The effect of GAOs (glycogen accumulating organisms) on anaerobic carbon requirements in full-scale Australian EBPR (enhanced biological phosphorus removal) plants

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Abstract Glycogen-accumulating organisms (GAOs) were present in six full-scale plants investigated and in all but one made a significant contribution to the amount of volatile fatty acid (VFA) taken up anaerobically. While most plants surveyed contain GAOs, it was demonstrated that it is possible for a full-scale plant to operate with an insignificant GAO population. "Candidatus Accumulibacter phosphatis" were the significant polyphosphate-accumulating organisms (PAOs) in all plants surveyed. "Candidatus Competibacter phosphatis" were found in all plants along with other possible GAOs that were observed but not identified. A significant GAO population will increase the carbon requirements by removing VFA that could otherwise have been used by PAOs. Process optimization minimizing GAOs in full-scale plants would lead to a more efficient use of VFA.

Keywords Enhanced biological phosphorus removal (EBPR), fluorescence in situ hybridisation (FISH), glycogen accumulating organism (GAO); polyphosphate accumulating organism (PAO); poly-β-hydroxyalkanoate (PHA); volatile fatty acid (VFA)

Introduction Removal of phosphorus (P) from wastewater can be achieved without the addition of chemical precipitants by enhanced biological phosphorus removal (EBPR). EBPR is frequently capable of good P removal (to less than 0.5 mg/L), but the process can be unreliable and occasionally fail. Not surprisingly, the fact that EBPR plants can fail is not widely reported in the literature, but this fact is well known in the wastewater industry. However, in one account, Hartley and Sickerdick (1994) surveyed 7 plants and reported sudden temporary process upsets leading to effluent levels of 2–3 mg/L P and in one plant, recovery did not occur for 4 months.

EBPR is an activated sludge process where the influent enters an anaerobic zone prior to the rest of the reactor. In the anaerobic zone, polyphosphate-accumulating organisms (PAOs) in the sludge have a selective advantage in volatile fatty acid (VFA) uptake, while ordinary heterotrophic organisms are unable to uptake VFA. PAOs derive the energy for VFA uptake from intracellular storage polymers, polyphosphate (polyP) and glycogen. Hydrolysis of some intracellular polyP results in the release of phosphate, while glycogen and VFA are simultaneously converted to intracellular poly-β-hydroxyalkanoate (PHA), most of it as poly-β-hydroxybutyrate (PHB). In the aerobic zone, PHA is oxidised, providing energy for growth and the regeneration of storage polymers within the cells. The cells aerobically accumulate polyP in excess of the anaerobic P release, and this provides the net P removal from the wastewater. Figure 1a shows a typical profile of extracellular P, acetate, PHA and glycogen in a selectively enriched PAO sludge.

Deterioration of P removal performance of lab-scale EBPR reactors has been reported and attributed to the proliferation of glycogen-accumulating organisms (GAO) (Mino et al., 1995). GAOs also have the ability to anaerobically uptake VFA, however they use glycogen as their energy source as they do not store polyP. Figure 1b shows a
The typical profile of extracellular P, acetate, PHA and glycogen in a selectively enriched GAO sludge.

The ratio of the rate of anaerobic P release to the rate of acetate uptake (P release to acetate uptake) in each cycle has been used to characterise the P removal efficiency of sludge, however, significant variation in this ratio has been observed (Mino et al., 1998). In some studies, a significant variation in the P release to acetate uptake ratio was attributed to varying pH (Smolders et al., 1994), and in others, this ratio was linked to temperature (Brdjanovic et al., 1997). It is hypothesised that the P release to acetate uptake ratio would also be affected by variation in the relative populations of PAOs and GAOs. Higher proportions of PAOs would maximise the amount of P released per acetate uptaken and a higher proportion of GAOs in the sludge would lower the P release to acetate uptake ratio, as less P would be transformed for the same amount of acetate taken up.

PAOs and GAOs can be microscopically observed by specifically staining the characteristic storage polymers, PHA (Sudan Black B) and polyP (methylene blue) but it is impossible to differentiate morphologically similar cells on this basis. Fluorescence in situ hybridisation (FISH) uses fluorescently labeled gene probes to label specific populations of microorganisms. Sludge can then be observed with epifluorescent or confocal laser scanning microscopes (CLSM) to identify microorganisms in the sludge and to observe their spatial arrangements in flocs (Amann, 1995).

