# CHAPTER III

# THE EFFECTS OF TEMPERATURE ON SYSTEM PERFORMANCE AND BACTERIAL COMMUNITY STRUCTURE IN EBPR SYSTEMS

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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$^{o}$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 acclimated EBPR sludges accumulated high concentrations of both PHA and glycogen at 20$^{o}$C, but accumulated more PHA and much less glycogen at 5$^{o}$C. Although the results could be interpreted as the consequence of changes in the PAO-GAO competition, comparisons of transmission electron microscopy examinations revealed no indication of the presence of GAO population under any temperature

condition. Regardless, mass balances of the glycogen data showed that the involvement of glycogen is less at cold temperature, even though PHA production and EBPR performance was greater. Unlike current EBPR models (e.g., the Mino model, 1987), the results suggest that involvement of glycogen metabolism in EBPR biochemistry is a requirement as suggested by Mino model (Mino *et al*. 1987) but its involvement depends on the environmental conditions. EBPR stoichiometry was presumed to be insensitive to temperature changes (Brdjanovic et al. 1997). However, this study showed that the stoichiometry of EBPR was sensitive to temperature The results also indicate that temperature not only causes selective pressure on the dominant organisms, but also may force them to use different metabolic pathways as temperature decreases.

**Keywords:** EBPR, temperature, PAOs, GAOs, PHA, glycogen, poly-P, electron microscopy, UCT.

## 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. Due to the differences in their 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 the studies have yielded 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 $^{o}$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$^{o}$C and a sludge retention time (SRT) of 5 days, whereas wash-out did not occur at 10$^{o}$C when the SRT was 15 days. Then, Mamais and Jenkins (1992) showed that there was a wash-out SRT for all temperatures over the range from 10 to 30$^{o}$C. This introduces 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 before other heterotrophic functions wash-out. More recently, John 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 temperatures, 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. There is an apparent contradiction in the literature regarding the effects of temperature on the reaction rates and performance of EBPR systems. The purpose of this research was to more closely examine this apparent contradiction.

The first isolation of EBPR bacteria grown on acetate was performed by Fuhs and Chen (1975) and *Acinetobacter spp*. were identified as the predominant group. The dominance of *Acinetobacter* was further demonstrated by Buchan *et al*. (1983); Lotter (1985); and Kerdashi and Healey (1987). A high *Acinetobacter* percentage (79%) was detected by Wentzel *et al*. (1988) when agar plate techniques were applied to bacterial cultures taken from a lab scale EBPR plant achieving very good P removal. However, when the same culture methods were used by Kavanaugh and Randall (1992), they showed that *Acinetobactor spp.* were less numerous than several other types of PAOs in EBPR activated sludge, and determined that one of the key reactions used to identify *Acinetobacter* in the previously mentioned studies was in error. Further, when techniques other than culture dependent identification methods were used for the isolation of EBPR bacteria no dominance of *Acinetobacter* was reported. (Brock, 1987; Cloete and Steyn, 1987; Hiraishi *et al*., 1989; Hiraishi and Morishita, 1990; Auglin *et al.*, 1991; Manz *et al*., 1994). Cloete and Steyn (1987) showed that less than 10% of total bacterial population accounted for *Acinetobacter* spp when fluorescent antibody staining technique was

applied to their EBPR sludge. The quinone profile of an EBPR sludge revealed that dominant quinones of EBPR bacterial community were detected as quinone-8 and mena quinone-8 which are quite different than the usual quinone type of EBPR bacteria (quinone-9) (Hiraishi *et al.*, 1989). Only a small portion (up to 6%) was detected as quinone-9. When a technique that uses a specific polyamine biomarker for *Acinetobacter spp* (diaminopropane; DAP) was applied to a working EBPR sludge by Auglin *et al.* (1991), the results showed no presence of DAP in polyamine pattern suggesting no involvement of *Acinetobacter* in the EBPR process. More recent studies using 16s-rRNA targeted oligonucleotide probe showed that only a small portion (10% or less) of the total bacterial population was *Acinetobacter spp*. (Wagner *et al*., 1994; and Bond *et al*., 1995).

The apparent dominance of *Acinetobacter* shown in early studies came about due to the use of culture dependent techniques, one of which was erroneous. Culture dependent techniques can seriously err because they tend to selectively grow bacteria according to the artificial growth medium and conditions selected for isolation and culture (Mino *et al*., 1998). The possibility of developing a pure culture EBPR system was investigated by Jenkins and Tandoi (1991) using a pure culture of *Acinetobacter*. The system failed and no phosphorus removal was achieved. So far, no successful operation of an EBPR process containing a pure bacterial culture has been reported (Bond and Rees, 1999). Therefore, it is now agreed that the EBPR microbial community is a diverse population rather than one or two species. Current techniques such as fluorescent in situ hybridization (FISH) and clone library analysis also indicate that EBPR bacteria are diverse and they contain mainly beta subclass Proteobacteria (Bond and Rees, 1999).

Good P removal is achieved when the activated sludge is enriched with a population of phosphorus accumulating organisms (PAOs) (Cech and Hartman, 1990; Mino *et al.*, 1998). The dominance of PAOs in EBPR sludge results in complete anaerobic substrate uptake and aerobic P removal by a sludge with a high P content (Randall et al., 1992; Liu *et al*., 1997). In an EBPR sludge with an enriched population of PAOs, a P content of up to 37 and 43 % as VSS was reported by Wentzel *et al*. (1987) and Copp and Dold (2001) respectively. It has also been shown that EBPR performance was inhibited under certain

conditions (Bond and Rees, 1999). The first observation of EBPR deterioration by population change was reported by Cech and 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" because of the designation of the flask they were grown in, but they have since been called glycogen accumulating organisms (GAOs) (Liu *et al*., 1997; Mino *et al*., 1998).

Both PAOs and GAOs accumulate PHA anaerobically. The main difference between them is that PAOs use P as an energy source under anaerobic conditions, but GAOs use only glycogen as an energy source 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). Even though both PAOs and GAOs store PHA anaerobically, the form of PHA is mainly poly-hydroxyvalarate (PHV) for GAOs and poly-hydroxybutarate (PHB) for PAOs. The differences in morphological characteristics and staining response of the two groups make them easily distinguishable (Cech and Hartman, 1993; Liu *et al*., 1997). PAOs are generally small and rod or oval shaped with large poly-P granules before anaerobic substrate uptake, whereas GAOs are large spheres and generally 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 and Hartman (1993). Matsuo (1994) reported inhibition of EBPR performance when a high anaerobic/ aerobic contact time ratio was applied. Liu *et al*. (1997) showed that a wastewater with a low P/C ratio (2/100) stimulates the growth of GAOs because there is insufficient P to support a large PAO population. P content of sludge decreased down to 1.5% TSS during steady state operation. In a recent study, 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.

## METHODS AND MATERIALS

### a. Experimental Design

PAO and GAO microbial community changes with temperature changes were investigated using mass balance determinations of internal storage products (i.e. PHA, glycogen, and Poly-P), and by the application of light and electron microscopy techniques. Unlike several of the preceding EBPR temperature studies reported in the literature, the systems were acclimated to their temperature of interest before steady-state data were collected for comparison and evaluation. The importance of temperature acclimation was further investigated by comparing short and long term temperature exposures on EBPR kinetic rates and system performance. In addition, the SRT values were adjusted as the temperature was decreased so that washout of EBPR was avoided.

### Stage I:

*Determination of EBPR system performance:*

The early phase of the study evaluated steady-state EBPR system performance at 20$^{o}$C. A UCT configuration system was used for this phase and this configuration was used throughout the study. This phase was followed by determination of the short-term temperature effects on EBPR performance. Temperature values of 18, 15, 10 and 5$^{o}$C

were used for the short-term investigation. Temperature acclimation was then accomplished at 5$^{o}$C and steady-state system performance was determined.. The systems were operated for 24 months and 12 months at 20 and 5$^{o}$C, respectively.

*Application of Electron and Light Microscopy Techniques:*

Neisser and Sudan Black staining techniques were used to identify and compare the morphological characteristics of steady state samples from the 20 and 5$^{o}$C systems. In addition, transmission electron microscopy (TEM) was used to further evaluate morphological characteristic of the EBPR sludges and to visualize internal storage products (PHA, glycogen, Poly-P). Most samples were taken from the last aerobic reactors in which maximum Poly-P and glycogen storages are achieved.

**Stage II:**

*Batch Test Experiments forEvaluation of the Competition between PAOs vs. GAOs:*

The stage I results of this study showed that cold temperatures improved EBPR system performance, and, based on the data, the improvement was attributed to an increased population of PAOs at the expense of the GAO population. It also was observed that there was a considerable decrease in PHV and glycogen storage at the colder temperatures, which suggested that GAOs cannot compete adequately for substrate at cold temperatures because they are mesophilic. Even though this explanation seemed logical, another possibility existed that deserved investigation. The possibility was that temperature may not only exert selective pressure on the bacterial populations, but may also shift the metabolic pathways available to the organisms. If so, some bacterial cells under cold temperature stress may use pathways other than the glycolytic pathway, and, even if they contained appreciable amounts of glycogen, they might not be able to utilize them. Several batch experiments were designed and performed to further investigate this possibility. The experimental procedure utilized is as follows:

An 0.8 L sample of anoxic mixed liquor sludge was obtained from each UCT system (20 and 5$^{o}$C) and equally divided into 2 volumetric flasks each. The flasks were numbered 1 through 4. Anoxic sludge from the 5$^{o}$C system was put into flasks 1 and 3, while flasks 2 and 4 contained 400 mL of anoxic sludge from the 20$^{o}$C system. Acetate was added to each flask before starting anaerobic batch tests at 20 and 5$^{o}$C. The purpose of the tests was to deplete the poly-P pool in the EBPR bacterial cells. Two of the flasks, one of each sludge, had anaerobic periods of 3 hrs at 20$^{o}$C., and the other two had 6 hr periods at 5$^{o}$C. Then each batch of sludge was washed with distilled water at 20 and 5$^{o}$C, at least three times. This removed any remaining acetate and P from the solution. Before starting the aerobic part of the batch test, 200 mg/L P was added to flasks 3 and 4. Micronutrients along with K, Mg, Ca were added to all four flasks prior to the aerobic batch test. Five and nine hour aerobic periods were provided to complete P uptake at 20 and 5$^{o}$C, respectively (the aerobic periods were selected based upon continuous system experience). Sludges without P addition (flasks 1 and 2) could not accumulate Poly-P at either temperature, but could accumulate glycogen. Following the aerobic period, the supernatant of each flask was poured out and the sludge in each flask was washed with distilled water at 20$^{o}$C. Flasks 1 and 3 were immediately placed in a 20$^{o}$C room. Each flask then received excessive acetate and micronutrients right before starting a second anaerobic batch test. Before chemical addition, $N_2$ gas was introduced to each flask in order to remove $O_2$ from the sludge and $N_2$ addition was continued throughout the anaerobic period. Acetate utilization, PHA production and glycogen consumption were measured for all samples. Solids measurements from each flask were also performed at the beginning and at the end of the anaerobic period.

In the second step, the above procedure was applied to EBPR sludges taken from the 20$^{o}$C system. This time the anaerobic batch test was performed at 5$^{o}$C to see whether or not the bacterial community would utilize acetate under temperature stress without a poly-P pool.

**b. Experimental Systems**

Two identical laboratory-scale UCT systems with two anaerobic, two anoxic and three aerobic reactors in series were operated using acetate, primarily, with supplemental yeast extract as the carbon source (450-500 mg/L COD). They were operated in two different constant temperature rooms at 20$^{o}$C and at 10 day SRT for several months. The systems, referred to as System 1 and System 2, were fed with the synthetic wastewater whose composition is given below. The synthetic feed composition was decided according to Punatranasin (1987). Micro-nutrient requirement was calculated based upon Grady et al. (1999).

Acetate 450 mg/L, yeast extract 50 mg/L COD, $(NH_4)_2SO_4$ 40 mgN/L, $K_2HPO_4$ and $KH_2PO_4$ 25 to 90 P mg/L, 125 mg/L alkalinity in the form of $NaHCO_3$, 210 mg/L $MgSO_4$, 44.4 mg/L $CaCl_2$, 1.11 mg/L $FeCl_3$, 0.66 mg/L $MnCl_2{\cdot}6H_2O$, 0.44 mg/L $ZnSO_4{\cdot}7H_2O$, 0.14 mg/L $CuSO_4{\cdot}5H_2O$, 0.14 mg/L $CoCl_2{\cdot}6H_2O$, 0.05 mg/L KI, 0.12 mg/L $H_3BO_4$, and 0.05 mg/L EDTA.

The schematic of the lab scale modified UCT units is given in Figure 1. The pH of the system in the influent was kept nearly constant (7.2-7.6). The phosphate solution was added separately to avoid microbial growth in the feed and chemical precipitation of the P. Thus, a small pump delivered the P separately and the other peristaltic pump delivered organic and inorganic micronutrients to the first anaerobic tank of each system. Combined influent was defined as the combination of both flow rates entering the system. The influent flow rates of both systems were set at 35 L/day and they produced a nominal detention time of 14 hours.

The operation of System 2 was started following 8 months of steady state operation for System 1. The seed for both systems were obtained from the Roanoke wastewater treatment plant, which partially achieves biological P removal. Sludge wasted from System 1 was added to the aerobic section of System 2 on a daily basis for about one week. This way the acclimation period was shortened and a great similarity in microbial

community was obtained. Following steady state data collection of System 2 at 20°C, the temperature of the room was dropped from 20°C to 5°C over a week's time. The system was exposed to intermediate temperatures of 18, 15 and 10°C for two days each as the temperature was dropped. Data were 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 during and following the period of acclimation. Steady state and short-term data included MLSS, MLVSS, COD, oxygen uptake rate (OUR), acetate, $NH_4^+$-N, $NO_2$-N, $NO_3$-N, soluble $PO_4$, total P, PHB, PHV and glycogen. Data collection locations are summarized in Table 1.

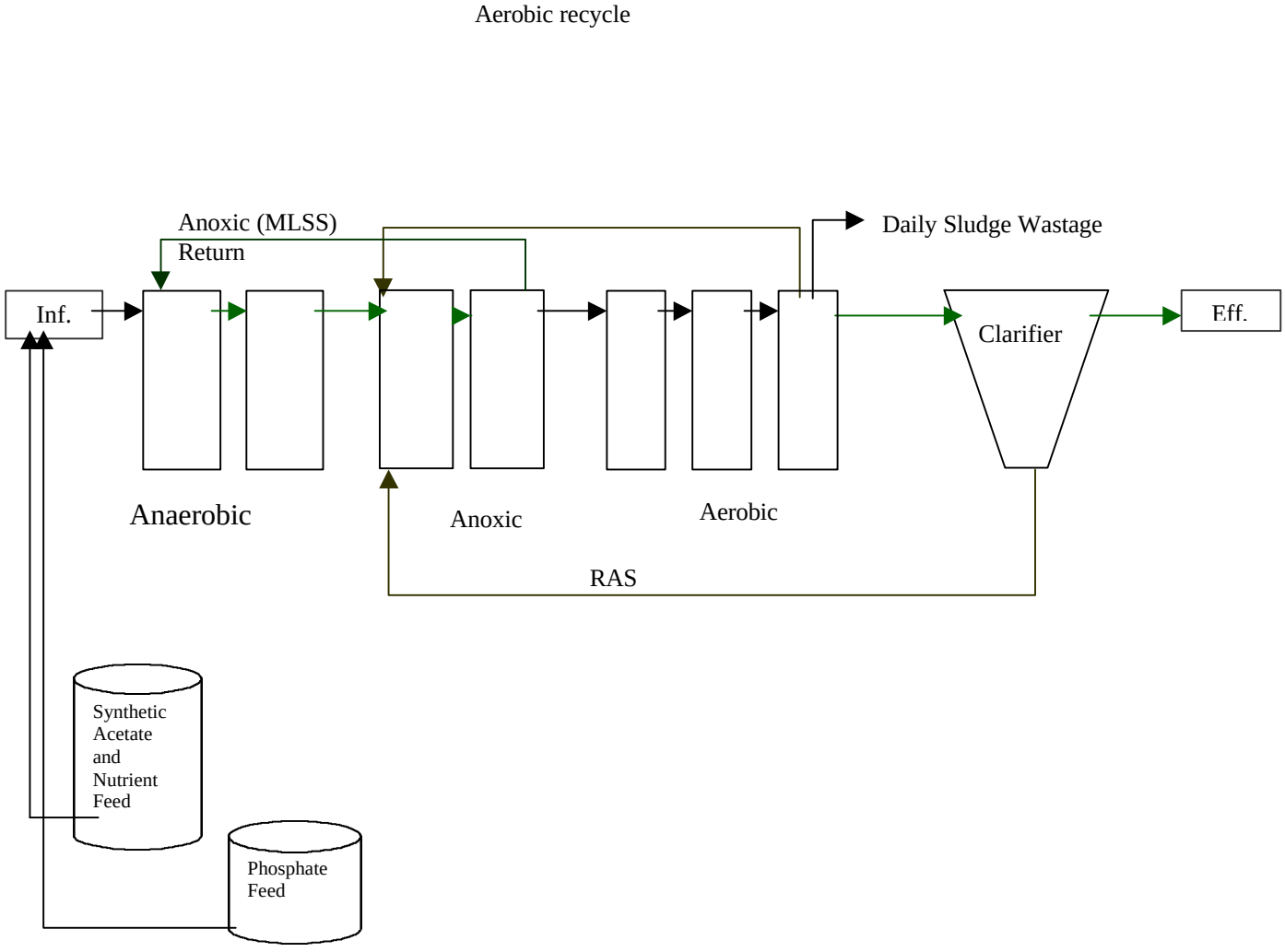



Figure 1. Flow schematic of the lab-scale UCT systems.

