

CHAPTER II
THERMAL ADAPTATION OF BACTERIA TO COLD TEMPERATURES IN AN
EBPR SYSTEM

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Abstract

Temperature is one of the key parameters that affects the reaction kinetics and performance of enhanced biological phosphorus removal (EBPR) systems. Although studies agree that decreases in temperature cause decreases in EBPR kinetic reaction rates, there are contradictory results in the literature regarding the effect of temperature on EBPR system performance. Early investigators reported better performance with lower temperatures (Sell, 1981; Ekama *et al.*, 1984; Daigger *et al.*, 1987), but more recent ones have reported partial or complete loss of EBPR functions at low temperatures (McClintock *et al.*, 1991; Brdjanovic *et al.*, 1997; Beatons *et al.*, 1999). Specifically, it has been shown that EBPR functions “washout” of biological nutrient removal (BNR) systems before COD removal functions (McClintock *et al.*, 1991; Mamais and Jenkins (1992). One speculation has been that deterioration in EBPR system performance at cold temperatures may be attributed to reduced fluidity and more rigid-like behavior of the cell membranes, which would reduce or prevent transport across the membrane. Most cells (not all) on the other hand have the ability to alter their membrane fatty acid composition as temperature changes in order to keep their membrane at nearly the same fluidity despite the temperature changes (Becker *et al.*, 1996). This unique ability is known as “homeoviscous adaptation”. In this study, homeoviscous adaptation by EBPR activated sludge was investigated for a series of temperatures ranging from 20°C to 5°C using a lab scale continuous flow EBPR system fed with acetate and supplemental yeast

extract. The fatty acid analysis results showed that the unsaturated to saturated fatty acid ratio increased from 1.40 to 3.61 as temperature dropped from 20 to 5°C. The increased cis-9-hexadecanoic acid (C16:1) at 5°C strongly indicated the presence of homeoviscous adaptation in the EBPR bacterial community. Thus the cell membranes of the EBPR community were still in a fluid state, and solute transport and proton motive force were operable even at 5°C. It was concluded that loss of EBPR performance at low temperatures is not related to the physical state of the cellular membranes, but is possibly related to the application of unsuitable operational conditions for the reduced kinetic rates, e.g. SRT less than critical, excessive electron acceptors, low anaerobic detention time, non-acclimated sludge, enzyme inactivation, etc. The study also showed that short chain volatile fatty acids (acetate and propionate) are transported through either facilitated or active transport, not through simple passive diffusion.

Keywords: activated sludge, EBPR, temperature effects, homeoviscous adaptation, membrane fluidity, fatty acid transport.

INTRODUCTION

Temperature is a fundamental factor that affects all living organisms. The major impact of reduced temperatures on any system is the reduction of molecular motion which causes the rate of biological or chemical reactions to slow down (Grout and Morris, 1987). In addition, the physical properties of water are very sensitive to temperature, and these properties show remarkable changes as temperature changes, especially to temperatures below 10°C (Morris And Clarke, 1981). Thermal stresses have strong impacts on the membrane lipids of organisms that influence membrane structure and function (Hazel, 1995). The transition from the fluid to the gel phase reduces the activity of membrane bound enzymes, slows the rate of lateral protein diffusion within the plane of the membrane bilayer, and induces cluster formation of integral membrane proteins (Hazel, 1995). Living organisms typically encounter temperature changes throughout their life cycle. Most microorganisms can tolerate a variety of changing conditions and

stresses in their surrounding environment, and adaptations to fluctuations in temperature are common because of the impact of temperature on the biochemical reactions of the cell. Organisms possess several defense mechanisms against temperature stresses such as production of cold and heat shock proteins and altering membrane fatty acid composition in a process known as “homeoviscous adaptation”. This is essential because the temperature in aquatic environments changes seasonally, requiring the microorganisms to carry out long term temperature adaptation.

