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# The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on system performance

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**Abstract** It is well known and firmly established that the rate of chemical and biochemical reactions slow down as temperature decreases. Nevertheless, several studies have reported that the efficiency of enhanced biological phosphorus removal (EBPR) improves as temperature decreases. However, several recent studies have reported that EBPR reaction rates decrease with temperature decrease in accordance with the Arrhenius relationship. This study was designed to more thoroughly investigate this controversy using two UCT plants fed with a synthetic wastewater consisting primarily of acetate as the COD form, and a small amount of supplemental yeast extract. Experiments were performed over temperatures ranging from 5 to 20°C. The results showed that, even though the kinetic rates decrease as temperature decreases, EBPR systems perform better at colder temperatures. The reason for better system performance is apparently related to reduced competition for substrate in the non-oxic zones, which results in an increased population of PAOs and, thus, greater EBPR efficiency. The proliferation of PAOs apparently occurs because they are psychrophilic whereas their competitors are not. The experiments showed that the EBPR sludges accumulated high concentrations of both PHA and glycogen at 20°C, but accumulated more PHA and much less glycogen at 5°C. Although the results could be interpreted as the result of changes in the PAO-GAO competition, Mann-Whitney non-parametric comparisons of transmission electron microscopy examinations revealed no indication of the presence of GAOs population under any temperature conditions. Regardless, mass balances of the glycogen data showed that the involvement of glycogen is less at cold temperature, even though EBPR was greater. Unlike current EBPR models (e.g. Mino model), the results suggest that glycogen metabolism is not a precursor for EBPR biochemistry. The results also indicate that temperature not only may cause selective pressure on the dominant organisms, but also may force them to use a different metabolic pathway as temperature decreases.

**Keywords** EBPR; electron microscopy; GAOs; glycogen; PAOs; temperature

## Introduction

Temperature is a fundamental factor that affects all living organisms. It influences the rates of enzymatically catalyzed reactions and affects the rate of diffusion of substrate into the cells (Grady *et al*., 1999). The diverse bacterial consortium responsible for EBPR processes in biological wastewater treatment systems consists of psychrophilic, pyschrotrophic and mesophilic heterotrophic bacteria. Because they have different optimum growth temperatures, the temperature of the wastewater-microbial mixture (mixed liquor) strongly influences the population composition of the consortium. The effects of temperature on the efficiency and the kinetics of excess biological phosphorus removal (EBPR) systems have been under investigation for the past two decades, but with contradictory results. Early researchers (Sell, 1981; Ekama, *et al*., 1984; Barnard *et al*., 1985) reported that EBPR efficiency was greater at lower temperatures than at higher temperatures over the range from 5 to 24°C. The first contradictory finding was reported by McClintock *et al*. (1992). They showed that EBPR functions would "wash-out" of

activated sludge systems before other heterotrophic functions at a temperature of 10°C and a sludge retention time (SRT) of 5 days, whereas wash-out did not occur at 10°C when the SRT was 15 days. Then, Mamais and Jenkins (1992) showed that there is a wash-out SRT for all temperatures over the range from 10 to 30°C. This introduced the paradox that, even though EPBR system performance becomes more efficient at lower temperatures, if the SRT-Temperature combination is below a critical value, EBPR ceases to function before other heterotrophic functions wash-out. More recently, Jones and Stephenson (1996), Brdjanovic *et al*. (1997 and 1998) and Beatons *et al*. (1999) have shown that EBPR biochemical reaction rates become slower with decreasing temperature, as is typical of biochemical reactions if the microbial population is unchanged. Thus, although temperature appears to affect EBPR reaction rates in a normal manner, a substantial body of evidence including full-scale experience indicates that many EBPR systems perform more efficiently as the temperature decreases. Therefore, an apparent controversy exists in previous temperature studies when the performance of the EBPR systems under different temperature conditions were studied.