Table 1. The sampling parameters and their locations

| Parameter | Sample Location | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CI | AN1 | AN2 | AX1 | AX2 | AE1 | AE2 | AE3 | Effluent | RAS Line |
| Acetate | x | x | X | x | x | x | x | x | x | x |
| Ammonia N | x | x | X | x | x | x | x | x | x | x |
| COD | x | x | X | x | x | x | x | x | x | x |
| DO | x | | | | | x | x | x | | |
| Glycogen | | x | X | x | x | x | x | x | | x |
| MLVSS, MLSS | | x | X | x | x | x | x | x | x | x |
| Nitrite, nitrate N | x | x | X | x | x | x | x | x | x | x |
| OUR | | | | | | x | x | x | | |
| pH | x | | X | | x | x | x | x | | x |
| PHB, PHV | | x | X | x | x | x | x | x | x | x |
| Soluble P | x | x | X | x | x | x | x | x | x | x |
| TKN | x | | | | | | | | x | x |
| Total P | | x | X | x | x | x | x | x | | x |

CI: Combined influent
AN: Anaerobic reactor
AX: Anoxic reactor
AE: Aerobic reactor
RAS: Return activated sludge

**c. Analytical Methods**

Mixed liquor solids (MLSS, MLVSS) and COD measurements were analyzed according to APHA (1995) as defined in sections 2540D, 2540E, and 5220C, respectively. Anions (acetate, PO4-P, $NO_2$-N and $NO_3$-N) were analyzed by a Dionex 2010I ion chromatograph with an IONPAC AS40A-SC column. Cations including $NH_4$-N were analyzed using a Dionex 120 ion chromatograph furnished with an electrochemical conductivity detector.

*PHB and PHV analysis*

The mixed liquor samples from designated reactors were placed into 15 mL plastic centrifuge tubes. They were immediately centrifuged at 10,000 rpm for about 10 minutes and clear supernatant was poured off. The tubes were immersed into liquid nitrogen for quick freezing. Frozen samples were then lyophilized and 25 to 50 mg homogeneous samples were weighed out into a 5 mL high pressure Wheaton "V-vial". A minimum of 8 external PHA standards (0 to 20 mg) were prepared. Two mL of methanol-sulfuric acid-benzoic acid solution was added to each vial. Benzoic acid served as an internal standard. Benzoic acid solution was prepared freshly by solubilizing 50 mg of benzoic acid into 100 mL of 3% sulfuric acid in a methanol solution (v/v). Before vials were tightly sealed, 2 mL of chloroform was added to each vial. The vials were then incubated in a drying oven at 100$^{o}$C for 3.5 hours. Following digestion, the vials were cooled to room temperature and 1 mL of distilled water was added to each vial. The vials were shaken about 10 minutes to separate methanol and chloroform layers. A sufficient volume of the chloroform phase (1 mL) was transferred into GC autosampler vials by Pasteur pipettes. The samples were injected automatically to a GC with a Reoplex 400 Chromosorb GAW column and a FID detector. The oven, injector and detector temperatures were set to 130, 160 and 200$^{o}$C, respectively.

*Glycogen analysis*

Glycogen measurements were performed according to a modification of the method outlined by Gerhardt *et al.* (1995). Glycogen is resistant to hydrolysis in alkali environment, but it is readily soluble in water and acid, and insoluble in ethanol (ASM Manual, 1981). Lyophilized biomass samples were weighed into screw-capped centrifuge tubes, and 1 to 2 mL of 30% wt/vol KOH was added depending on the quantity of the biomass solids. The samples were digested in a 100ºC drying oven for 3 hours to break down the cells and to solubilize the glycogen homopolymer. Following digestion, the samples were taken out and left to cool to room temperature. Then, to each tube 3mL water and 8 mL ice-cold ethanol was added to precipitate the glycogen. The opaque solution formed after addition of ethanol was centrifuged for 15 min at 10 000 rpm. The pellet was washed with 60% (vol/vol) ice-cold ethanol. The remaining precipitate was dried at 60ºC. To breakdown and solubilize glycogen, dried solids were further digested at 100ºC in 3 mL of 6N HCl for 1 hour. Measurement of soluble glucose was done using the Phenol method (Gerhardt *et al.*, 1995). The straw yellow color developed was measured to determine the glucose in the samples. The absorbance of each sample and the standards was read with a Spectronic 20 spectrometer set at 490 nm. Glycogen content was then reported as mg glucose per mg of dry solids.

*Electron microscopy analysis*

Electron microscopy samples taken from the anaerobic and aerobic stages of the systems operated at 20, 10 and 5$^{o}$C were prefixed using 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.05% sodium cacodylate buffer. The samples were immediately delivered to the morphology lab of the Veterinary Medicine Department of Virginia Tech. The post-fixation of the samples was performed using a 1-2% solution of buffered osmium tetroxide ($OsO_4$). The osmium tetroxide stains specific components of the cell, making them more electron-dense. Graded ethanol solutions with increasing concentrations (15%, 30%, 50%, 70% and 95%) were applied to the specimen during dehydration. Following dehydration samples were infiltrated by a 50:50 solution of propylene oxide. Samples then were stained with uranyl acetate and lead citrate. Samples

were embedded in freshly prepared Poly/Bed 812in flat embedding molds. Microtome, glass and diamond knives were used during cutting and sectioning and they produced very thin sections (800-900 $A^{o}$). The micro-graphs were visualized using a Jeol-100CX II transmission electron microscope operating at 75 kV accelerating voltage.

*Data analysis and statistical comparison*

Data quality was determined according to Standard Methods (APHA 1995). Duplicate samples were taken for PHA, glycogen and total P measurements. The standard deviations of the mean values were included in tables and figures.

## RESULTS

The results of the short-term temperature effects are shown in Figure 2. 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, respectively, which is in good agreement with the results of previous short-term studies (Brdjanovic *et al*., 1997 and 1998; Beatons *et al.*, 1999).

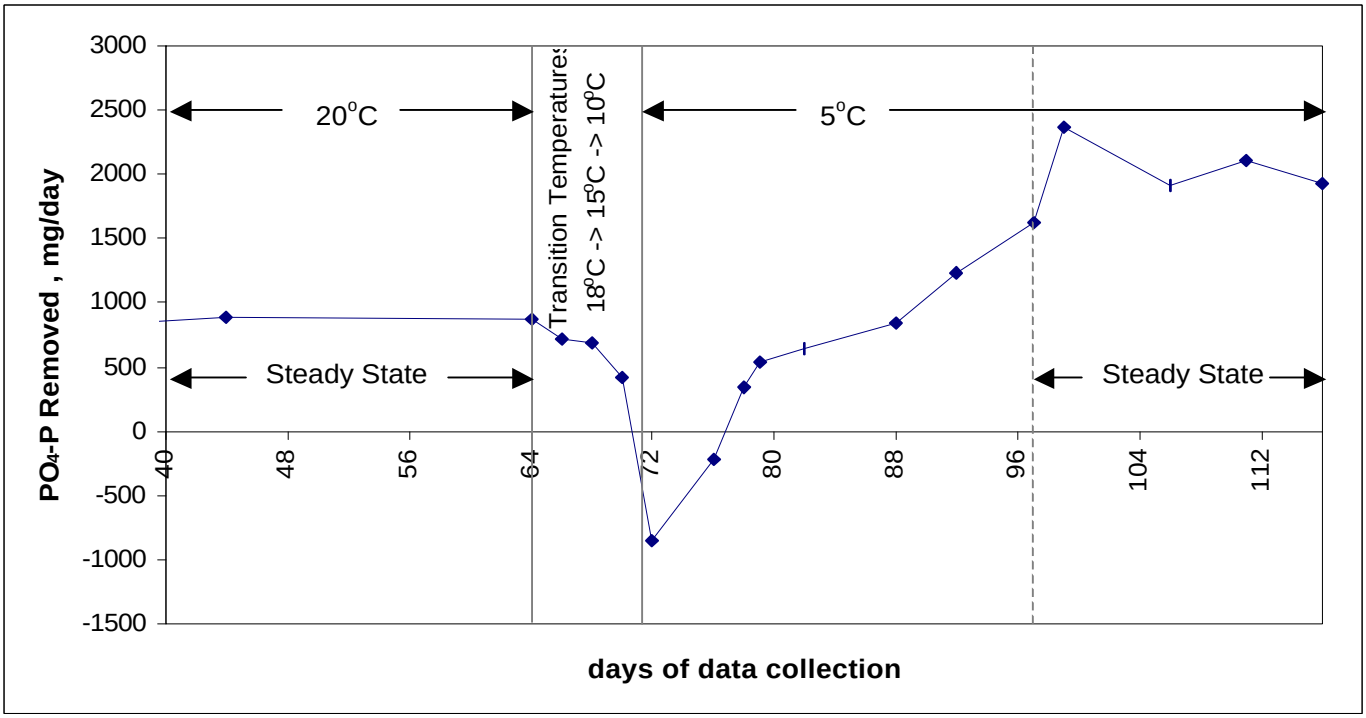



Figure 2. Change in net $PO_4$-P removal throughout the temperature study. Transition period covers the period of stepwise temperature decrease from 20°C to 18, 15, 10 and 5°C at 2-day steps.

Phosphorus mass balance calculations (Table 2) showed that net P removal was reduced by 57% at 10$^{o}$C, and no P removal was initially observed at 5$^{o}$C. In addition, acetate utilization was incomplete in the non-oxic reactors at 5$^{o}$C despite the presence of greater anaerobic times in the anoxic stages 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$^{o}$C (Figure 2). The influent phosphorus had to be increased as the system acclimated to 5$^{o}$C to keep the effluent phosphorus concentration greater than 1 mg/L, so that the P removal capacity could be determined. The influent concentration had to be increased to more than 60 mg/L by the time that steady state acclimation was complete. The average effluent P concentrations at steady state were 1.1 and 5.7 mg/L for system 1 and 2, respectively (Figure 3). During this period no breakthrough of acetate to the aerobic stage was observed (Figure 4). Steady state performance was lost thereafter, however, because of a big loss of biomass from the clarifier caused by excessive filamentous organism growth. The system was re-seeded and operated until steady state was reestablished at 5$^{o}$C. The system performances were similar and average P removals were 62 and 67 mg/L for the initial and later steady state periods, respectively. The later system performance is not included in this chapter but it will be presented in Chapter 4.

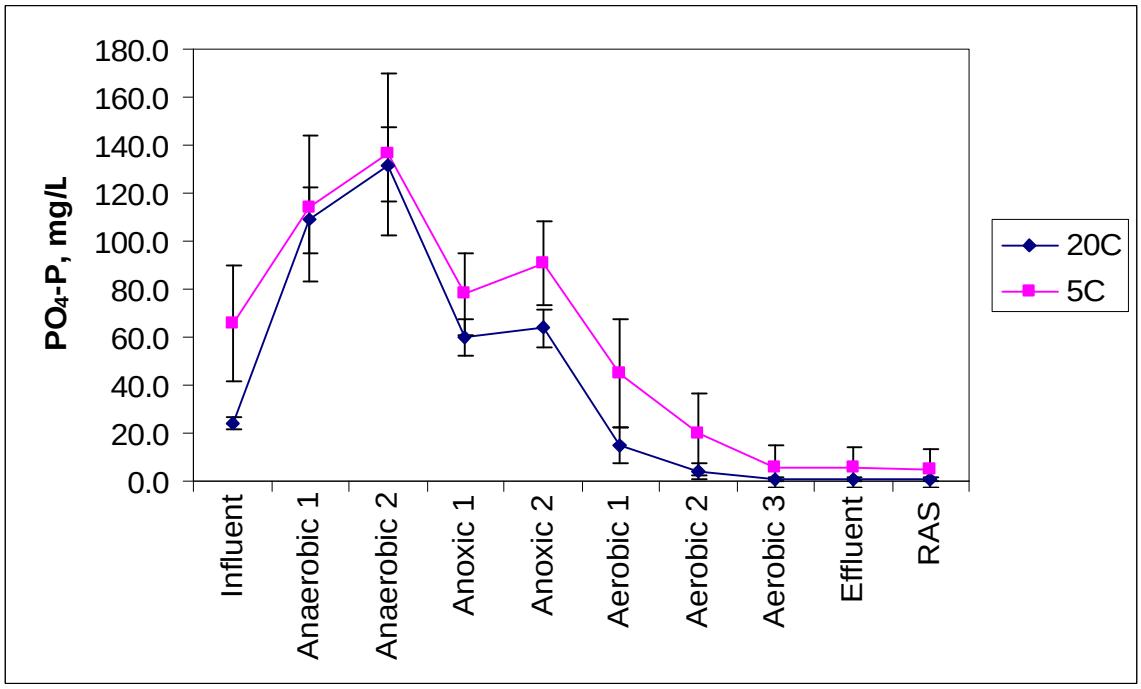



Figure 3. Comparison of steady state P profiles at 20 and 5$^{o}$C.

Table 2. Mass balance of P through the anaerobic, anoxic and aerobic stages.

| **Date** | **Temp.** | 7-day SRT | Anaerobic | Anoxic | Total | Aerobic | Anoxic | Total | Net P Removed |
|---|---|---|---|---|---|---|---|---|---|
| | **°C** | d | P-Relased mg/d | P-released mg/d | P-released mg/d | P-uptake mg/d | P-Uptake mg/d | P-uptake mg/d | mg/d |
| 11/12/00 | 20* | 9.9 | 8972 | 579 | 9551 | 7925 | 2499 | 10424 | 873 |
| 11/29/00 | 20* | 9.92 | 7185 | 392 | 7577 | 6282 | 1942 | 8224 | 647 |
| 12/04/00 | 20* | 9.96 | 7017 | 485 | 7502 | 6820 | 1414 | 8234 | 732 |
| 12/26/00 | 20* | 9.78 | 7140 | 587 | 7727 | 7797 | 821 | 8618 | 891 |
| 01/15/01 | 20* | 9.03 | 6342 | 740 | 7082 | 6966 | 993 | 7959 | 877 |
| 01/17/01 | 18 | 9.03 | 5392 | 965 | 6357 | 6905 | 167 | 7072 | 715 |
| 01/19/01 | 15 | 9.36 | 5078 | 749 | 5827 | 6301 | 220 | 6521 | 694 |
| 01/21/01 | 10 | 10.2 | 4870 | 1284 | 6154 | 6528 | 37 | 6565 | 411 |
| 01/23/01 | 5 | 12 | 1905 | 3166 | 5071 | 4214 | 0 | 4214 | -857 |
| 01/27/01 | 5 | 18.8 | 1631 | 3552 | 5183 | 4965 | 0 | 4965 | -218 |
| 01/29/01 | 5 | 23.7 | 1245 | 4500 | 5745 | 6092 | 0 | 6092 | 347 |
| 01/30/01 | 5 | 25.3 | 1819 | 3530 | 5349 | 5894 | 0 | 5894 | 545 |
| 02/01/01 | 5 | 25 | 1848 | 3240 | 5088 | 5728 | 0 | 5728 | 640 |
| 02/08/01 | 5 | 38.9 | 1903 | 5176 | 7079 | 7920 | 0 | 7920 | 841 |
| 02/12/01 | 5* | 23.8 | 3464 | 5416 | 8880 | 10116 | 0 | 10116 | 1236 |
| 02/17/01 | 5* | 16.9 | 3386 | 3666 | 7052 | 8674 | 0 | 8674 | 1622 |
| 02/19/01 | 5* | 16.5 | 4718 | 3087 | 7805 | 10176 | 0 | 10176 | 2371 |
| 02/26/01 | 5* | 16 | 5355 | 3778 | 9133 | 11042 | 0 | 11042 | 1909 |
| 03/03/01 | 5* | 16.1 | 5718 | 3556 | 9274 | 11382 | 0 | 11382 | 2108 |
| 03/08/01 | 5* | 15.9 | 6093 | 4305 | 10398 | 12327 | 0 | 12327 | 1929 |

* EBPR sludge acclimated to temperature

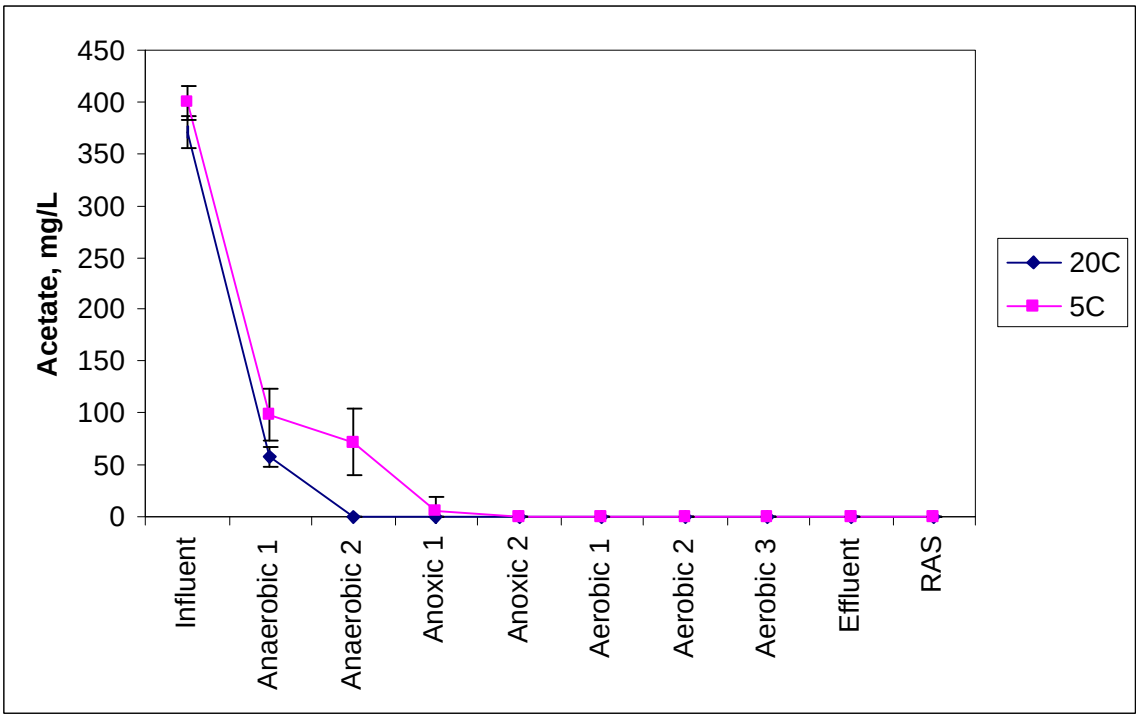



Figure 4. Acetate utilization during steady state operation at 20 and 5°C.