The membrane of a bacterial cell does not simply define the boundaries of the cell and delineate its compartments, but also serves like a brain for specific functions such as regulating movement of substances into and out of the cell and its compartments (Becker *et al.* 1996). A membrane is a hydrophobic permeability barrier consisting of hydrophobic phospholipids and hydrophobic proteins. Fatty acids are an integral part of the membrane structure because their long hydrocarbon tails form an effective hydrophobic barrier to the diffusion of polar solutes. Membrane fatty acids normally contain even-numbered hydrocarbon chains which are either fully saturated or contain varying numbers of *cis* double bonds which makes them unsaturated (Pringle and Chapman, 1981). The heterogeneity of the fatty acid structure results in a bend in the hydrocarbon tail due to *cis* or *trans* formation, and this confers unique thermodynamic properties to cells, such as setting the transition temperature. The transition temperature is a critical temperature below which the membrane is rigid, and above which the membrane is fluid. The fluidity of the membrane mainly depends on the length of the fatty acids present and the degree of unsaturation of their side chains (number of double bonds present). Membrane lipids with saturated fatty acids pack together very tightly whereas lipids with unsaturated fatty acids do not pack together well because the *cis* double bonds cause bends in the chains that interfere with packing (Becker *et al.*, 1996). The illustration of *cis* double bond formation is given in Figure 1 (Bruice, 1995). However, the resulting bends decrease the van der Waals interactions and therefore lower the transition temperature (Voet and Voet, 1995). Consequently, the larger the number of double bonds (greater degree of unsaturation), the lower the melting point of the acyl chains. The melting point also decreases as the acyl chain length decreases. The

relationship between the degree of unsaturation and its effect on the transition temperature is illustrated in Figure 2 (Becker *et al.*, 1996).

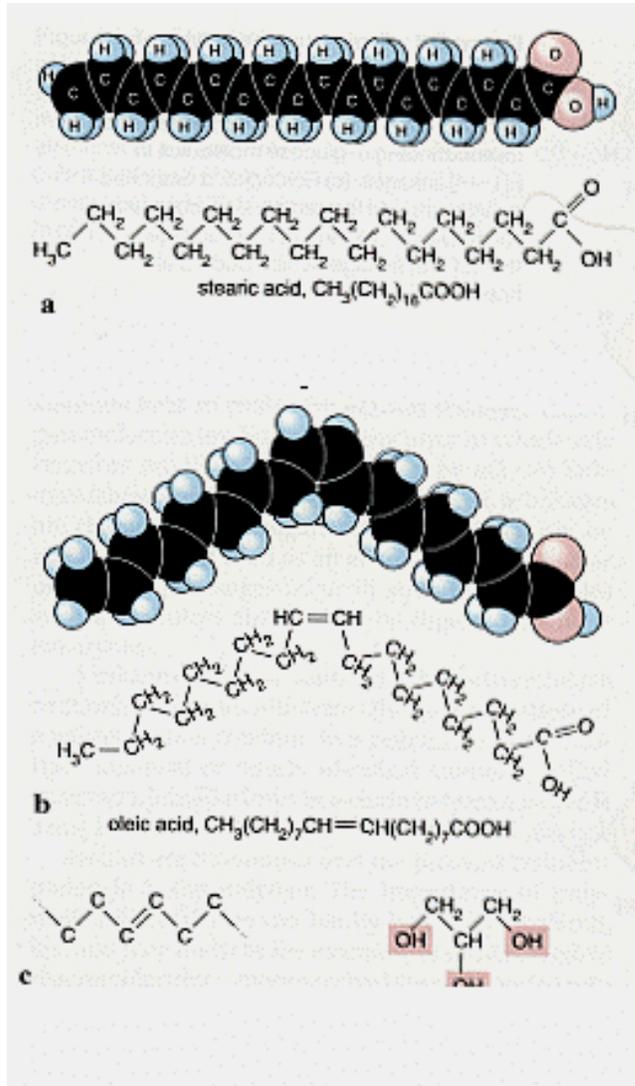


Figure 1. The comparison of saturated and unsaturated fatty acid with 18C fatty acids. (a: stearic or octadecanoic acid; b: cis formation in oleic or 9-octadecanoic acid (Bruce, 1995)