It is known that the EBPR microbial community is a diverse population rather than one or two species. Good phosphorus (P) removal is achieved when the activated sludge is enriched with a population of phosphorus accumulating organisms (PAOs) (Cech and Hartman, 1993; Mino *et al*., 1998). It also has been shown that EBPR performance is inhibited under certain conditions (Seviour and Blackall, 1999). The first observation of EBPR deterioration by population change was made by Cech and Hartman (1993) when their microscopic investigations detected clusters of large Gram-negative and Gram-positive coccoid cells, usually arranged in tetrads, that accumulated glycogen but did not store polyphosphate in excess. They named them G bacteria because of the flask they were grown in, but they have since been called glycogen accumulating organisms (GAOs) (Liu *et al*., 1996; Mino *et al*., 1998). Both PAOs and GAOs accumulate PHA anaerobically. However, the main difference between PAOs and GAOs is that PAOs use P as an energy source under anaerobic conditions, but GAOs use only glycogen as an energy source under anaerobic conditions. Liu *et al*. (1996) showed that a wastewater with a low P/C ratio stimulates the growth of GAOs because there is insufficient P to support a large PAO population. More recently, Filipe *et al*. (2001) proposed that a pH value of 7.25 or below favors GAOs over PAOs and causes EBPR efficiency to decrease. The complete or partial loss of EBPR performance at cold temperatures has been reported by McClintock *et al*. (1992), Beatons *et al*. (1999), Jones and Stephenson (1996), Brdjanovic *et al*. (1997), and several other researchers. Such deterioration of EBPR performance might be caused by a population shift from PAOs to GAOs or to other heterotrophs. However, no temperature study that actually investigated PAO-GAO population shifts has been performed. In addition, no solid evidence has been presented that explains why EBPR systems perform either more or less efficiently at cold temperatures. This research was designed to develop evidence that could be used to resolve the EBPR temperature paradox.

PAO and GAO microbial community changes with temperature changes were investigated using mass balance determinations of internal storage products (e.g. PHA, glycogen, and Poly-P), and by the application of light and electron microscopy techniques. Unlike several of the EBPR temperature studies, the systems were acclimated to the temperatures before comparison data was obtained. The importance of temperature acclimation is further demonstrated by comparing short and long term temperature exposures on EBPR system performance. In addition, the SRT values were adjusted such that washout of the EBPR sludge was eliminated.

## Methods and materials

A laboratory-scale UCT system with two anaerobic, two anoxic and three aerobic reactors in series, receiving acetate and supplemental yeast extract as the carbon source (450 mg/L COD) was operated at 20°C and at 10 day SRT for more than 8 months. Following steady state data collection at 20°C, the temperature of the room was dropped from 20°C to 5°C in a week. The system was exposed to intermediate temperatures of 18, 15 and 10°C for two days each as the temperature was dropped. Data was collected during this period to evaluate short-term temperature effects. Next the system was operated at 5°C until steady-state EBPR was established at an SRT of 18 days, with data collection both during and subsequent to the period of acclimation. Steady state and short-term data included MLSS, MLVSS, COD, acetate, $NH_4^+$-N, $NO_2$-N, $NO_3$-N, soluble $PO_4$, total P, PHB, PHV and glycogen. All ions were measured with a DIONEX ion chromotograph. The PHV and PHB content of the sludge was measured as outlined in the paper by Kisoglu *et al*. (2000). Glycogen measurements were performed according to Erdal (2002). All other measurements were performed according to *Standard Methods* (APHA, 1998). The pH of the system was kept between 7.1 to 7.6 throughout the experimental period. Target SRTs of 15 and 20 days were selected to prevent washout of the biomass at 10 and 5°C, respectively. Effluent VSS carryover reduced the SRT to 18 days at 5°C. The Neisser and Sudan Black staining techniques are applied to anaerobic and aerobic samples in order to detect poly-P and PHA granules according to the methods outlined in Seviour and Blackall (1999). In addition, samples taken from the aerobic section of the experimental system were fixed and sectioned for transmission electron microscope (TEM) in order to compare changes in morphological structure at 20 and 5°C. Osmium tetraxide and lead citrate were used for prefixation of the samples according to Hayat (1986). The Mann–Whitney non-parametric test outlined in Zar (1999) was applied for comparison of the TEM grids.