When lab-scale sludges were enriched for PAOs by pushing the EBPR performance to the limit, a bacterium closely related to Rhodocyclus called “Candidatus Accumulibacter phosphatis” (henceforth called Accumulibacter) dominated the microbial community (Hesselmann et al., 2000). FISH probes were developed for Accumulibacter (Croce et al., 2000) and a strong positive correlation was established between the proportion of

![Figure 1](https://iwaponline.com/wst/article-pdf/47/11/37/422187/37.pdf)

**Figure 1** Profiles of extracellular phosphate-P (●), acetate (○), PHA (▲), and glycogen (▲) during the anaerobic and aerobic reactor cycle stages of a typical PAO sludge (A) and GAO sludge (B). Adapted from Bond et al. (1999).
Accumulibacter and the sludge P content in lab-scale reactors. Although Accumulibacter have been detected in many plants, their significance to full-scale EBPR has not been established, though to date no other PAO has been found in EBPR sludges. The only GAO identified has been a short, rod-shaped bacterium named “Candidatus Competibacter phosphatis” (henceforth called Competibacter). The organism was identified by cloning and sequencing the 16S rDNA from EBPR-type reactor operations where no P removal was observed. FISH probes were designed (Crocetti et al., submitted) but the presence of the Competibacter has not been reported in full-scale sludges.

Analysis of digital images from the microscopes has been used to enumerate these specific populations as a ratio of the entire bacterial population (Bouchez et al., 2000). FISH can be linked with chemical stains to provide information on the function of the organisms thus identified.

In addition to microscopic examination, PAOs and GAOs can be differentiated through the storage polymers that are anaerobically produced. Although both organisms produce PHA, PAOs produce mainly PHB when fed acetate, with little or no poly-β-hydroxyvalerate (PHV) production (Satoh et al., 1992; Smolders et al., 1994; Mino et al., 1998). In contrast, GAOs produce significant quantities of both PHB and PHV when fed acetate (Satoh et al., 1994). Glycogen is produced aerobically by both PAOs and GAOs, however, GAOs produce and utilize glycogen in much higher quantities (Figure 1). The relative rate of acetate uptake and the amount of total PHA produced gives an indication of the combined population of PAOs and GAOs.

VFAs are often the limiting substrate in EBPR and can be supplemented by prefermentation or external carbon dosing. GAOs serve only to remove VFA and are therefore an unwanted competitor with PAOs for VFA. This PAO-GAO competition could be a factor in contributing to process upsets and failures. Additionally, process optimization to minimize GAOs would lead to more efficient use of VFA and potentially reduce plant costs. This study will demonstrate the significance of anaerobic carbon utilization by GAOs in full-scale plants.

**Methods**

A batch test was performed using sludge from six Australian EBPR plants in a sequencing batch reactor (SBR). The plants chosen for this study included a number of different configurations such as oxidation-ditch (carousel), Johannesburg, modified University of Cape Town (UCT), and UniFed sequencing batch reactors. In each plant, the sludge was obtained from the zone preceding the anaerobic zone, which was usually the return activated sludge (RAS) line, and this sludge was diluted to a volume of 4 L in the SBR using plant effluent. The sludge was subjected to sequential 1.5 hour anaerobic, 1 hour anoxic and 1.5 hour aerobic periods in the SBR. Sodium acetate was added as a VFA source at the beginning of the anaerobic period in a sufficient quantity to determine an acetate uptake rate. At the beginning of the anoxic period, nitrate was added to a concentration of 10 mg NO₃-N/L in the SBR, while air was sparged through the reactor to provide oxygen during the aerobic period. A multi-probe (Grant/YSI 3800 Water Quality Logger) was used to log the pH, dissolved oxygen, redox, conductivity and temperature in the SBR at all times during each study. Phosphorus and acetate concentrations were monitored off-line through flow injection analysis (FIA) and gas chromatography (GC), respectively, using standard methods (APHA et al., 1995). FIA and GC samples were taken every 3–5 minutes during the first half hour of each period, and every 10 minutes for the remainder of each period. PHA and glycogen analysis was performed as in Bond et al. (1999) at the start and end of the anaerobic period.

Microscopic observation of chemical staining of cell smears with Sudan Black B (for
lipophilic granules in this study referred to as PHA granules) monitored the presence of PHA (Jenkins et al., 1984) and methylene blue staining monitored presence of polyP (Murray et al., 1994). FISH was performed as in Amman (1995). Bacteria were probed using FITC or Cy5-labelled EUBMIX probe (Daims et al., 1999) and Accumulibacter were probed using Cy3-labelled PAO421, PAO651 and PAO846 (Crocetti et al., 2000) together in the same hybridisation (henceforth called “PAOMIX”) at 35% formamide. Competibacter cells were probed with GAQQ989 (Crocetti et al., submitted) at 35% formamide. Samples were visualised with a BioRad Radiance 2000 CLSM using an Olympus 60×, oil immersion objective collecting 8-bit, 512 × 512 pixel images with 0.2 µm pixel size and 0.6 µm z-depth, sequentially. FITC, Cy3 and Cy5 were excited with an Ar laser (488 nm), HeNe laser (543 nm) and red diode laser (637 nm) and collected with 500–530 nm BP, 550–625 nm BP and 660 LP emission filters, respectively.