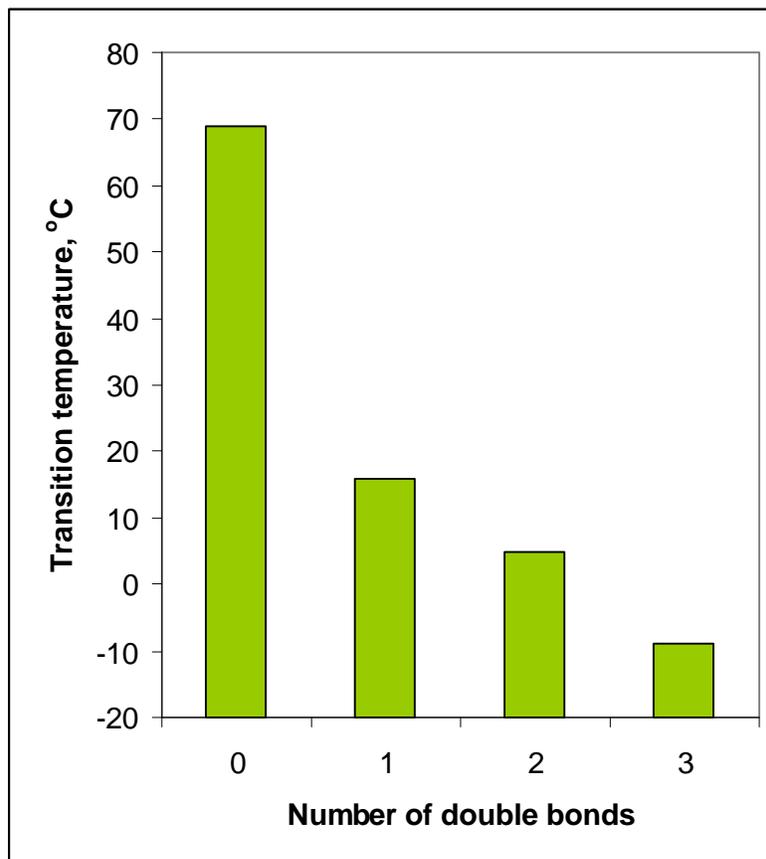


Figure 2. Effect of unsaturation on the transition temperature of fatty acids with 18 carbon atoms (Becker *et al.* 1996).

Most prokaryotic organisms (not all) are able to compensate for temperature changes by altering the lipid composition of their membranes, thereby regulating membrane fluidity. This ability is called “homeoviscous adaptation” because the main goal of such regulation is to keep the viscosity of the membrane approximately the same despite the change in temperature (Becker *et al.*, 1996). Small differences in temperature are usually tolerated by adjusting the length of the hydrocarbon tails in accordance with the temperature. This process is called elongation of membrane fatty acids and relatively little is known about fatty acid elongation in prokaryotic organisms (Thompson, 1992). A decrease in chain length of fatty acids was reported by Fulco (1973) when the growth temperature of *B. licheniformis* was reduced from 35 to 20°C. An increase in growth temperature in *B. megaterium* leads to a greater ratio of iso- to anteiso-fatty acids which proves that the adjustment in fatty acid chain length is reversible (Shinitzky, 1984). The increase in acyl chain length was thought to be controlled by a membrane bound elongase enzyme, which introduces a C₂ subunit to C₁₆ and C₁₈ fatty acids (Morris and Clarke, 1981).