PHA mass balance (Table 3) showed that PHA production in the non-oxic reactors decreased by 50% and 62% at 10 and 5°C, respectively, when compared to that at 20°C. Glycogen utilization (Table 4) in the anaerobic zone and production in the aerobic zone decreased significantly as the temperature decreased. After steady state was reached at 5°C, 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 performance at 20°C.

Carbon balance calculations (Figure 5) 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 metabolism at 5°C (362±73.4 mg/d) compared to 20°C (195±29.2 mg/d). The poly-P content of the sludge greatly increased and was as 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.

Table 3. Mass balance of PHA through the 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 Upt. 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 |

Table 4. Mass balance of Glycogen through the 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 |

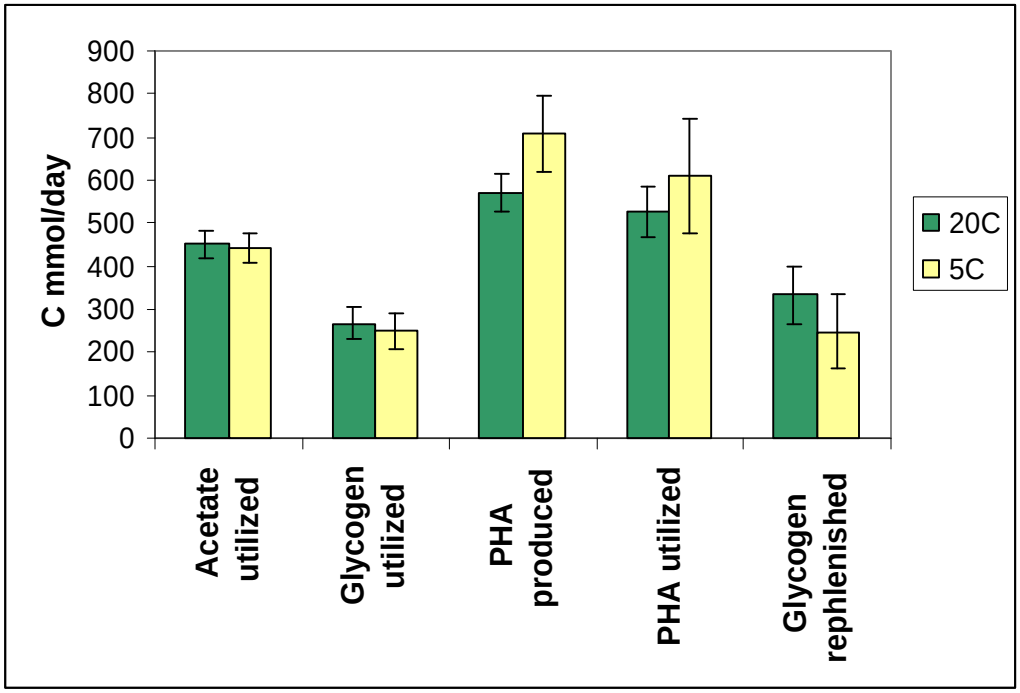



Figure 5. Carbon mass balance of acetate, PHA and glycogen.

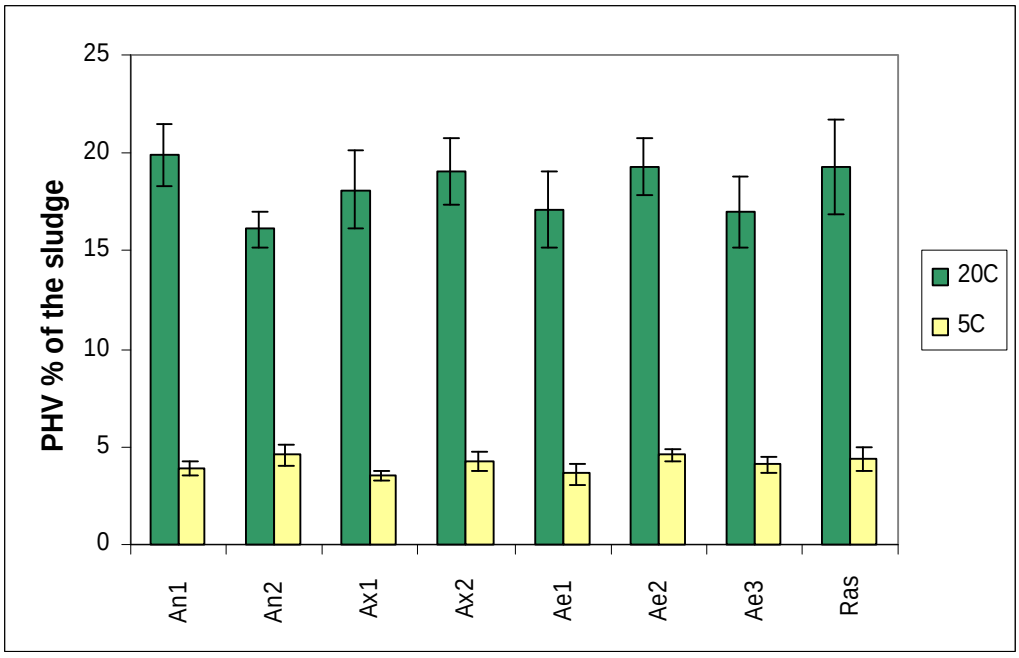



Figure 6. PHV content of the sludge under steady state conditions at 20 and 5$^{o}$C.

The steady state samples from the last aerobic section of the UCT system were Neisser positive at both 20 and 5$^{o}$C. No distinct coccoid cells with tetrad arrangement were observed under either temperature conditions suggesting an absence of G bacteria or GAOs. The individual cells in each grid of the TEM samples obtained from the aerobic sections during steady state operation at 20 and 5$^{o}$C showed a striking difference: the 5$^{o}$C sample had a much greater poly-P accumulation compared to those observed at 20$^{o}$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 were similar with respect to their internal storage products.

*Morhphological characteristic of EBPR sludge under both temperature conditions*

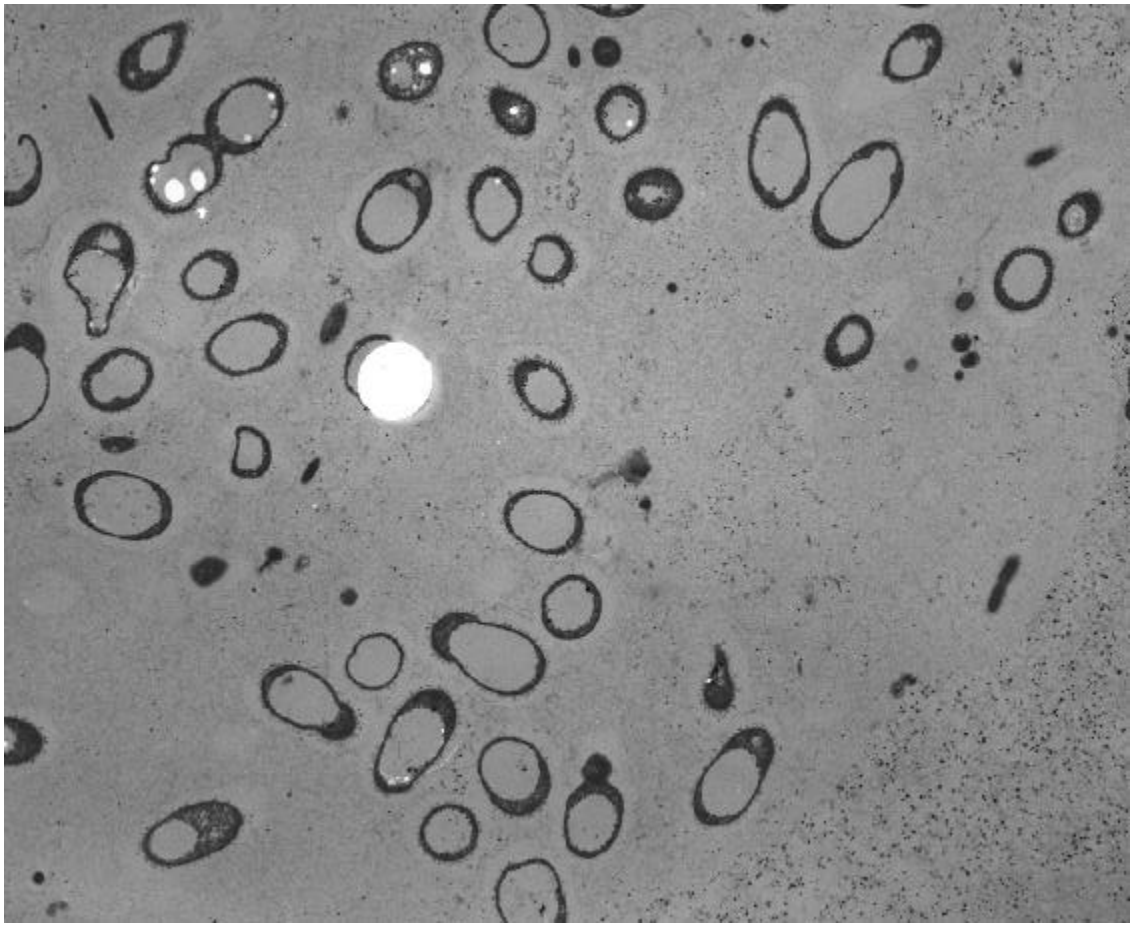
The steady state samples from the last aerobic section of the UCT system were Neisser positive at 20 and 5$^{o}$C. No distinct coccoid cells with tetrad arrangement were observed using the light microscope under either temperature conditions. This strongly suggests the absence of G bacteria or GAOs. A better comparison of bacterial community was performed using a transmission electron microscope (TEM). The individual cells in each grid of the TEM samples obtained from the aerobic, anaerobic and anoxic sections of the UCT systems during steady state operation at 20 and 5$^{o}$C showed striking differences:

- The 5$^{o}$C samples had much greater poly-P accumulation compared to the 20$^{o}$C samples. The poly-P granules were extremely large and they were removed during sectioning. Another striking feature of the 5$^{o}$C samples compared to the 20$^{o}$C samples is that individual cells showed greater similarity with respect to poly-P and glycogen accumulation (Micrograph 1 through 6). In addition, the size and the shape of each cell were nearly identical suggesting the presence of a single population.

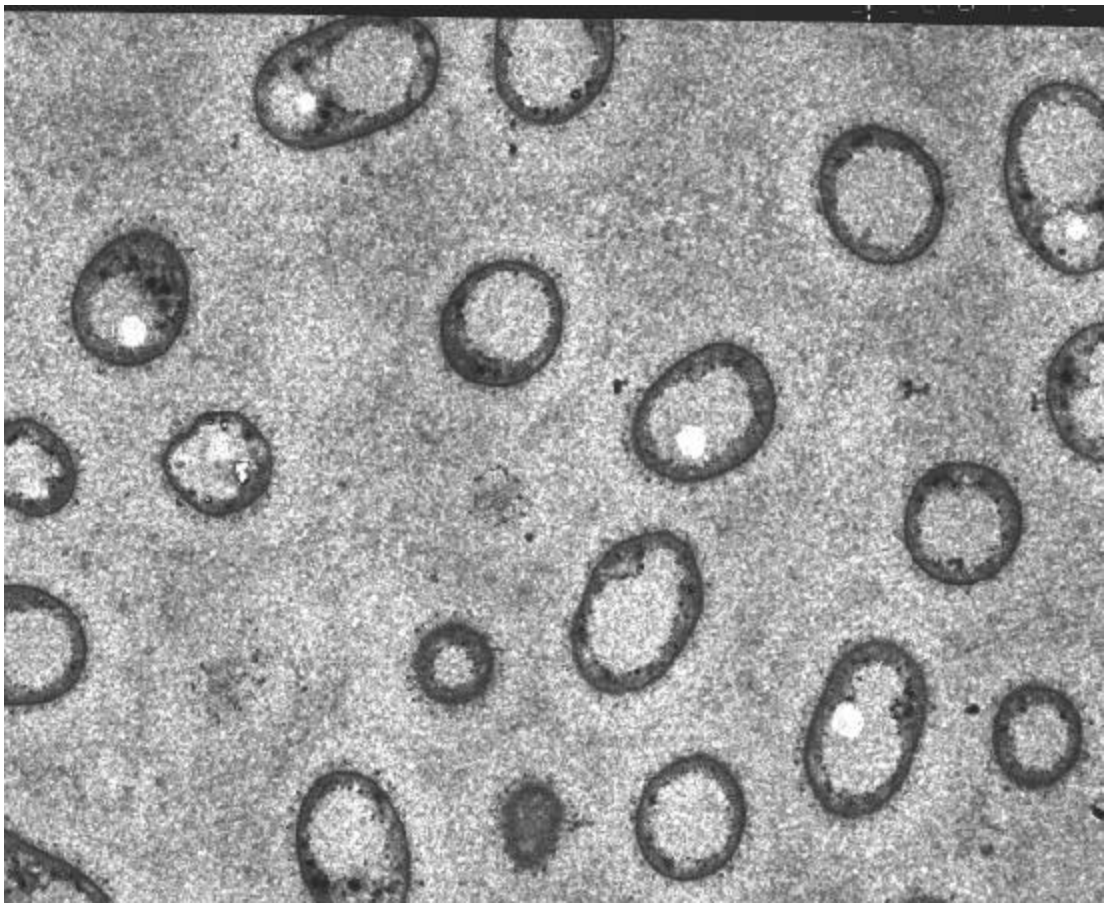
- The bacterial community was found to be more diverse at 20$^{o}$C and at least two different major groups (one with great poly-P accumulation, the other with very little or no poly-P accumulation) of bacterial cells were identified (Micrographs 7 and 8).

The cluster or tetrad formation of bacterial cells was never observed at 5$^{o}$C. This formation however was observed in 20$^{o}$C samples (Micrographs 5 and 6). In one grid of a sample taken from the first anoxic reactor of the UCT system, bacterial cells with some glycogen storage ability formed clusters and they stored PHA without the presence of any poly-P inclusions. They were initially thought to be GAOs. However, the observed amount of glycogen storage was less than that observed at 5$^{o}$C. In addition, micrographs of samples taken from the 20$^{o}$C aerobic stage showed similar bacterial cells with no poly-P storage ability. These individual cells stored very insignificant amounts of glycogen, and the main storage material was PHA. In this respect, it is questionable to classify such bacteria as glycogen accumulating organisms, but they can be classified as non-poly-P organisms. It was concluded that two major groups of bacteria compete for substrate in the anaerobic zone and these are poly-P and non-poly-P organisms.

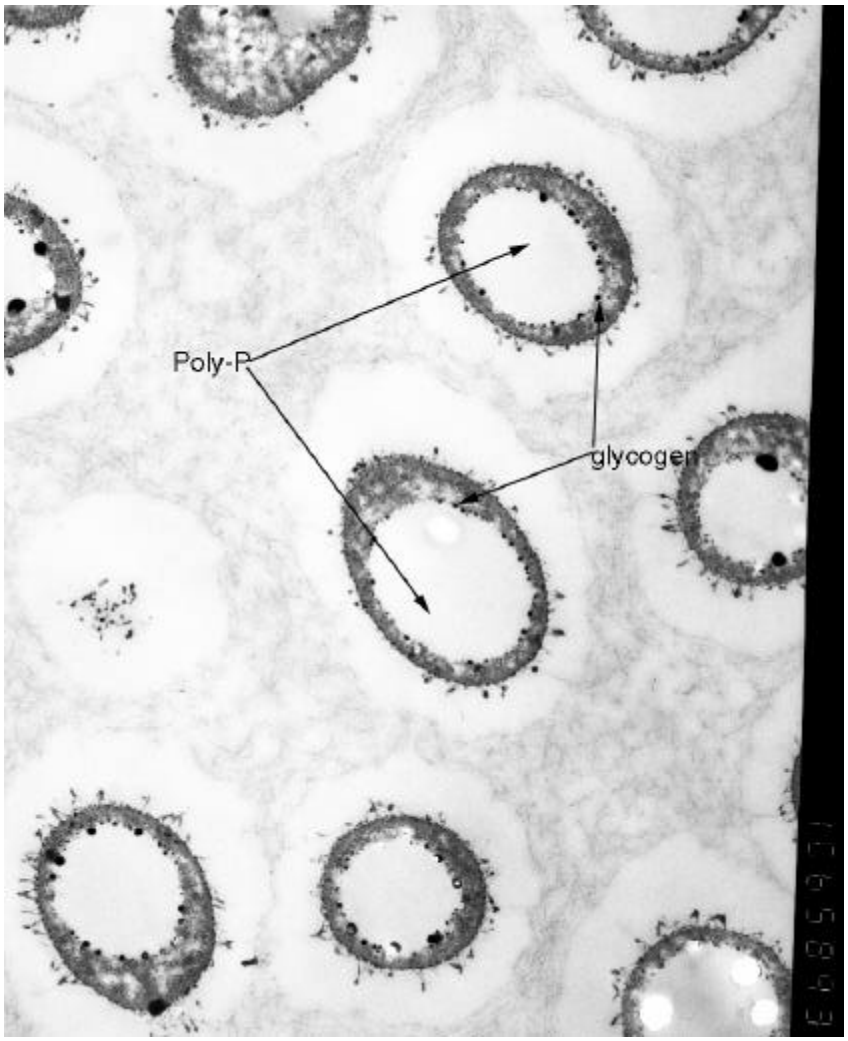
Liu *et al.* (1997) observed deterioration of EBPR performance and proliferation of GAO when they limited P in their influent (P/C=2/100). To further evaluate potential PAO and GAO competition, one of the EBPR systems was operated at 10$^{o}$C for three weeks without P addition to the influent, after the P stores in the sludge were emptied through excessive release without subsequent uptake. Analytical results indicated that PHA formation (23% as VSS) was high even in the absence of phosphorus metabolism, thereby suggesting possible GAO proliferation (Z. K. Erdal, 2002). However, electron microscopy pictures (Micrograph 15 and 16) of indivudual cells growing in the aerobic reactor of the system showed no significant glycogen accumulation. This was in very good agreement with the analytical results that showed low glycogen replenishment through the aerobic stages. The electron micrographs also clearly showed that bacterial cells did not utilize all the PHA they stored during the non-oxic stages. Despite providing favorable conditions for GAO proliferation, no evidence of GAO proliferation was found through electron microscopy and analytical results.