For quantification, FISH was performed with the following modifications. Microscope slides (coated in Teflon to form 7 mm wells) were repeatedly spotted with sludge such that the entire well was covered. Slides were then coated in 0.5% molten agarose and cooled on ice before FISH was performed. A series of 20 images was collected on the CLSM. The area containing Cy3-labelled specific probe (PAOMIX or GAQQ989) cells was quantified as a percentage of the area of Cy5-labelled bacterial probe (EUBMIX) within each image using Image Pro Plus 4 software (Media Cybernetics). Large, non-bacterial autofluorescent particles were removed from the images before analysis. Then, several images were examined to determine the optimum threshold for each channel. The thresholds are then applied to all images in the series to separate the background signal from the countable signal. The area of the specific cell signal is expressed as a mean percentage of the area of bacterial cell signal for the 20 images with a standard error based on a t-distribution.

Results and discussion
Table 1 shows the P release to acetate uptake ratio obtained for each plant as well as the microbial quantitation data. Table 2 shows the breakdown of PHA synthesis from each plant. The PHV fraction is actually representing a total of both poly-β-hydroxyvalerate and poly-β-hydroxymethylbutyrate, which are isomers of the same molecule. The total PHA production, acetate uptake rates and glycogen consumption are standardised per milligram of mixed-liquor suspended solids (MLSS) to provide a comparison basis for each test. The PHB, PHV and total PHA results shown were the total amount produced in the anaerobic period of the batch tests. The glycogen results shown were the total amount consumed during the anaerobic period of these tests.

The results shown in Tables 1 and 2 illustrate major differences in the sludge characteristics between plant A and plant B. Plant A exhibits a large amount of P release per acetate taken up as compared to the other sludges tested, and is very close to the theoretical P release to acetate uptake ratio obtained from lab scale enriched P sludges at a similar pH of 40.

**Table 1** P release to acetate uptake ratio and FISH quantitation

<table>
<thead>
<tr>
<th>Plant</th>
<th>Pmol/Cmol</th>
<th>Accumulibacter</th>
<th>Competibacter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pmol/Cmol</td>
<td>Mean SE mean</td>
<td>Mean SE mean</td>
</tr>
<tr>
<td>A</td>
<td>0.49</td>
<td>7 0.6</td>
<td>&lt;1 &lt;0.5</td>
</tr>
<tr>
<td>B</td>
<td>0.33</td>
<td>12.0.9</td>
<td>12 1</td>
</tr>
<tr>
<td>C</td>
<td>0.29</td>
<td>8 0.8</td>
<td>4 &lt;0.5</td>
</tr>
<tr>
<td>D</td>
<td>0.40</td>
<td>11 0.7</td>
<td>3 &lt;0.5</td>
</tr>
<tr>
<td>E</td>
<td>0.51</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>F</td>
<td>0.41</td>
<td>present</td>
<td>present</td>
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6.5–7 (Smolders et al., 1994). This suggests that the sludge has a minimal GAO population coupled with a significant PAO population. Microbial analysis supports this hypothesis further, as FISH probing showed that the plant A sludge contained a significant PAO population (7% *Accumulibacter*) and a very low GAO population. FISH quantitation showed <1% *Competibacter*, while the absence of other GAOs was confirmed using a Sudan Black B stain. Acetate was the sole VFA source in the anaerobic period of the batch test and yielded the production of 100% PHB and 0% PHV. Although both organisms produce PHA, PAOs produce mainly PHB when fed acetate, with little or no poly-β-hydroxyvalerate (PHV) production (Satoh et al., 1992; Smolders et al., 1994; Mino et al., 1998). In contrast, GAOs produce significant quantities of both PHB and PHV when fed acetate (Satoh et al., 1994). The glycogen consumption for plant A was also very low, which again points toward a low GAO population. Taking into account all of these factors, it is quite clear that the plant A sludge has a minimal GAO population. The effect of this low GAO population is that all the acetate is taken up anaerobically by the PAOs, and no VFA is wasted on organisms that contribute nothing to EBPR. The low acetate uptake rate in the anaerobic period indicates that the sludge does not require as much VFA as do other plants and therefore has efficient carbon utilization.