More commonly, adaptation to temperature involves alteration in the degree of unsaturation rather than in the chain lengths. Different types of organisms have different strategies and regulations for controlling the desaturation process (Aloia *et al.*, 1988). These strategies can be classified into two groups, which are:

a. Desaturation under anaerobic conditions

The pathway for the biosynthesis of saturated and unsaturated fatty acids in *E. coli* is given in Figure 3 (Thompson, 1992). According to the above pathway, the fatty acid synthetase system produces both saturated and unsaturated fatty acids because the branch point is localized in the cyclic elongation pathway at the level of the Beta-OH-decanoyl-ACP intermediate (Aloia *et al.*, 1988). This C-10 intermediate can either be dehydrated to a trans-2-unsaturated isomer, which contributes to the saturation of the fatty acids, or a cis-3-unsaturated fatty acid isomer leading to an unsaturated fatty acid such as

palmitoleic acid. (Aloia *et al.*,1998). The ratio of saturated to unsaturated fatty acid synthesis in *E.coli* is partially controlled by the activity of the enzyme B-hydroxydeconoyl thioester dehydrase (Cronan, 1974). It also has been suggested that B-ketoacyl ACP synthetases I and II play a role in this regulation (Cronan, 1978).

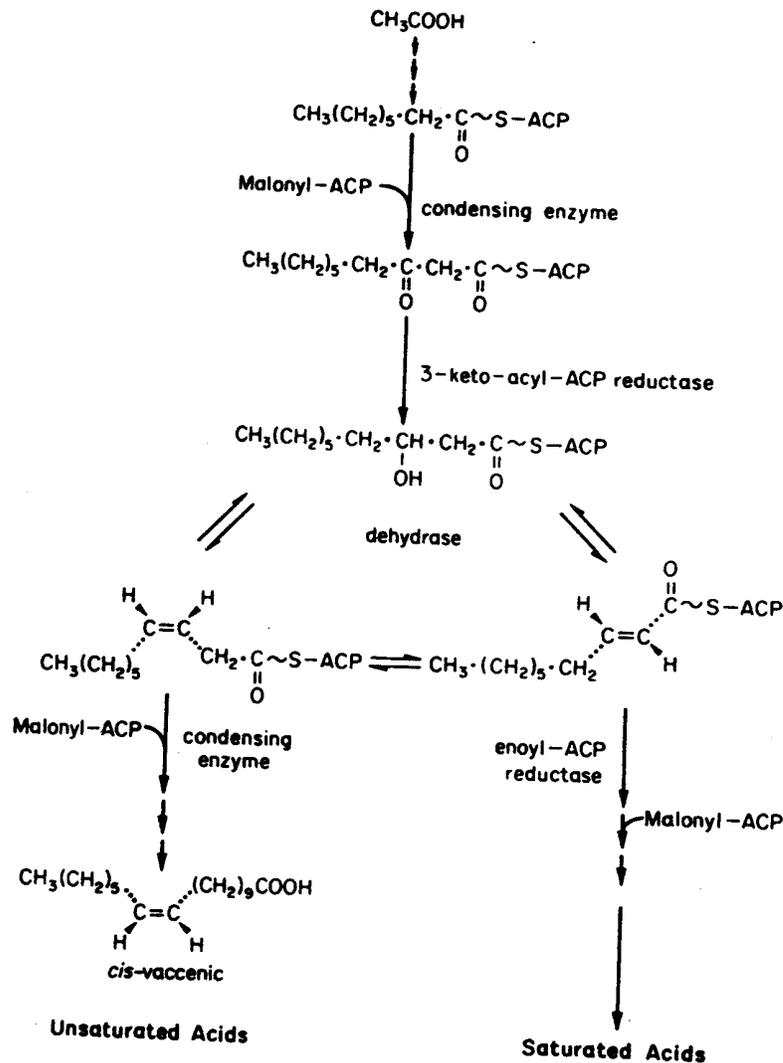


Figure 3. The pathway for the biosynthesis of saturated and unsaturated fatty acids in *E.coli*. (Thompson, 1992).

b. Desaturation under aerobic conditions

Introduction of a double bond into saturated long chain fatty acids is an oxygen dependent process in aerobic bacteria. The most common products are palmitoleic and oleic acids. The fatty acid desaturation pathway for the aerobic bacteria *Bacillus megaterium* is given in Figure 4, and it is mainly affected by three factors: (Thompson, 1992).