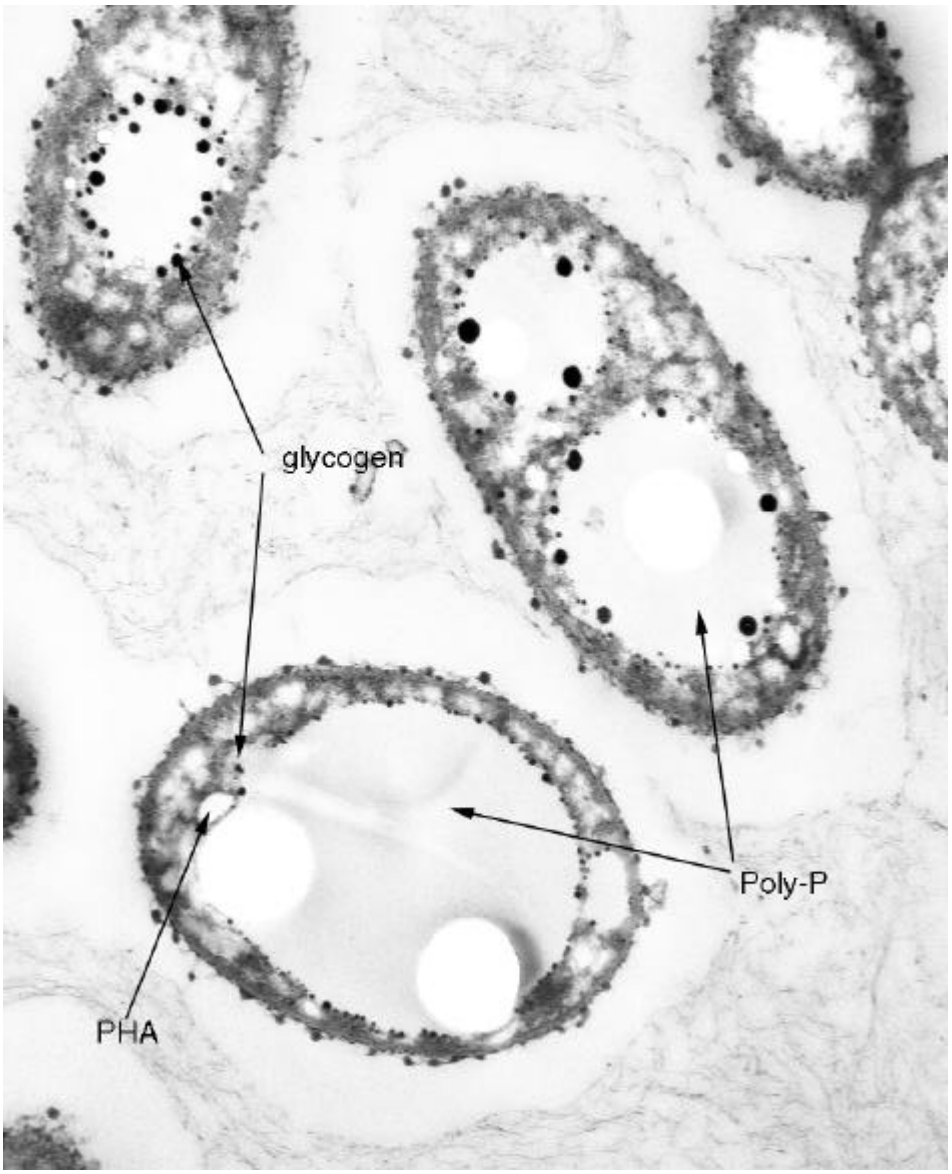
Micrograph 1. Aerobic sludge from System 2 at 5°C with 7,500X total magnification. The clear areas in the cells were occupied by polyphosphate granules before sectioning. The very white spots were caused by excess chemical during photographic processing.



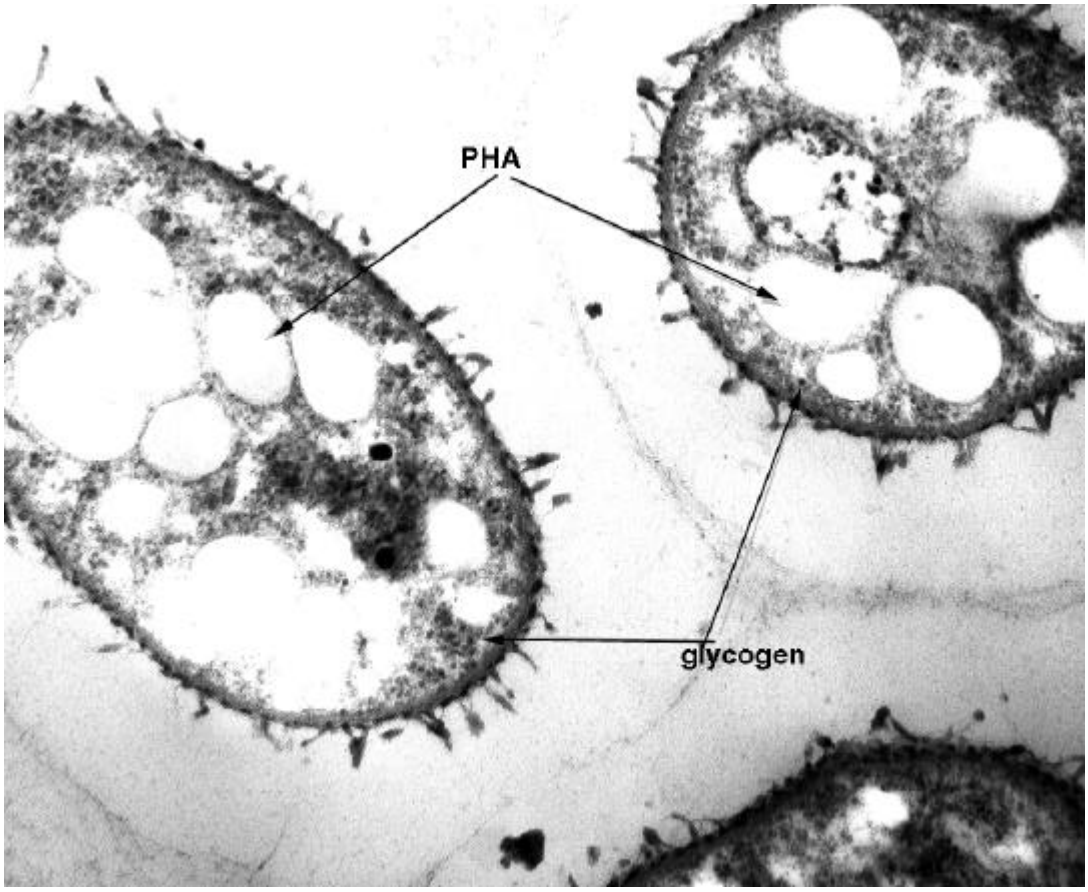
Micrograph 2. Aerobic sludge from System 2 at 5°C with 15,000X total magnification.



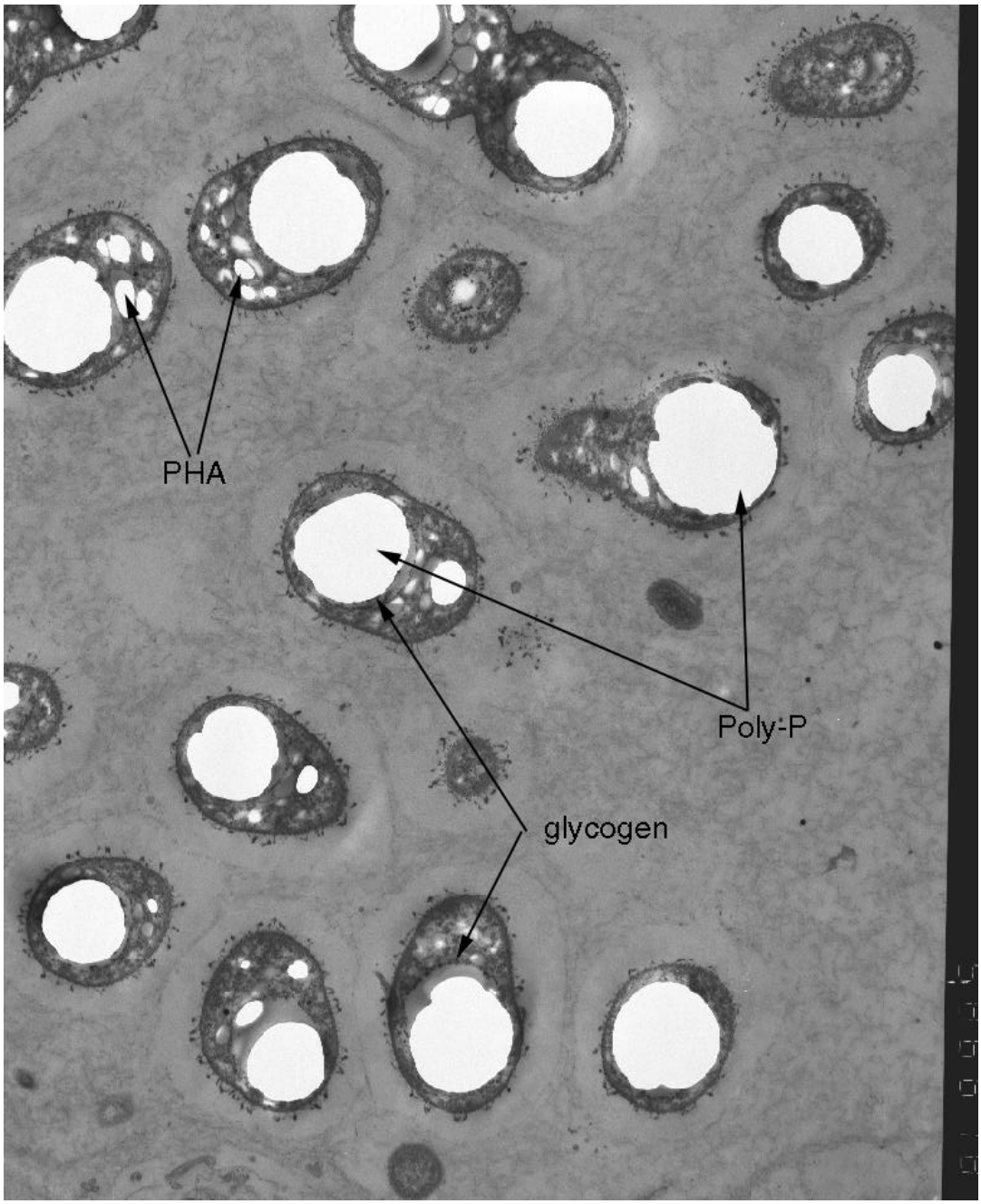

Micrograph 3. Aerobic sludge from System 2 at 5°C with 26,000X total magnification.



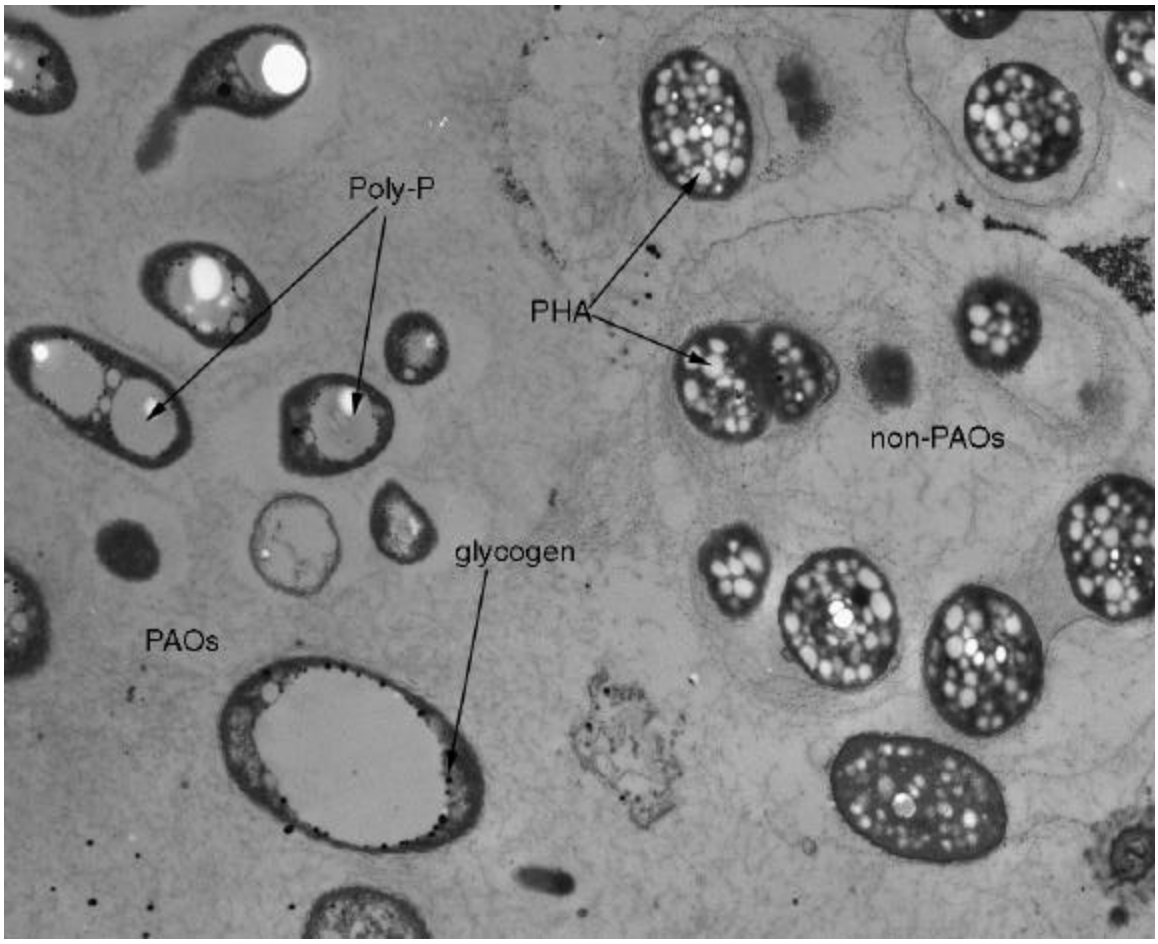

Micrograph 4. Aerobic sludge from System 2 at 5°C with 48,400X total magnification.