Plant B yielded extremely different results from that of plant A. In plant B there was a high number of *Competibacter* (12%) and other possible GAOs observed using a Sudan Black B stain. There was also a large portion of PAOs observed by FISH probing in plant B, even more than that observed in plant A. The high total PHA production also indicates that there are a large amount of both PAOs and GAOs present in the sludge. The microbial results are further supported by the fact that the biomass produces a significant fraction of PHV when fed acetate, and consumes a large amount of glycogen, both indicative of a significant GAO population in the biomass. From these observations, it comes as no surprise that the P release to acetate uptake ratio of plant B is significantly lower and the acetate uptake rate markedly higher than that of plant A. This is due to a large fraction of the acetate being metabolized by the competing GAO population. It is therefore clear that a high GAO population leads to a significant level of VFA used by these organisms and less efficient carbon utilization for EBPR.

The other plants studied exhibited variable results between the two extremes of plant A (minimal GAO population) and plant B (large GAO population).

The results of Plant E are similar to that of plant A. The P release to acetate uptake ratio and low PHV production were very consistent between these two plants. There was also a relatively low PHA production and acetate uptake rate, showing that plant E is not likely to have a high GAO population.

The results for plant F are more similar to that of plant B. There was a significant amount of PHV produced from acetate, which would suggest a relatively high GAO population. The high overall PHA production and high acetate uptake rate are indicative of a higher combined fraction of PAOs and GAOs in the biomass.

### Table 2 PHA production, acetate uptake rate and glycogen consumption

<table>
<thead>
<tr>
<th>Plant</th>
<th>PHA production fractions</th>
<th>PHA prod/MLSS (µg/mg)</th>
<th>HAc uptake rate/MLSS (µg/mg/hr)</th>
<th>Glycogen/MLSS (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 0 3.3 12.9 1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>76 24 31.7 48.3 17.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>90 10 3.9 10.2 12.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>not determined not determined not determined 23.2 2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>97 3 5.1 15.3 4.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>80 20 10.3 33.3 not determined</td>
<td></td>
<td></td>
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</table>
Indeed, while present in varying amounts, *Competibacter* were observed microscopically in plants B, C, D and F using FISH, and this was reflected in the lower P release to acetate uptake ratios for these plants. PHV production was variable among these sludges, but the fact that PHV is produced with acetate as the sole VFA source, supports the presence of GAOs in these plants.

The study also assessed the significance of specific genera, *Accumulibacter* and *Competibacter*, in full-scale plants. The presence of these organisms is shown in Figure 2. *Accumulibacter* were present in significant numbers in all of the plants studied (Table 1). They have been detected previously in many full-scale plants and this survey further supports the assertion that they are the most important microorganism facilitating EBPR. Cells other than *Accumulibacter* displaying the PAO phenotype were not observed in any of the samples.

The presence of *Competibacter* was also investigated and it was found to be present in all plants. In agreement with the overall low number of GAOs present in plant A, less than 1% of *Competibacter* were detected. The *Competibacter* population was high in plant B (Figure 2b) accounting for 12% of the bacterial cells, while in other plants *Competibacter* were present, though in numbers lower than those present in plant B. Microorganisms other than *Accumulibacter* and *Competibacter* capable of anaerobically accumulating PHA (differentiated on the basis of morphology) were observed in the sludges and require further investigation.

Plants E & F contained extremely high numbers of the stalked ciliate *Vorticella*, whose strong autofluorescence made the FISH quantification method inaccurate. However, FISH was able to identify *Accumulibacter* and *Competibacter* in plants E & F.

**Conclusions**

The batch test performed using sludge from six full-scale Australian EBPR plants clearly showed that GAOs are significant in full-scale plants. Furthermore, it was demonstrated that a GAO population increases the carbon requirements for P removal by removing VFA that could have otherwise been used by the PAOs. Efficient carbon utilization by selectively optimizing the PAO population over the GAOs could reduce the VFA requirements and lower operational costs of EBPR plants.

![Figure 2](https://iwaponline.com/wst/article-pdf/47/11/37/422187/37.pdf)

**Figure 2** CLSM images of sludge FISH-probed with PAOMIX (cyan – shows bright) and EUBMIX (blue – shows dark grey) probes. (A) No GAOs were present in Plant A, while (B) additionally plant B contains *Competibacter* cells (GAOQ989-magenta – shows mid-grey). Bar = 10 µm
Accumulibacter were significant PAOs in all plants surveyed. Competibacter (the only GAO identified to date) were found in all plants. Other GAOs were observed, which requires further investigation. Identification of these bacteria would allow for more comprehensive probing of EBPR sludges by FISH and assist in sludge characterisation.

The results have also demonstrated that while most plants contain GAOs, it is possible for a full-scale plant to operate with an insignificant GAO population. A useful future investigation would involve the determination of operational factors that optimize the efficiency of VFA utilization for P removal through minimization of GAOs in EBPR sludges.

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