1. An irreversible loss of desaturase enzyme activity at high temperature.
2. Desaturase synthesis stops at high temperature, but then resumes rapidly when cells are transferred from a high to a low temperature.
3. A protein modulator may regulate the transcription of the desaturase gene by governing the desaturase mRNA.

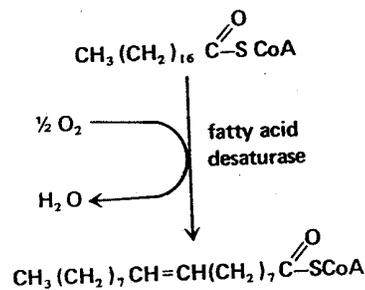


Figure 4. The fatty acid desaturation pathway for the aerobic bacteria *Bacillus megaterium*. (Thompson 1992).

Desaturation enzymes in *B. megaterium* were reported to be induced by a decrease in temperature and repressed at increased temperatures (Morris and Clarke, 1981). It was

shown that the desaturation enzyme was absent in *B. megaterium* at 30°C. However, decreasing the temperature to 20°C resulted in de novo synthesis of desaturation enzyme (Morris and Clarke, 1981).

Even though the mechanism of homeoviscous adaptation is not yet fully understood, researchers agree that there is a temperature that triggers the synthesis of desaturase enzymes that introduce double bonds into hydrocarbon chains of fatty acids (Becker *et al.*, 1996). When temperature increases, however, it was observed that desaturase enzymes are inhibited allosterically. As a result of allosteric inhibition, one or more double bonds are removed from the unsaturated fatty acid to prevent the membrane from becoming very leaky (Becker *et al.*, 1996).

Several studies have shown that homeoviscous adaptation does exist and is the main mechanism that controls membrane viscosity under thermal stresses. For example: McElhaney and Sauza (1976) investigated the relationship between temperature, cell growth and the fluidity and physical state of membrane lipids in *Bacillus stearothermophilus*. It was reported that this organism possesses a sensitive homeoviscous adaptation mechanism which maintains a relatively constant degree of membrane fluidity over a wide range of temperature. Sato and Murata (1980) showed that a sudden downward temperature shift rapidly altered the membrane fatty acid composition of *A. variabilis*. While the total amount of lipids stayed at a constant level, a decrease in 16:0 and a concomitant increase in 16:1 VFAs occurred, suggesting the presence of homeoviscous adaptation. Okuyama *et al.* (1986), observed the preservation of membrane fluidity at 10 and 0°C in the psychrophilic bacteria, *Vibrio* Strain ABE-1. The adaptation was attributed to the bacteria having an extremely high content of hexadecanoic acid (16:1, i.e, 16 carbons with one double bond) in the membrane phospholipids. Hamamoto *et al.* (1995) investigated the cellular fatty acid composition of psychrophilic and psychrotrophic vibrios isolated from deep-sea sediments. The presence of eicosapentanoic acid (20:5) played a key role in low temperature adaptation.

On the other hand, Svobodova and Svoboda (1988) investigated membrane fluidity in *Bacillus subtilis* under 37 and 15°C, and insignificant changes were observed in the membrane fatty acid compositions when both temperatures were compared. It was concluded that *B. subtilis* has no ability to homeostatically control bulk lipid fluidity, thereby suggesting no homeoviscous adaptation. Fodor *et al.* (1997) investigated the lipid compositions of two symbiotic photosynthetic bacteria, *Xenorhabdus nematophilus* and *Photorhabdus liminescens*, at 28 and 18°C. Lipid fatty acid composition from primary and secondary cultures of both bacterial species grown at 18°C were more ordered (i.e. less fluid) than those grown at 28°C. It suggested that these particular bacterial species were unable to perform homeoviscous adaptation. In light of the available information, it is clear that homeoviscous adaptation is not universal among bacterial species.