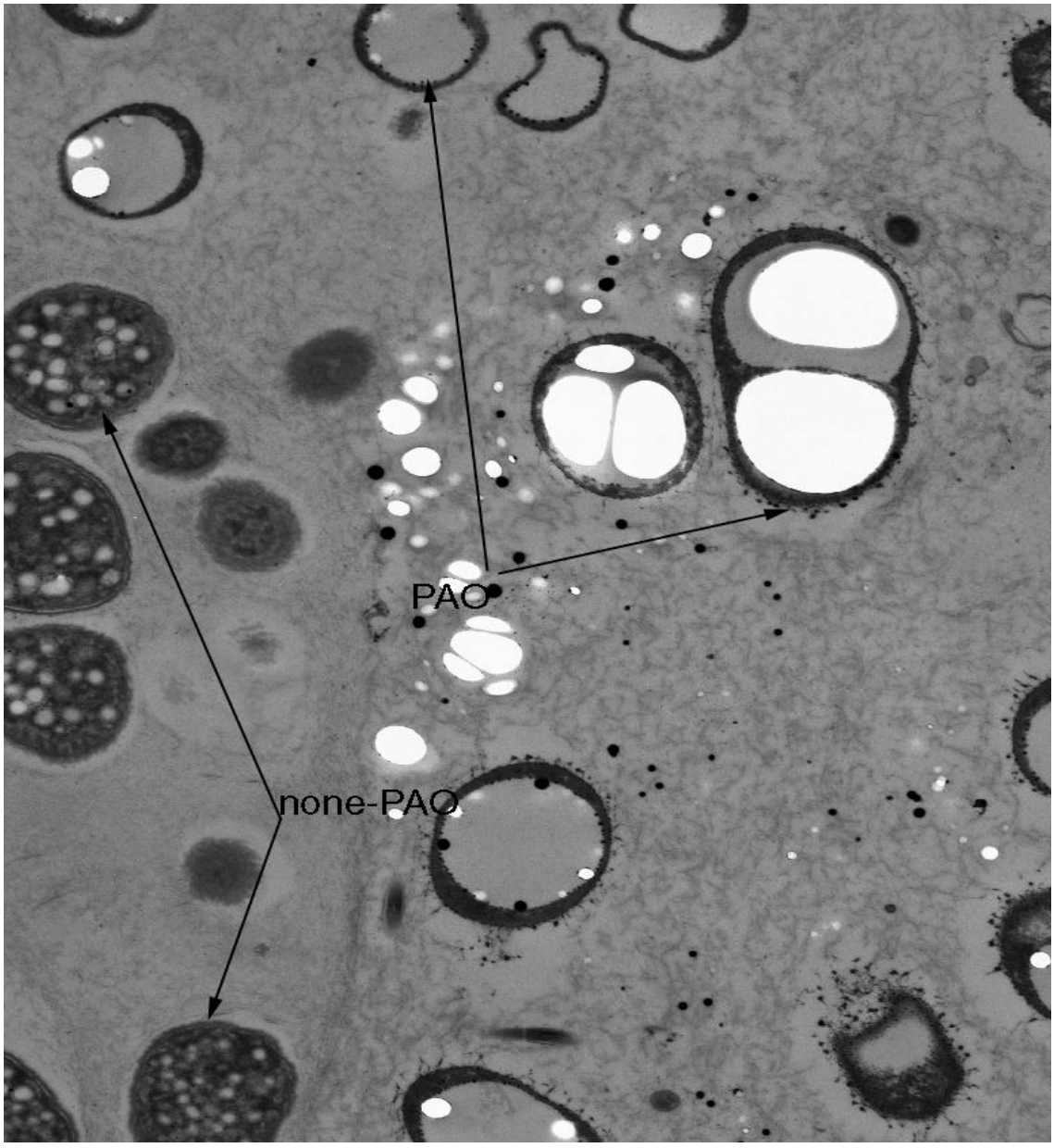

Micrograph 5. Anoxic sludge of system 2 at 5$^{o}$C with 75,400X total magnification.



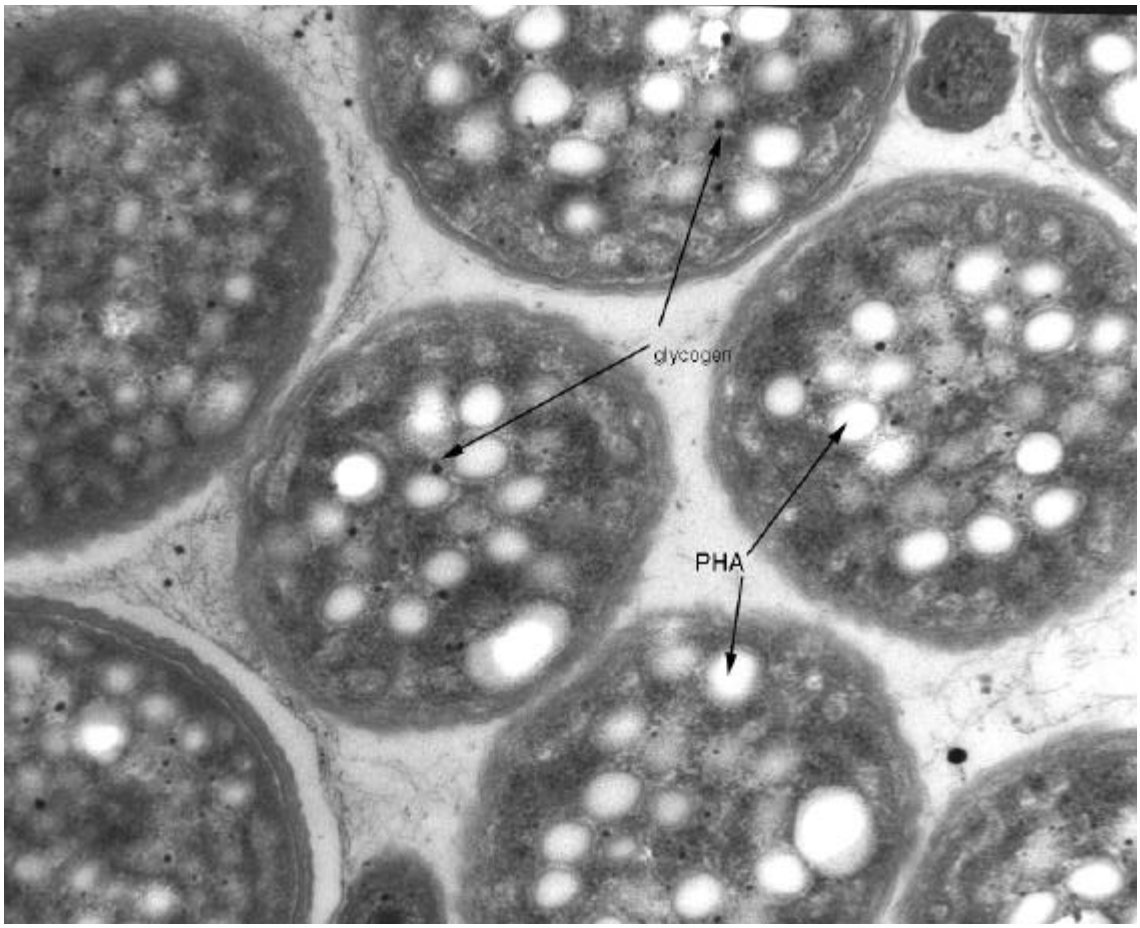

Micrograph 6. Anaerobic sludge from System 2 at 5°C with 15,000X total magnification.



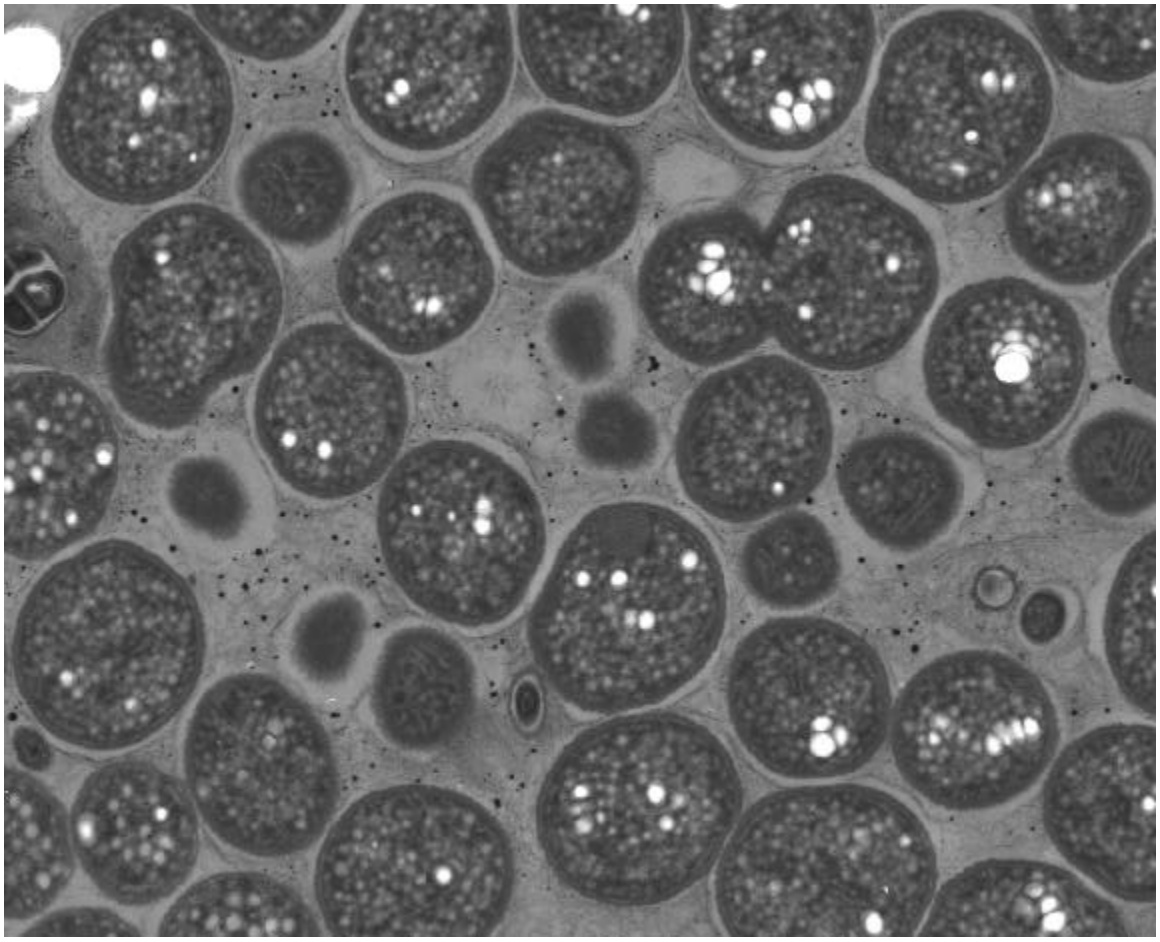

Micrograph 7. Aerobic sludge from System 1 at 20°C with 15,000X total magnification (bacterial cells with empty circular granules are PAO (in the left) and bacterial cells without poly-P accumulation are non-PAOs (in the right).



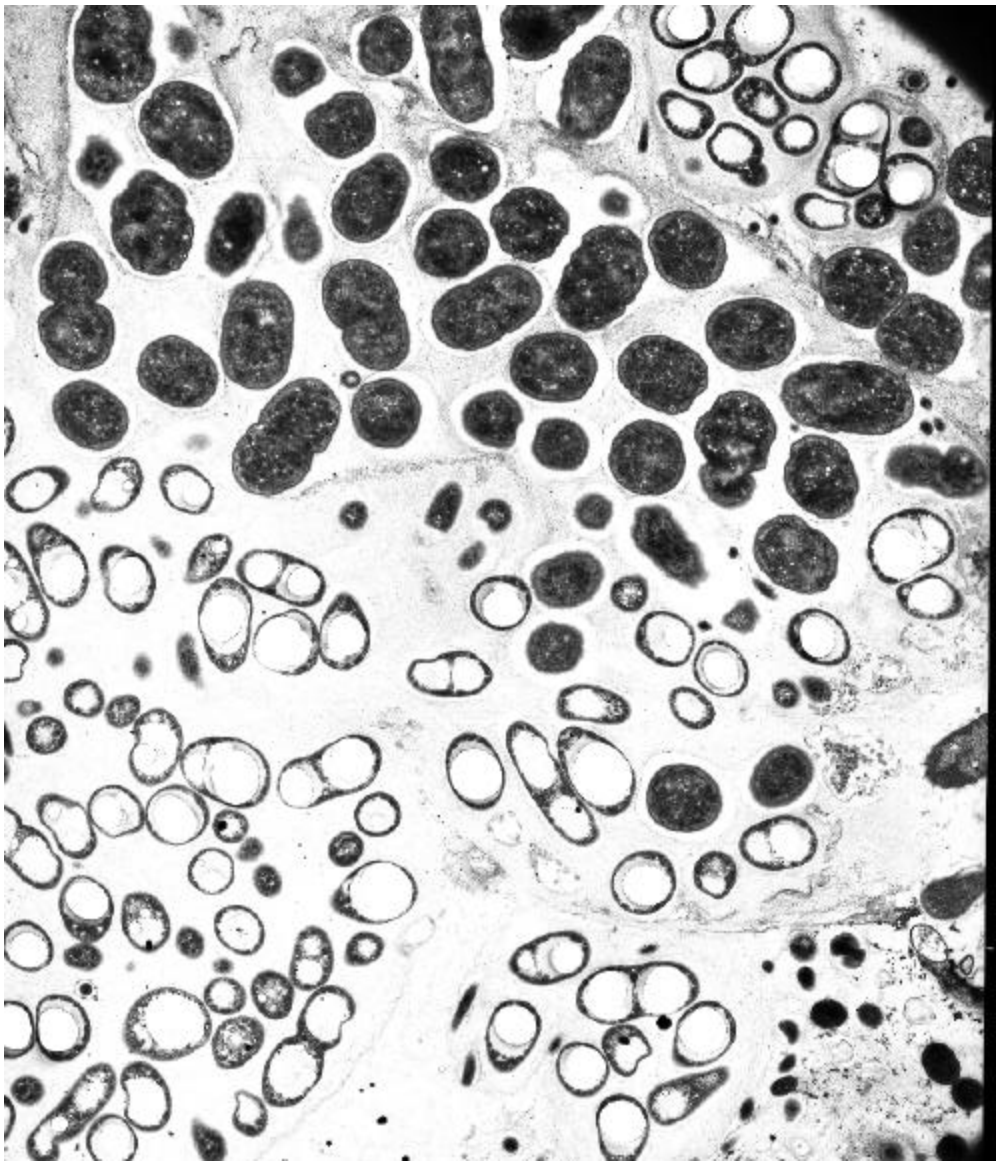

Micrograph 8. Aerobic sludge from System 1 at 20°C with 20,000X total magnification.



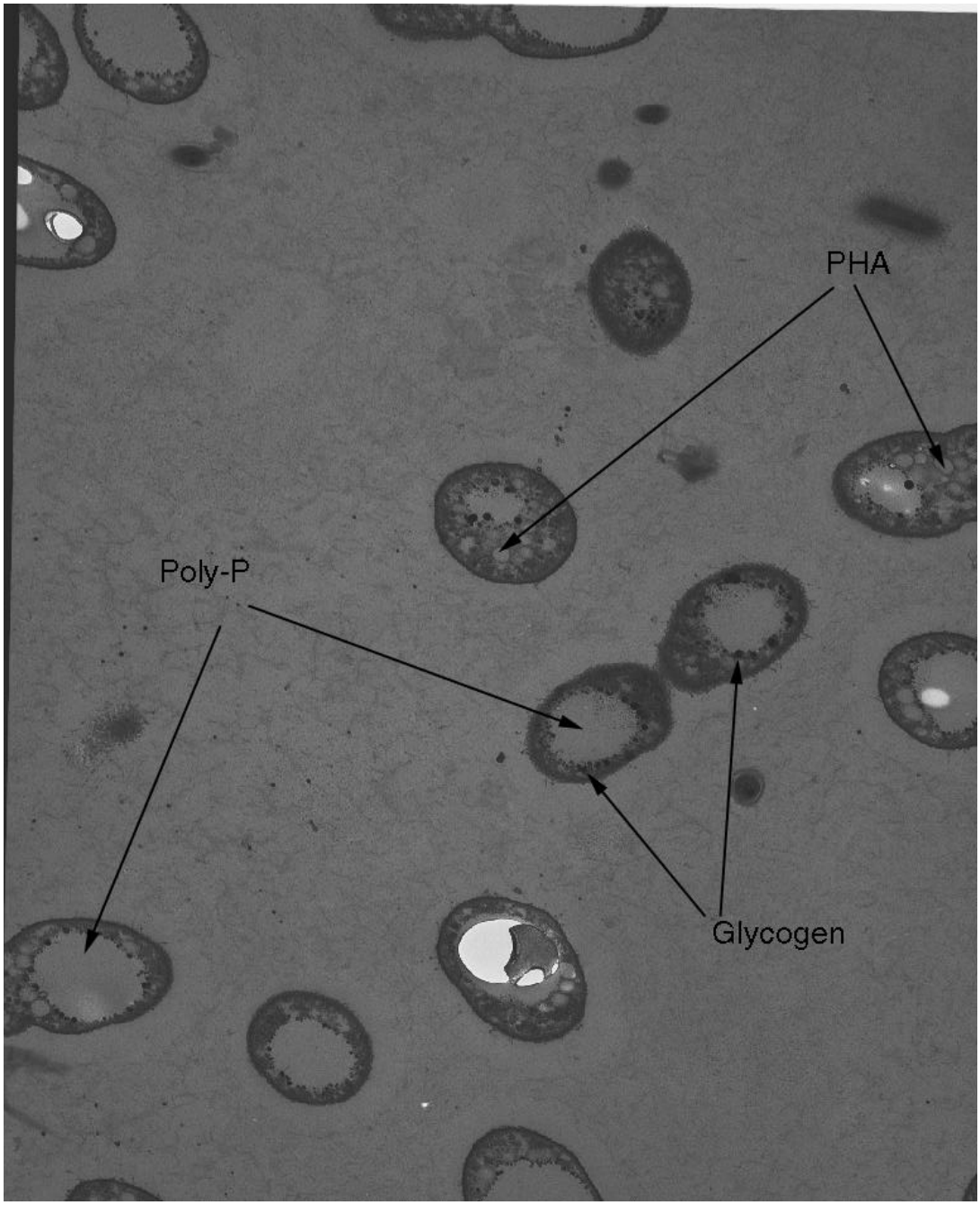

Micrograph 9. Anoxic sludge from System 1 at 20°C with 48,400X total magnification.