## Results

The results of the short-term temperature effects are shown in Figure 1. There was a general decreasing trend in P removal performance as temperature decreased. Average P removals were 23, 22.8 and 21 mg/L, at 20,18 and 15°C, which was in good agreement with the results of previous short-term studies (Brdjanovic *et al*., 1997 and 1998; Beatons *et al*., 1999). Phosphorus mass balance calculations showed that net P removal was reduced by 57% at 10°C, and no P removal was initially observed at 5°C. In addition, acetate utilization was incomplete at 5°C despite the presence of greater anaerobic times in the anoxic stages

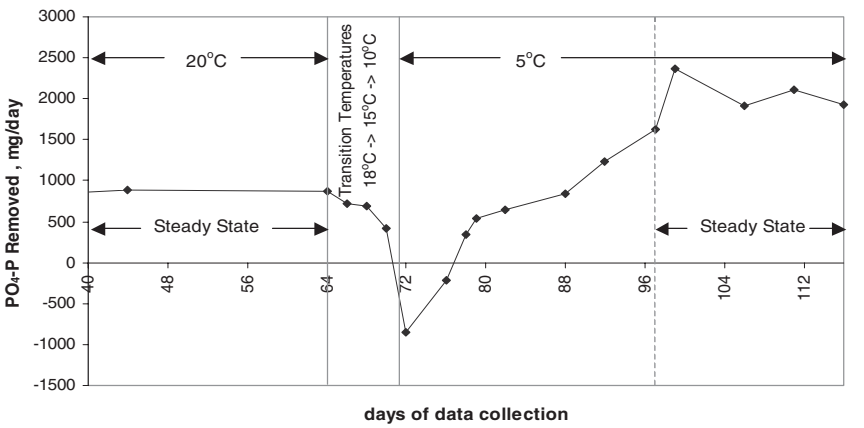


**Figure 1** Change in net $PO_4$-P removal throughout the temperature study. Transition period covers the period of stepwise temperature decrease from 20°C to 18, 15, 10 and 5°C in 2-day steps.

due to the loss of nitrification. The system discharged excess P for a period of 6 days as the system adjusted to the temperature. Then the system began to remove P with increasing efficiency until removal was more than double what it was at 20°C.

PHA mass balance (Table 1) showed that PHA production in the non-oxic reactors decreased by 49.8% and 62% at 10 and 5°C compared to 20°C. Glycogen utilization (Table 2) in the anaerobic zone and production in the aerobic zone decreased significantly as the temperature decreased. After steady state was reached at 5ºC, P removal averaged 74 mg/L (Figure 1), and the average PHA formation was 23.9% greater and glycogen formation was 25% less compared to 20°C. Carbon mass balances of acetate, PHA and glycogen are given in Figure 2.



**Table 1** Mass balance of PHA through anaerobic, anoxic and aerobic stages

| Temp. °C | Anaerobic PHA prod. mg/d | Anoxic PHA prod. mg/d | Total PHA prod. mg/d | Aerobic PHA uptake mg/d | Anoxic PHA uptake mg/d | Total PHA uptake mg/d | Net PHA production mg/d |
|---|---|---|---|---|---|---|---|
| 20* | 11379 | 1245 | 12624 | 10296 | 1431 | 11727 | 897 |
| 20* | 12809 | 418 | 13227 | 11902 | 572 | 12474 | 753 |
| 20* | 10965 | 728 | 11693 | 9269 | 2541 | 11810 | –117 |
| 20* | 12181 | 939 | 13120 | 11355 | 1582 | 12937 | 183 |
| 20* | 10848 | 1720 | 12568 | 11188 | 815 | 12003 | 565 |
| 18 | 10231 | 1012 | 11243 | 9298 | 582 | 9880 | 1363 |
| 15 | 9064 | 787 | 9851 | 8113 | 233 | 8346 | 1505 |
| 10 | 8507 | 650 | 9157 | 6292 | 155 | 6447 | 2710 |
| 5 | 3004 | 4373 | 7377 | 4787 | 0 | 4787 | 2590 |
| 5 | 4261 | 7841 | 12102 | 12440 | 0 | 12440 | –338 |
| 5 | 2960 | 4994 | 7954 | 6792 | 0 | 6792 | 1162 |
| 5 | 5802 | 8282 | 14084 | 13011 | 0 | 13011 | 1073 |
| 5 | 4968 | 9021 | 13989 | 13802 | 0 | 13802 | 187 |
| 5* | 7864 | 7362 | 15226 | 14460 | 0 | 14460 | 766 |
| 5* | 7788 | 10807 | 18595 | 15792 | 0 | 15792 | 2803 |
| 5* | 9668 | 6701 | 16369 | 15792 | 0 | 15792 | 577 |
| 5* | 10791 | 5595 | 16386 | 14058 | 0 | 14058 | 2328 |
| 5* | 9466 | 5970 | 15436 | 14917 | 0 | 14917 | 519 |
| 5* | 12350 | 3771 | 16121 | 15792 | 0 | 15357 | 764 |
| 5* | 11464 | 5081 | 16545 | 15476 | 0 | 15476 | 1069 |

* Asterisks indicate steady-state data (long-term temperature effects).

