shift phenomenon but rather was related to inhibition of the alternative energy generation pathway at cold temperatures. These results clearly suggest that even though poly-P cleavage is the main and preferred energy generation pathway for PAOs, they have the ability and the enzymatic machinery to generate energy using the glycolytic pathway. It is not preferred under normal conditions because it is not energetically efficient and involves slower ATP/NADH generation steps relative to poly-P cleavage.

**DISCUSSION AND CONCLUSIONS**

Many EBPR studies have documented the co-existence of PAOs and GAOs in EBPR systems (Seviour & Blackall 1999; Filipe et al. 2001). In recent studies, technological advances (i.e., FISH) have been used to identify organisms labeled as “PAOs” and “GAOs” in EBPR sludge. Composition of feed characteristics, COD/P ratio, pH and temperature were found as the major factors that influence PAO/non-PAO competition. To current date, all EBPR researchers (Wentzel et al. 1986; Comeau et al. 1987; Mino et al. 1987; Smolders et al. 1994; Pereira et al. 1996; Filipe et al. 2001) have agreed that both PAOs and GAOs accumulate PHA anaerobically. However, they have concluded that PAOs use poly-P as the sole energy source, while GAOs are using glycogen as a sole energy source under anaerobic conditions. Glycogen can serve as a reducing power for PAOs during PHA formation under anaerobic conditions. Therefore, GAOs can take up organic material in the anaerobic stage without P release (Mino et al. 1998). The results of this study agree with the literature findings, but additionally propose that PAOs have the ability to use the glycolytic pathway to generate energy under anaerobic conditions. The ability of PAOs to alter (i.e.; activate the most energetically efficient or preferred) energy generation pathway and store excessive amount of glycogen is particularly important since all EBPR models have assumed that glycogen use by PAOs is for providing reducing power only during PHA generation.

The results clearly indicate that PAOs can alternatively use glycogen as an energy source and accumulate significant amounts of glycogen. Although the mechanism of such metabolic shift is not clear yet, it appears that energy shortage can trigger a shift where a less favorable and energy generation pathway (glycolytic pathway) is used. Temperature, herein, functions as a regulator which controls the use of the glycolytic pathway. Some other stress conditions (pH, COD/P ratio, etc.) may also have similar impacts leading to metabolic shifts and
therefore deserve more investigation. Many pilot and full-scale studies indicate that EBPR performance is better at cold temperatures than at warm temperatures (Sell 1981; Daigger et al. 1987; Choi et al. 1998; Erdal et al. 2002, 2003a). The reduced performance of enhanced EBPR facilities operated at warm temperatures may not be a result of GAO proliferation; but instead may be related to more efficient use of the glycolytic pathway by PAOs thus establishing them to store more glycogen thereby reducing phosphorus removal performance of the facility. The possibility of a population shift from PAOs to GAOs or other non-PAOs was ruled out in this study, given the short batch test periods (<12 hrs). This conclusion was further justified with the electron microscopy results.

In a review study, Mino et al. (1998) has postulated that PAOs and GAOs may be the same organism. Observing GAO-like characters by the enriched PAO sludge in this study strongly favor Mino and his coworkers’ presumption. More study, however, needs to be done to identify and compare characteristics of deteriorated EBPR sludge communities and PAO exhibiting GAO like behavior similar to that observed in this study. The results presented in this study also showed that organisms responsible from EBPR have the capability to alter their biochemical metabolism based on the environmental conditions. They were already shown to be capable of “homeoviscous adaptation” of their cell membranes for adaptation to cold temperatures (Erdal et al. 2003b). However, modeling and design assumptions made based on one set of environmental condition will not necessarily be suitable for another set of conditions if the active bacterial community undergoes full acclimation. Also, it is not correct to assume unchanged glycogen involvement under changing temperature conditions, or decreased phosphorus removal at low temperatures merely because of the slowing biochemical reaction kinetics.

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