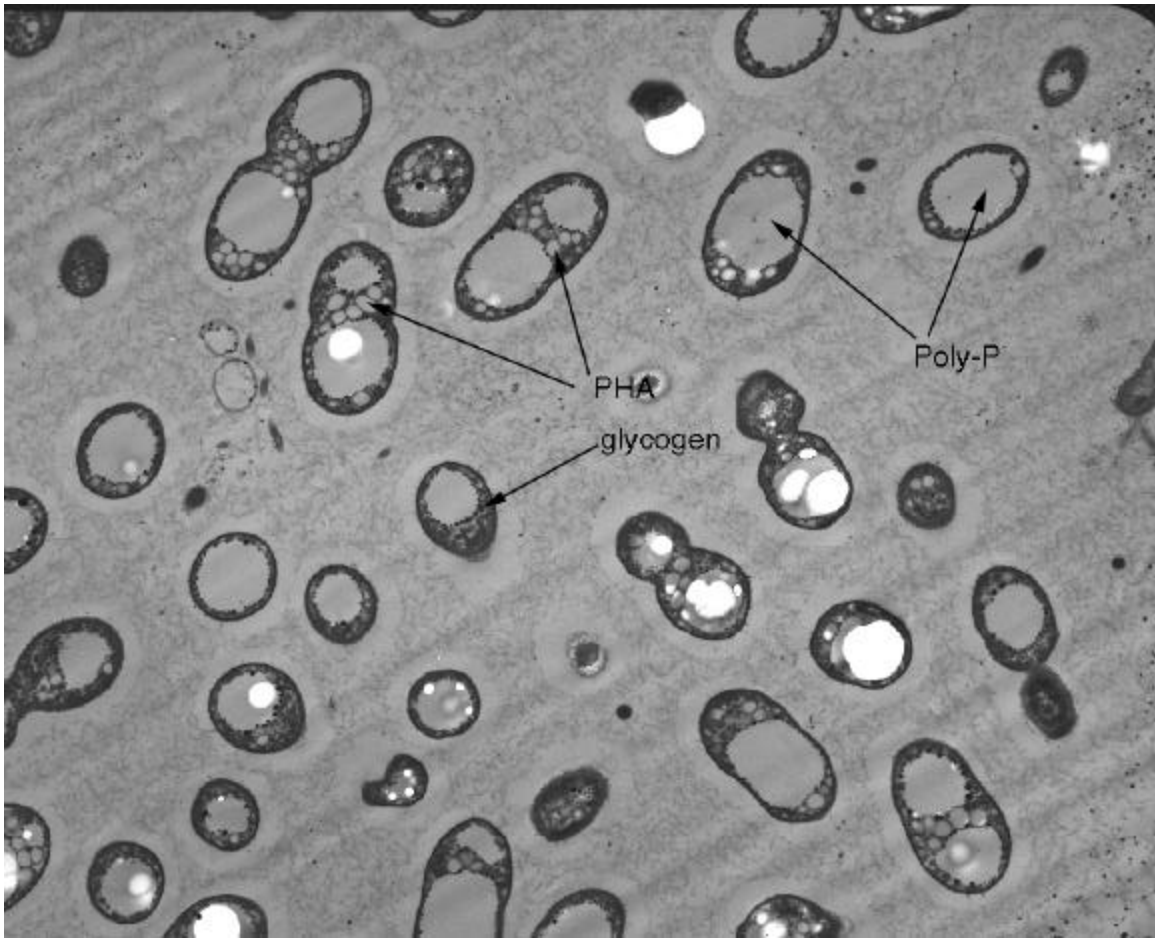
Micrograph 10. Non-PAOs in anaerobic sludge from System 1 at 20°C with 15,000X total magnification (Bacterial cells have very limited PHA (small light circles)).



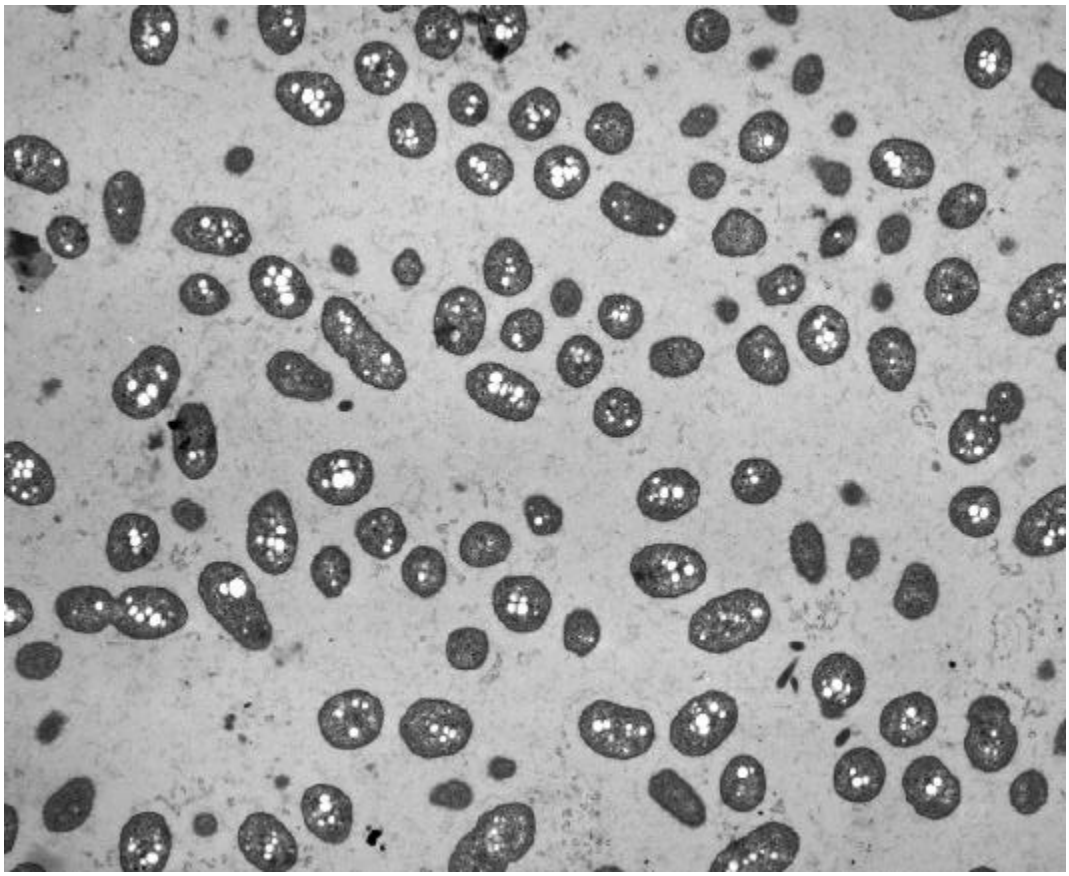
Micrograph 11. Aerobic sludge from system 1 at 10$^{o}$C with 7,500X  total magnification.



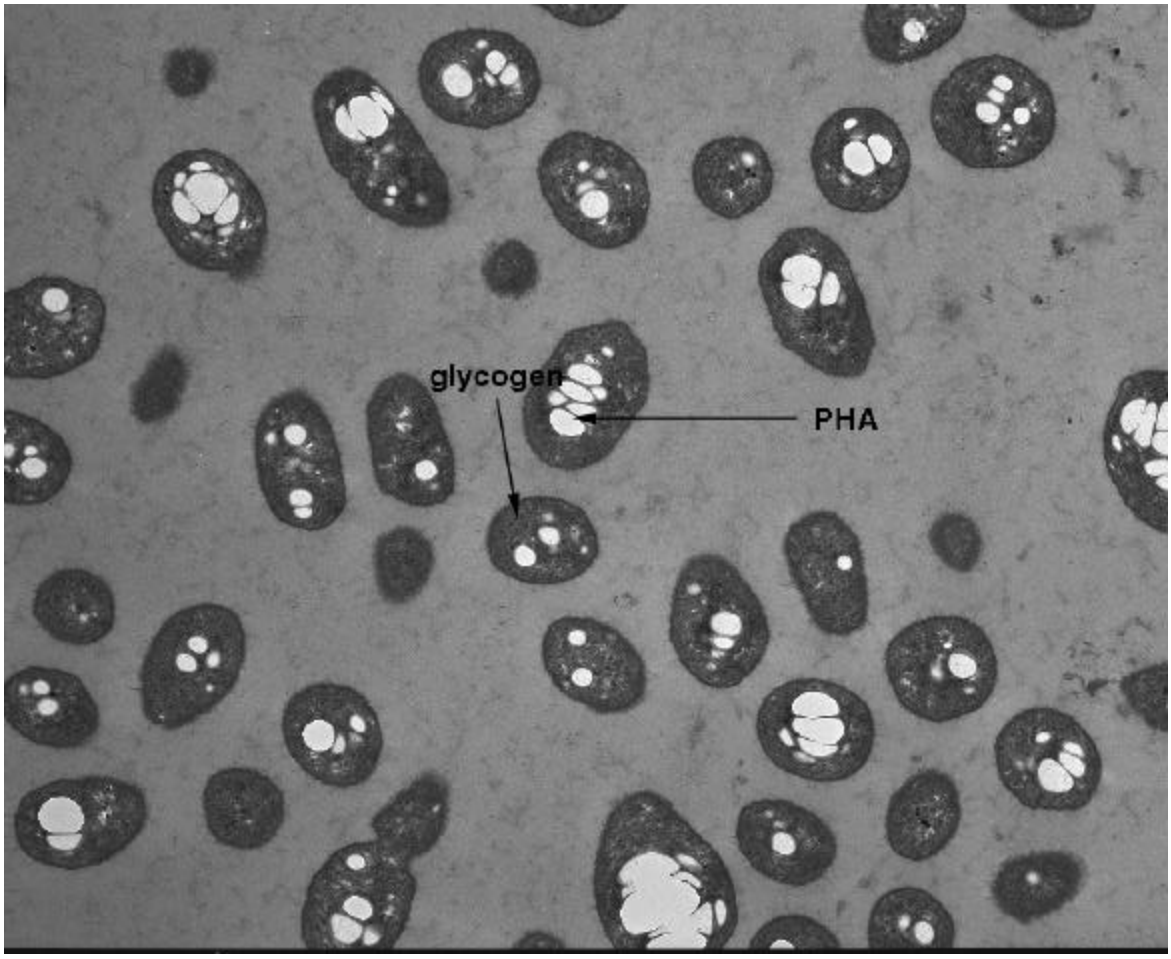

Micrograph 12. Aerobic sludge from system 1 at 10$^{o}$C with 10,500 total magnification.




Micrograph 13. Aerobic sludge from system 1 at 10$^{o}$C with 9,360X magnification.



Micrograph 14. Aerobic sludge of phosphorus limited A/O process at 10$^{o}$C with 7,300X total magnification. (Sludge has no EBPR ability; no poly-P granules were detected).




Micrograph 15. Aerobic sludge from phosphorus limited A/O process at 10$^{o}$C with 15,000X total magnification.

*The assessment of PAO and GAO competition*

The potential existence of PAO and GAO competition was evaluated using a series of anaerobic batch tests as defined in the methods section. Acetate uptake and PHA storage and glycogen utilization behavior of the activated sludge samples were determined under enriched and depleted poly-P pool conditions. The results of the first anaerobic batch test illustrated in Figure 7 showed that significant acetate was taken up even when the Poly-P reserves of the cells were nearly depleted (Flasks 1 and 2). In this condition, an insignificant amount of P was released to solution (Figure 8), as expected. Cells with an enriched Poly-P pool utilized more acetate and concomitantly released large amounts of P to solution (Flasks 3 and 4).

Because complete acetate uptake was observed in flask 3, indicating that the added acetate was not excessive, a second batch test was performed with a higher acetate concentration. The results of the second batch test are illustrated by Figures 9 through 12. These figures show that greater PHA productions can be achieved when EBPR sludge contains high amount of poly-P (enriched poly-P pool). The sludge in flask 3 produced the greatest amount of PHA accompanied with the least glycogen uptake. Even though approximately the same amount of acetate was taken up in flasks 3 and 4, a lesser PHA production was observed in flask 4. These results indicated that the UCT system operated at 20$^{o}$C (system 1) had a considerable non-PAO population. If this non-PAO portion of the sludge mainly consisted of GAOs - as was initially thought based on the high glycogen and PHV observed in system 1 - it would have stored more PHA than was observed during these batch tests. However, all the acetate taken up by the 20$^{o}$C sludge was not stored as PHA to the extent it was stored by the 5$^{o}$C sludge taken to 20$^{o}$C. These results are in good agreement with our analytical and electron microscopy results.

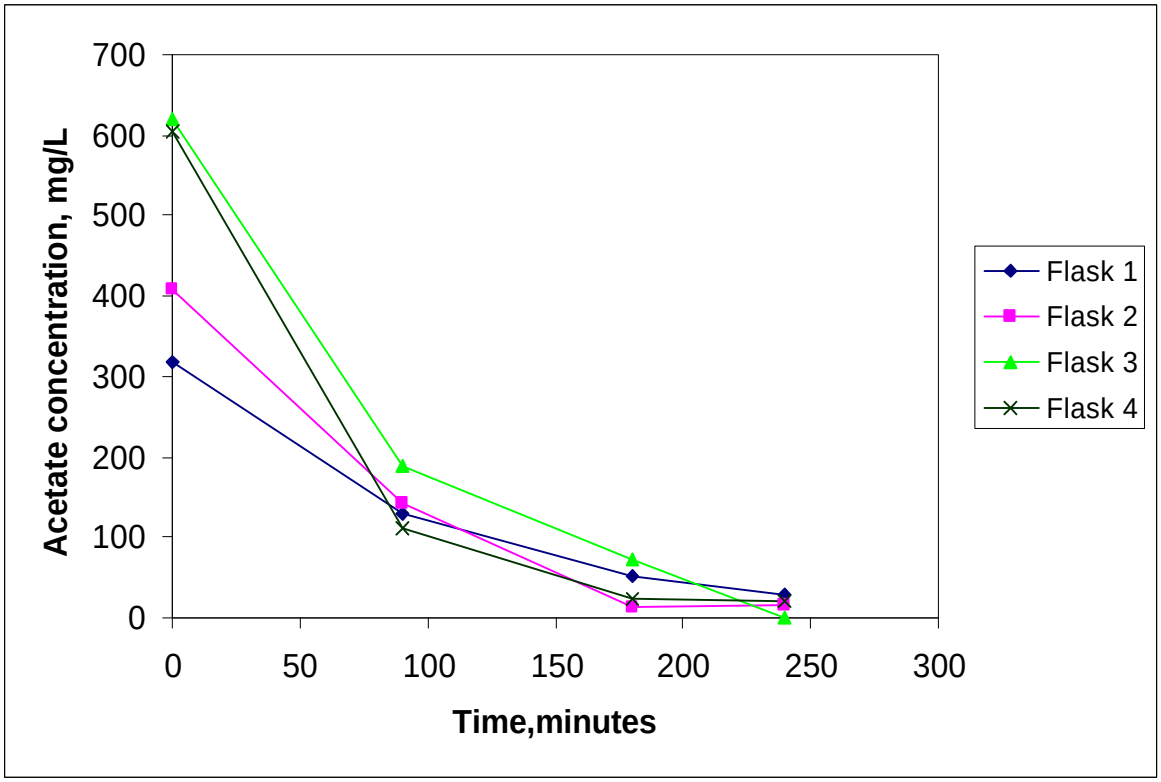



Figure 7. Batch test 1, acetate utilization with and without depleted poly-P pool at 20$^{o}$C (Flask 1: anoxic sludge taken from system 2 at 5$^{o}$C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20$^{o}$C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5$^{o}$C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20$^{o}$C with enriched poly-P pool).

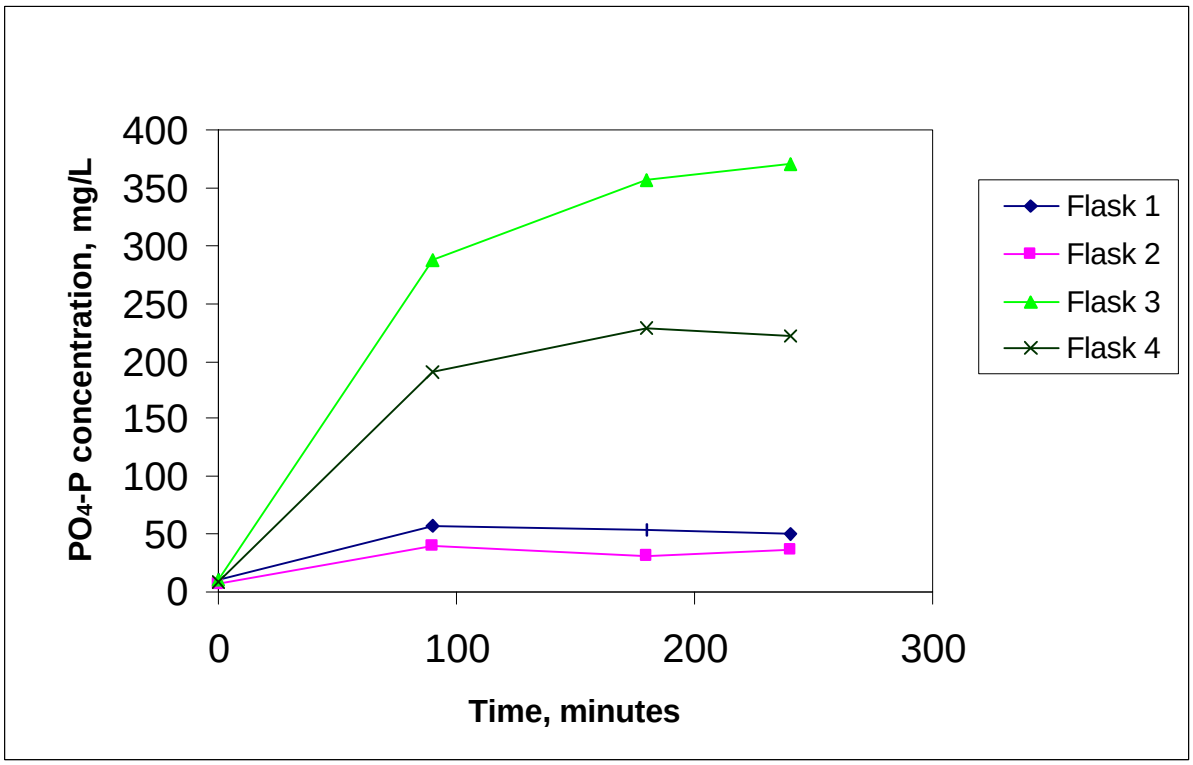



Figure 8. Batch test 1, P uptake with and without depleted poly-P pool at 20°C (Flask 1: anoxic sludge taken from system 2 at 5°C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20°C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5°C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20°C with enriched poly-P pool).