**Table 2** Mass balance of glycogen through anaerobic, anoxic and aerobic stages

| Temp. °C | Anaerobic Gly utilized mg/d | Anoxic Gly utilized mg/d | Total Gly utilized mg/d | Aerobic Gly stored mg/d | Anoxic Gly stored mg/d | Total Gly stored mg/d | Net Gly stored mg/d |
|---|---|---|---|---|---|---|---|
| 20 | 8215 | 251 | 8466 | 9952 | 982 | 10934 | 2468 |
| 20 | 8133 | 88 | 8221 | 11293 | 1357 | 12650 | 4429 |
| 20 | 7232 | 2143 | 9375 | 8908 | 1546 | 10454 | 1079 |
| 20 | 7895 | 1766 | 9661 | 9114 | 1480 | 10594 | 933 |
| 20 | 6568 | 1906 | 8474 | 8492 | 1866 | 10358 | 1884 |
| 18 | 5897 | 920 | 6817 | 7725 | 2137 | 9862 | 3045 |
| 15 | 5138 | 1139 | 6277 | 7571 | 233 | 7804 | 1527 |
| 10 | 4737 | 821 | 5558 | 6024 | 157 | 6181 | 623 |
| 5 | 2684 | 3177 | 5861 | 4275 | 0 | 4275 | –1586 |
| 5 | 1654 | 3505 | 5159 | 4759 | 0 | 4759 | –400 |
| 5 | 2069 | 3672 | 5741 | 6432 | 0 | 6432 | 691 |
| 5 | 2305 | 5473 | 7778 | 8158 | 0 | 8158 | 380 |
| 5 | 2467 | 5492 | 7959 | 8016 | 0 | 8016 | 57 |
| 5 | 4941 | 2521 | 7462 | 7712 | 0 | 7712 | 250 |
| 5 | 4272 | 2789 | 7061 | 7172 | 0 | 7172 | 111 |
| 5 | 4927 | 2713 | 7640 | 7667 | 0 | 7667 | 27 |

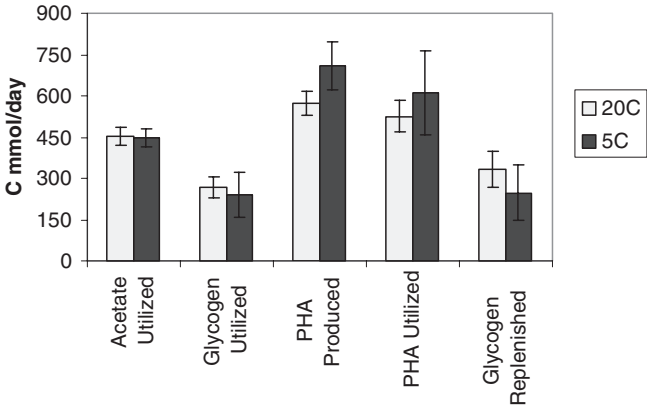


**Figure 2** Carbon mass balance of acetate, PHA and glycogen.

Carbon balance calculations (Figure 2) showed that less carbon was converted to PHA in the non-oxic stages at 20°C compared to 5°C. On the other hand, more carbon was associated with cell growth, maintenance energy and poly-P metabolisms at 5°C (362±73.4) compared to 20°C (195±90.2). Poly P content of the sludge greatly increased and was high as 37% P as VSS at 5°C operation. The average PHV content of the sludge decreased from 18% at 20 to 4% at 5°C. The steady state samples from the last aerobic section of the UCT system were Neisser positive at both 20 and 5°C. No distinctly coccoid cells with tetrad arrangement were observed under both temperature conditions suggesting no presence of G bacteria or GAOs. The individual cells in each grid of the TEM samples (micrographs not shown) obtained from the aerobic sections during steady state operation at 20 and 5°C show a striking difference: the 5°C sample had greater poly-P accumulation compared to those observed at 20°C. On the other hand, glycogen granules were highly abundant under both temperature conditions. Although no quantitative information can be gained with electron microscopy, these results are in good agreement with the analytical results. Individual cells in each micrograph were compared with each other in order to understand whether they are similar with respect to their shapes and internal storage products. A non-parametric Mann–Whitney test also suggested that there is no real difference among the bacterial cells when 4 and 10 grids (not shown) were examined at 20 and 5°C, respectively.