Bacterial cells in flask 3 ($5^oC$ sludge) showed the lowest demand for glycogen, even though they stored the greatest amount of PHA. Demand for glycogen increased sharply in sludges with exhausted poly-P pools (flasks 1 and 2). It can be deducted that glycogen served as the sole energy source for them, and it became limiting in the absence of the other energy source, poly-P. The limitation of glycogen, therefore, caused acetate utilization and PHA production to cease. This limitation was even more severe in flask 1 due to lesser glycogen replenishment observed during the prior aerobic cycle at $5^oC$.

The third batch test was performed at $5^oC$ using the EBPR sludge obtained only from system 1 ($20^oC$). The results of the third batch test showed that negligible acetate was taken up in the absence of poly-P. Despite the presence of a high glycogen content in the sludge, a small amount of glycogen was used and in turn insignificant amount of PHA was stored. This basically suggests that glycogen metabolism is not completely blocked at cold temperatures, but it operates significantly slower when an adaptation of the glycolytic enzymes is not allowed (e.g. an increase in the quantity of the related enzymes, or an increase in the affinity of the enzymes for their substrate – i.e. lowered $K_m$ values).

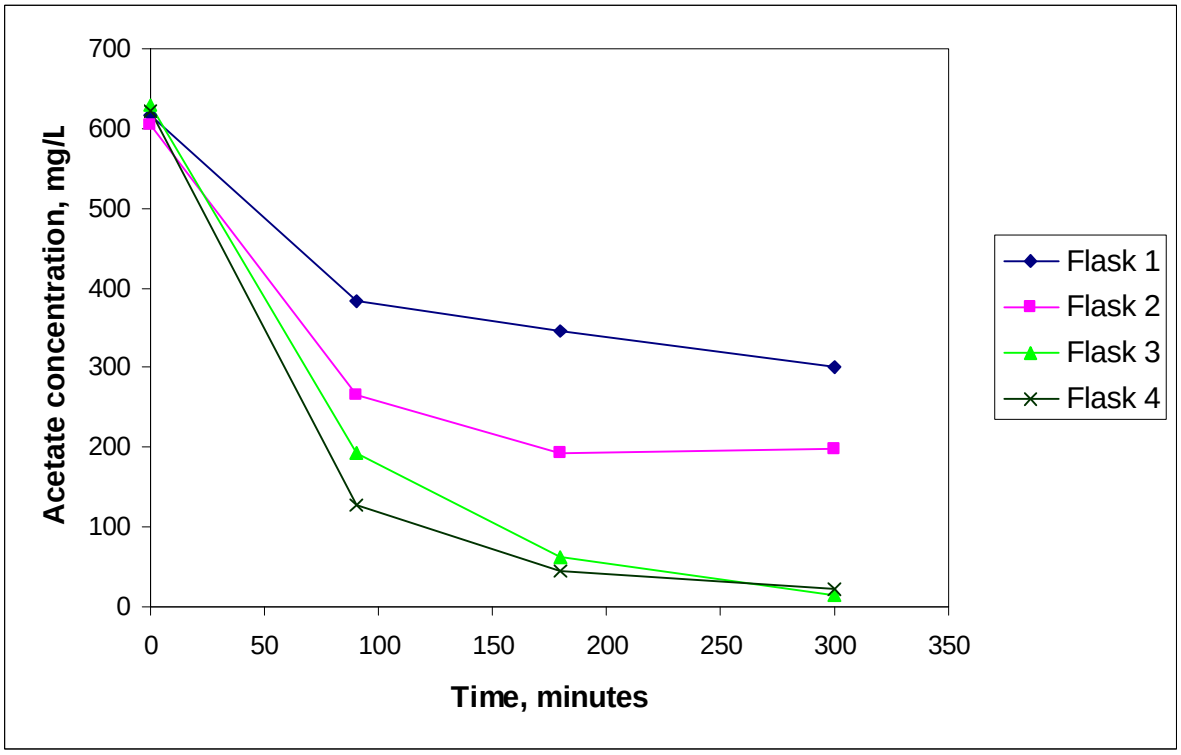



Figure 9. Batch test 2, acetate utilization with and without depleted poly-P pool at 20$^{o}$C (Flask 1: anoxic sludge taken from system 2 at 5$^{o}$C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20$^{o}$C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5$^{o}$C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20$^{o}$C with enriched poly-P pool).

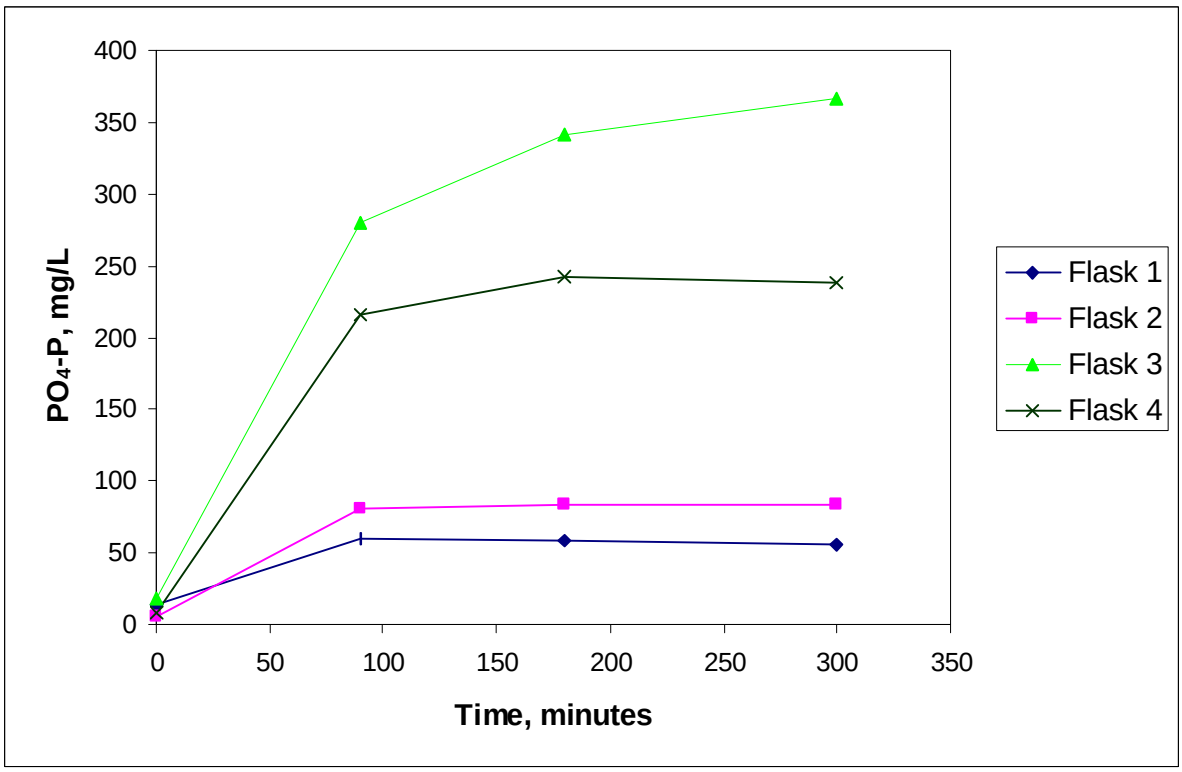



Figure 10. Batch test 2, acetate utilization with and without depleted poly-P pool at 20$^{o}$C (Flask 1: anoxic sludge taken from system 2 at 5$^{o}$C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20$^{o}$C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5$^{o}$C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20$^{o}$C with enriched poly-P pool).

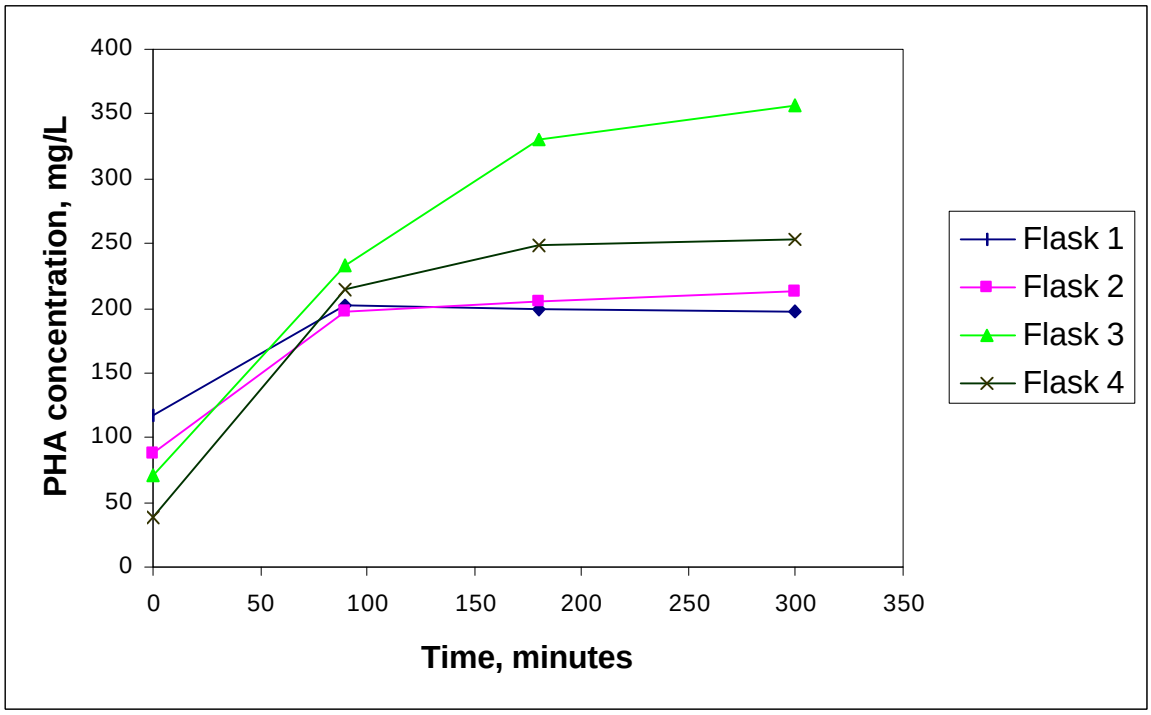



Figure 11. Batch test 2, PHA production with and without depleted poly-P pool at 20$^{o}$C (Flask 1: anoxic sludge taken from system 2 at 5$^{o}$C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20$^{o}$C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5$^{o}$C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20$^{o}$C with enriched poly-P pool).

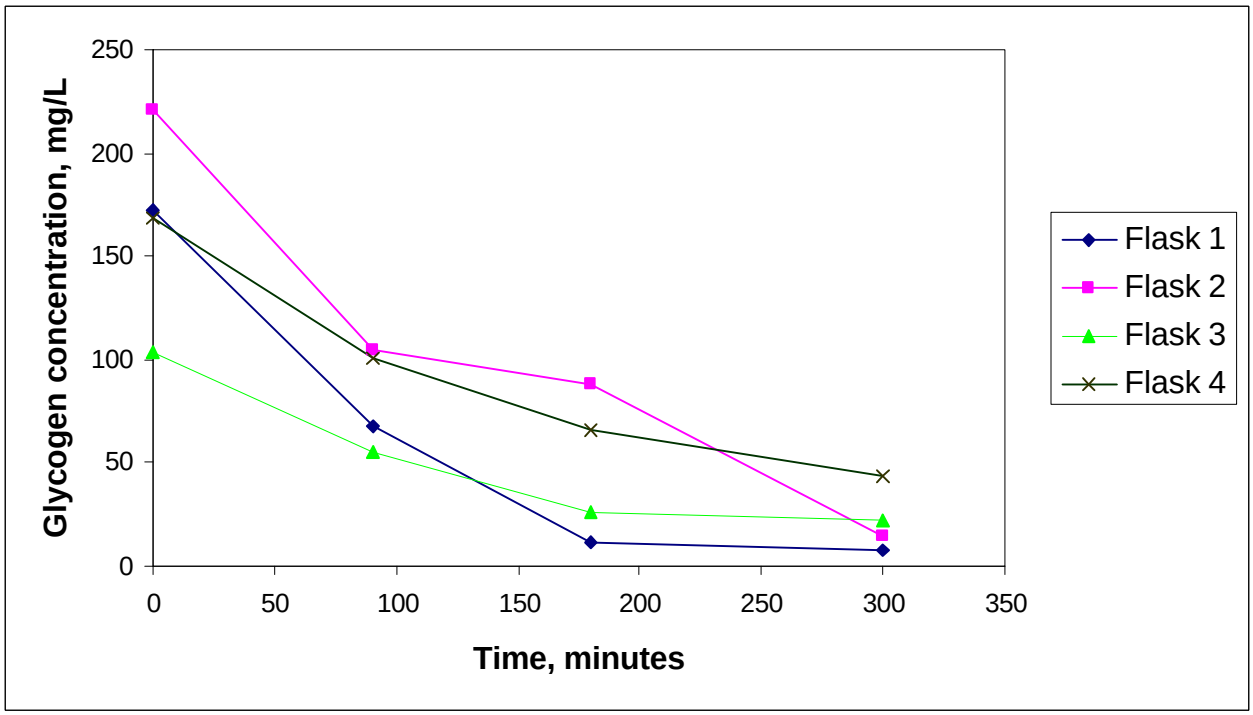



Figure 12. Batch test 2, Glycogen utilization with and without depleted poly-P pool at 20$^{o}$C (Flask 1: anoxic sludge taken from system 2 at 5$^{o}$C with depleted poly-P pool, Flask 2: anoxic sludge taken from system 1 at 20$^{o}$C with depleted poly-P reserve, Flask 3: anoxic sludge taken from system 2 at 5$^{o}$C with high poly-P pool, Flask 4: anoxic sludge taken from system 1 at 20$^{o}$C with enriched poly-P pool).

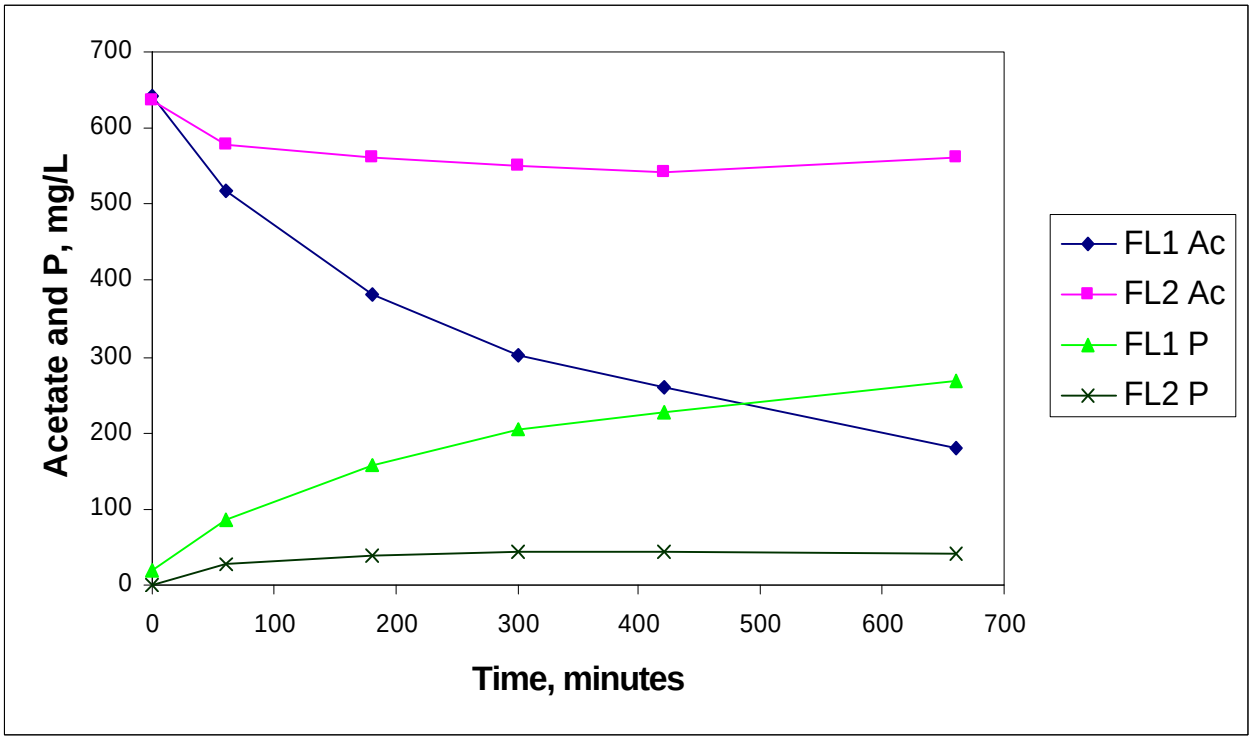



Figure 13. Acetate utilization and P release during batch test 3 performed at 5$^{o}$C. (flask 1: anoxic sludge of System 1 at 20$^{o}$C with enriched poly-P; flask 2: anoxic sludge of System 1 at 20$^{o}$C with depleted poly-P).

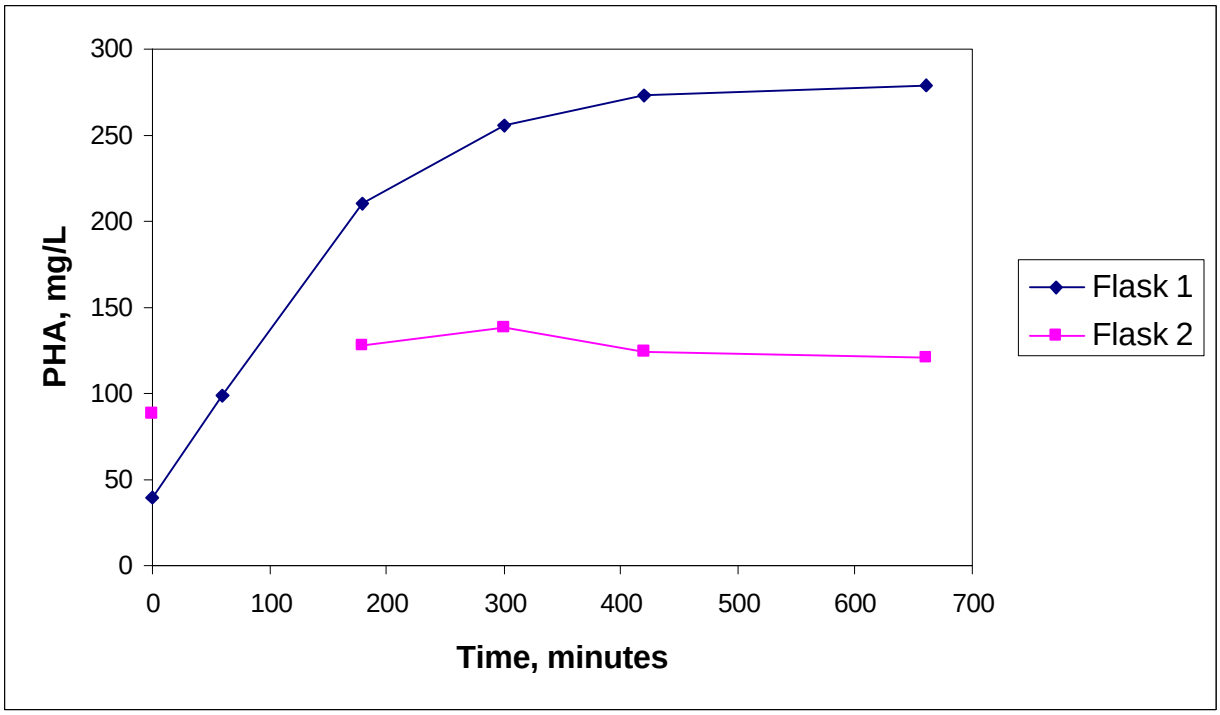



Figure 14. PHA production during batch test 3 performed at 5$^{o}$C. (flask 1: anoxic sludge of System 1 at 20$^{o}$C with enriched poly-P; flask 2: anoxic sludge of System 1 at 20$^{o}$C with depleted poly-P).

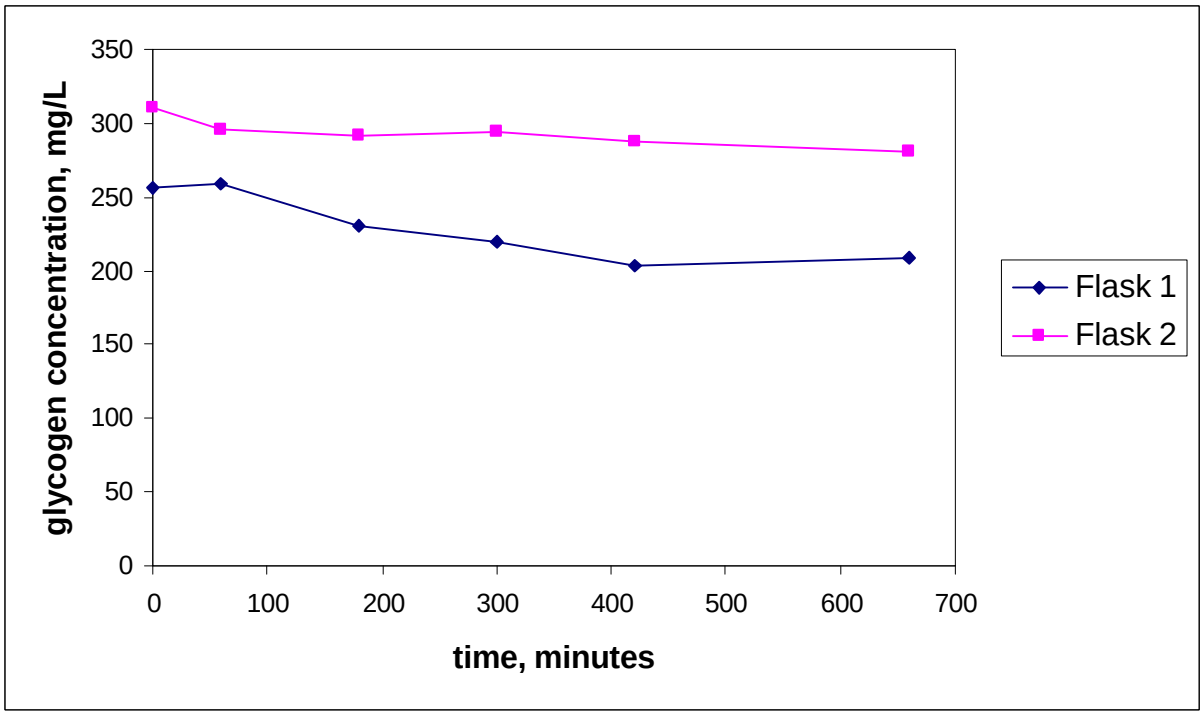



Figure 15. Glycogen utilization during batch test 3 performed at 5$^{o}$C. (flask 1: anoxic sludge of System 1 at 20$^{o}$C with enriched poly-P; flask 2: anoxic sludge of System 1 at 20$^{o}$C with depleted poly-P).

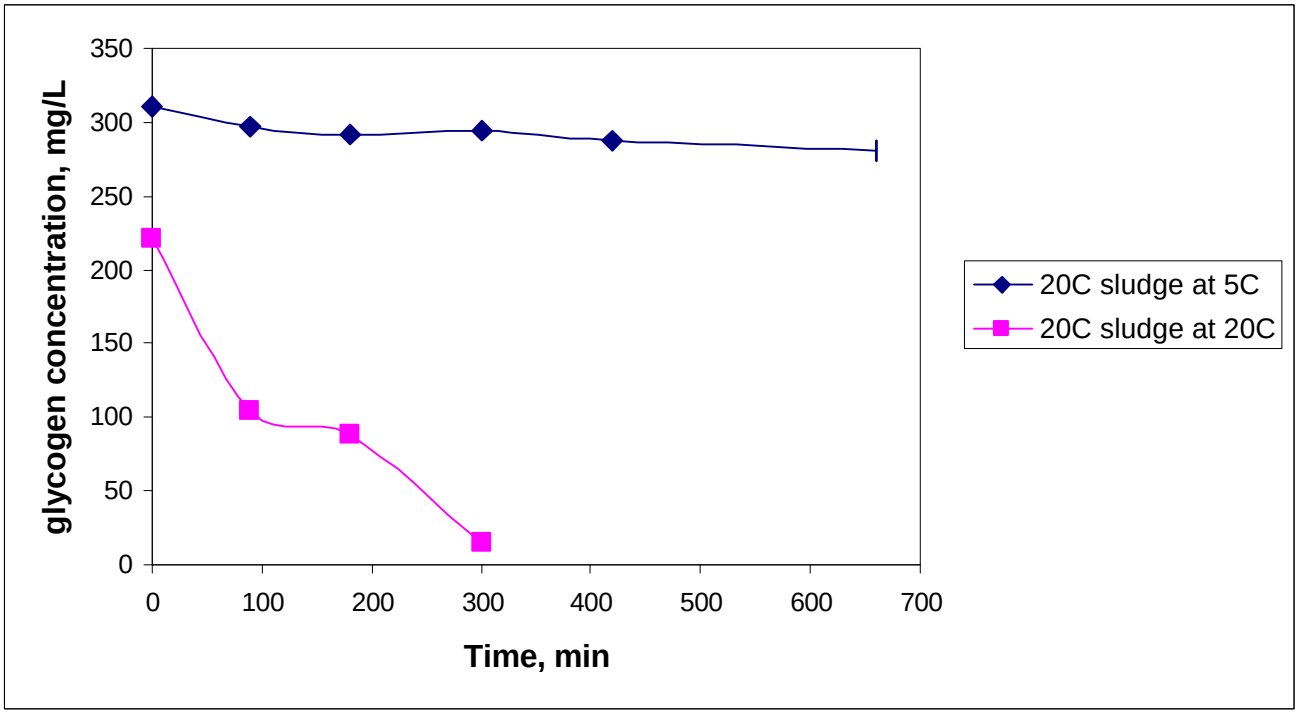



Figure 16. Comparisons of glycogen utilizations during batch test 2 at 20$^{o}$C and batch test 3 at 5$^{o}$C. (Both experiments were performed using anoxic sludge of system 1 acclimitazed to 20$^{o}$C.

## DISCUSSIONS

It has been shown that EBPR biomass can rapidly adjust cellular wall fluidity under decreasing temperature conditions (Erdal 2002 b). However, bacterial communities need time, on the order of 2 to 3 SRTs, to reach population steady state after large changes in temperature. Some of the temperature studies reported in the literature, however, used unacclimated activated sludge. For example, Brdjanovic *et al.* (1997) and Beatons *et al.* (1999) studied the effects of temperature change on unacclimated activated sludge, therefore, their results can be compared to the short term effects of this study, but not to the acclimated sludge results. The loss and then recovery of P removal following the temperature decrease to 5$^{o}$C clearly showed that temperature acclimation is a prerequisite 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, which was considerably greater than the 20$^{o}$C SRT of 10 days, to ensure that EBPR functions would not washout of the system as predicted by Mamais and Jenkins (1992). The disappearance of nitrification and the increase of the MLVSS concentration resulted in complete acetate uptake during the non-oxic stages, despite the slower acetate uptake rates at 5$^{o}$C. This occurred because COD was limiting rather than phosphorus, which favored the growth of PAOs. 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$^{o}$C. It was concluded that the steady-state PAO population was considerably higher, while the non-PAO population was considerably less, at 5$^{o}$C compared to 20$^{o}$C.

It seems likely that the lower P removal at 20$^{o}$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$^{o}$C sludge (18% of PHA) increased the probability that GAOs were part of the non-PAO present. Because of the lower glycogen (9.5%) and PHV (4%) concentrations at 5$^{o}$C, it was postulated that the GAO population became relatively insignificant at the colder temperature, probably because of their non-pyschrophilic, 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 in the non-oxic zones was not significant, and the glycogen content of the sludge remained in a narrow range (5 to 7% of VSS). In addition, the electron microscopy results indicated that the individual cells were very similar at 5$^{o}$C; i.e., not diverse compared to 20C as suggested by Brdjanovic *et al.* (1997). Comparison of several electron microscopy grids of the sludge maintained at 5$^{o}$C revealed no distinct organism with high glycogen and low poly-P content. A more diverse population was observed at 20$^{o}$C. Some cells of this diverse population accumulated poly-P to a great extent and some apparently had no ability to store poly-P. In addition, some cells with greater glycogen accumulation ability also stored poly-P (these cells were not considered to be GAOs, since GAOs have no ability to store poly-p). On one occasion, cells with cluster formation were observed. This formation was presumed to be indicative of GAOs. The batch test experiments revealed that despite similar acetate utilization by the sludges grown at the two temperatures, nearly 30% less PHA was produced by the sludge taken from the 20$^{o}$C reactor, indicating that GAOs were very small fraction of the population at 20$^{o}$C (Park et al. 2002). If the difference in P removal was caused by the presence of GAOs, the PHA content should have been higher. This finding is important, because it suggests that PAO-GAO competition is insignificant when EBPR systems are operated under COD limiting conditions. The decreased P removal efficiency at the warmer temperature can then be attributed to the presence of fermentative or other non-poly-P bacteria that are capable of utilizing substrate under anaerobic conditions.

Detailed micro-ecological studies have suggested that the taxonomic features of PAO and GAO are quite similar (Seviour and Blackall, 1999). The possibilty that they may be the same bacteria was first proposed by Mino *et al.* (1998). However, no comprehensive study was performed to prove the validity of this theory. In this study, electron microscopy pictures suggested that single cells of EBPR bacteria have the ability to simultaneously store PHA, glycogen and poly-P (Micrograph 6, 12 and 13). No evidence

for the presence of a bacteria type that does not store poly-P but excessively stores glycogen (GAO) was found. Based upon our analytical results and micrographs, it is highly likely that the biomass cultivated in this study consisted of bacterial groups that did not include GAO-type organisms. It is evident that the EBPR bacteria stored more PHA with less glycogen utilization at 5$^{o}$C whereas the same bacteria stored less PHA with higher glycogen utilization at 20$^{o}$C. Temperature possibly inhibits or induces some of the key enzymes of different energy metabolisms forcing the EBPR bacteria to use alternative pathways. It is proposed that, cold temperature slows down glycolysis resulting in low glycogen accumulation that in turn increases the performance of the EBPR process. This statement may also be true under various environmental stresses (e.g. pH). This is of great importance since it indicates that such changes in environmental conditions may alter the active metabolic pathways of the EBPR process as observed in this study, rather than resulting in drastic population shifts. Therefore decreased system performance with increased glycogen accumulation may not be related to GAO proliferation as suggested by Filipe *et al.* (2001). Current models (Satoh *et al.*, 1994 and Filipe *et al*., 2001) consider that GAO metabolism is an integral part of EBPR metabolism and the performance of EBPR processes depends on the PAO/GAO fraction in the EBPR system. Even though EBPR performance was completely lost in the A/O unit because of very low phosphate feed (less than 1 mg/L), GAO proliferation was not observed. Therefore such an important concept should be further investigated before it is included in EBPR models. The arbitrary selection of GAO parameters without any evidence of GAO presence introduces an undefined factor to the models rather than expanding them to include well-defined mechanisms.

Brdjanovic *et al*. (1997) suggested that EBPR process stoichiometry was insensitive to temperature changes. However, in this study, the observed stoichiometric values of PHA storage per unit glycogen utilization were clearly different (2.14 and 2.84 for 20 and 5$^{o}$C, respectively). In addition, observed aerobic stoichiometry between PHA utilization and glycogen replenishment also varied at different temperatures (0.63 and 0.40 for 20 and 5$^{o}$C, respectively). Therefore, temperature not only affects the kinetics of EBPR but also

affects EBPR stoichiometry. The reason for the changes in stoichiometry, and hence EBPR efficiency at different temperatures, is proposed to be related to three main factors:

1. Population shift: Two different populations were observed at 20$^{o}$C (micrograph 7 and 8) and they were identified as poly-P and non-poly-P populations because of their storage products. Cold temperature greatly reduced the mesophilic bacterial population. The ability of the poly-P organisms to adapt to cold temperatures made them dominant at 5$^{o}$C, and greatly increased P removal performance ($P_x$=37% as VSS at 5$^{o}$C vs. 14% as VSS at 20$^{o}$C).

2. Temperature can alter the metabolic pathways of EBPR process: Batch test 3 clearly showed that glycogen utilization was very slow at 5$^{o}$C. Enzyme assay tests performed by Z. K. Erdal (2002) showed that, even though bacterial cells growing at 5$^{o}$C possess phosphofructokinase (PFK) enzyme (one of the key enzymes of the EMP pathway), the activity of this enzyme was very low at 5$^{o}$C. The decreased contribution of glycogen to EBPR mechanisms at cold temperature suggests that although glycogen is required for EBPR operation, the EBPR microorganisms have the ability to adapt their metabolic pathways to environmental conditions and greatly reduce their need for glycogen.

3. Reduced nitrification: The reduced nitrification at cold temperatures lowered the mass of nitrate entering the anoxic stage. Thus, less acetate was utilized for denitrification, and more acetate was used for PHA metabolism

## CONCLUSIONS

- Temperature affects 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 relative to non-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 significantly co-existed in the EBPR sludge community under any of the temperature conditions for the COD limited systems. Even a highly favorable condition for GAO proliferation (no phosphorus in the feed) did not lead to prolific growth of GAO population.
- EBPR-P bacteria have the ability to store both poly-P and glycogen. This suggests that GAOs may not exist in activated sludge under all conditions. Therefore, GAO inclusion in the current models (Satoh *et al*., 1994; Filipe *et al*., 2001 and Copp and Dold 2001) needs further examination.
- It was presumed by Brdjanovic *et al*. (1997) that EBPR stoichiometry was insensitive to temperature. However, the stoichiometry of EBPR processes was shown to be sensitive to temperature.
- The decreased contribution of glycogen to EBPR mechanisms at cold temperature suggests that although glycogen is required for EBPR operation, PAOs have the ability to adapt their metabolic pathways to environmental conditions and drastically change their need for glycogen.
- As has been suggested by previously developed models, glycogen utilization is a consequence of the attempt of the organisms to balance reducing equivalents. However, there is more than one route of glycogen consumption or production, and based on temperature the most efficient route will be selected.

- It has been confirmed that PAOs consist of psychrophilic bacteria and that temperatures of 10$^{o}$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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