## Discussion

It was shown that EBPR activated sludge can rapidly adjust cellular wall fluidity for temperature changes (Erdal *et al.*, 2003). However, bacterial communities need time to reach steady state after large changes in temperature, i.e. on the order of 2 to 3 SRTs. This essential requirement, however, was ignored in many temperature studies of EBPR research (Brdjanovic *et al.*, 1997; Beatons *et al.*, 1999). The loss and then recovery of P removal performance in this study after the temperature was lowered to 5°C clearly showed that temperature acclimation is a prerequisite for a full understanding of EBPR system performance. The other key parameter that affects system performance is SRT. Following EBPR recovery, (no wasting was performed during the recovery period) the system was operated at 18 days SRT. The disappearance of nitrification and the increased MLVSS concentration resulted in complete acetate uptake during the non-oxic stages despite the slower acetate uptake rates at 5°C. This occurred because COD was limiting rather than P. But, even though nearly the same amount of acetate was taken up anaerobically under both temperature conditions, almost 50 mg/L more P was removed at 5°C. It was concluded that the steady-state PAO population was considerably higher at 5°C than at 20°C, while the

non-PAO population was considerably less at 5°C. It seems likely that the lower P removal at 20°C occurred because non-PAO bacteria successfully competed with the PAOs for substrate in the anaerobic stage. The high glycogen (13% glycogen as VSS) and PHV content of the 20°C sludge (18% of PHA) increased the probability that GAOs were present. Because of the lower glycogen and PHV concentrations at 5°C, it was postulated that GAO populations became insignificant at cold temperature, probably because of either their non-pyschrophilic or slow growing nature as reported by Mino *et al.* (1998). Thus, it initially was concluded that the PAO fraction substantially increased because they had very little competition for the substrate, resulting in the large increase in P removal efficiency at the lower temperature. However, careful examination of the glycogen data suggested that the total decrease in glycogen mass was insignificant and remained in a narrow range (5 to 7% of VSS). In addition, the electron microscopy results indicated that the microbial communities were very similar and not very diverse as mentioned by Brdjanovic *et al.* (1997). Comparison of 10 grids of the 5°C micrograph revealed that no distinct organism with high glycogen and low poly-P content was present. The same conclusion was drawn when individual cells of each 20°C grid were compared. However, a more diverse population was observed at 20°C. This finding is important because it suggests that PAO-GAO competition is insignificant when EBPR systems are operated with COD limiting conditions. The decreased P removal efficiency at the warmer temperature perhaps can be attributed to the presence of fermentative or other non-Poly P bacteria that are capable of utilizing substrate under anaerobic conditions.

Another explanation of the conclusion that PAOs are the only dominant group in EBPR systems is that lower temperatures may force the PAOs to use one or more different metabolic pathways. This possibility deserves further investigation. More importantly, the insignificant involvement of glycogen under the colder temperature suggests that glycogen metabolism is not a precursor for EBPR biochemistry as suggested by some current EBPR models (Mino *et al.*, 1987; Pereira *et al.*, 1998). It is speculated that temperature not only exerts selective pressure on bacterial growth by affecting rates of reactions, but also influences the metabolic pathways of the EBPR process at 5°C.

## Conclusions

- Temperature does affect EBPR reaction rates consistent with other biochemical and chemical reactions, and washout can occur at low temperatures and SRTs. However, reduced competition for substrate in the non-oxic zones at low temperatures results in an increased population of PAOs and greater EBPR efficiency at steady state if the SRT of the system is above the critical washout SRT for the prevailing temperature.
- Even though high PHV content of the EBPR sludge suggested the potential presence of GAOs at the warmer temperature, the rest of the evidence did not confirm that GAOs co-exist in EBPR sludge communities under any temperature conditions when the system is COD limited.
- The insignificant contribution of glycogen to EBPR mechanisms at cold temperature suggests that glycogen metabolism is not a precursor required for EBPR biochemistry.
- It has been confirmed that PAOs are psycrophilic bacteria and that temperatures of 10°C or less give them a growth advantage relative to the non-PAOs in activated sludge systems.
- It is postulated that cold temperatures suppress some of the metabolic pathways of EBPR organisms, either partially or completely, without altering the dominant population.

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