Complete loss of EBPR performance has been reported in several studies under downward temperature shifts (McClintock *et al.*, 1991; Mamais and Jenkins, 1992; Brdjanovic *et al.*, 1997; Beatons *et al.*, 1999), although most EBPR bacteria are thought to be psychrophilic. Such deterioration may be related to the inability of EBPR bacteria to perform homeo-viscous adaptation. However, no study has been performed to investigate the effects of cellular membrane changes on the observed temperature paradoxes of EBPR activated sludge systems. In this study, the membrane fluidity of an activated sludge bacterial community that had been enriched with phosphorus accumulating organisms (PAOs) was investigated at 20, 18, 15, 10 and 5°C to determine whether PAOs lose their ability to take-up substrate at low temperatures, or whether they can adapt.

The mechanism(s) of SCVFA transport into PAO cells has never been demonstrated and is still unknown. Despite this uncertainty, the transport of acetate is generally assumed to occur by either carrier mediated or active transport (Becker *et al.*, 1996). This assumption is probably made because volatile fatty acids are considered to be charged molecules in the neutral pH range and cell membranes are impermeable to charged molecules. It also is known that cellular membranes have low permeability to protons and the major route of proton entry is via energy-transducing transporters rather than by general leakage

(Mitchell, 1961). It is apparent that even very small charged molecules (H^+ ions) cannot enter the cell via simple diffusion. Bacterial membranes also are very impermeable to large molecules even when they are non polar or neutral species (Becker *et al.* 1996). Such large molecules need specific transporters to cross the cell membrane and this type of transport is termed as facilitated or carrier mediated passive transport (Becker *et al.* 1996). Glucose transport is a typical example of facilitated diffusion (Becker *et al.* 1996). The role of transport proteins is to facilitate the diffusion of polar or charged solutes across an otherwise impermeable barrier. Facilitated diffusion does not require energy as does simple passive diffusion because the solute is transported from a higher concentration to a lower concentration (exergonic; always negative free energy values) (Becker *et al.* 1996). The kinetics for facilitated diffusion depends on a limited number of transporters on the cell membrane (White, 1995). Therefore, the rate of solute entry is directly proportional to the quantity of transporters that is occupied with solute. More transporters can bind solute as the external concentration of solute increases. The rate of transport increases to a maximum rate (V_{max}), when no unloaded transporter is left (White 1995). Thus a saturation curve (hyperbola) must be observed as solute concentration is plotted against solute uptake rate. A saturation curve cannot be observed during simple diffusion because in the absence of transporters the rate of solute entry is relatively slow and does not approach a maximum, even at very high concentrations of solute (White 1995).

Unlike passive diffusion, active transport always occurs against the concentration gradient and needs a metabolic energy input (always endergonic) (Becker *et al.*, 1996). Energy is usually provided by the hydrolysis of high energy phosphate bonds. Proton and sodium-potassium pumps are common examples of active transport mechanisms. It is catalyzed by uniporters, symporters and antiporters that use electro-chemical gradient to accumulate solutes (White 1995). Therefore, during active transport of solutes, a saturation curve can also be observed when the substrate uptake rate is plotted against solute concentration.

Baronofsky *et al.* (1984) showed that the transport of acetic acid into *Clostridium thermoaceticum* was by passive diffusion over a concentration range of 0 to 150 mM and over a pH range of 5 to 7. Hume *et al.* (1993) found that uptake of acetate in the caecum and colon of prairie voles was primarily passive over the acetate range of 10-50 mmol/L. However, acetate uptake was found to be carrier-mediated in both regions when the acetate concentration was higher, i.e., 100 mmol/L. Carroll (1997) determined the transport mechanism of labeled acetate (^{14}C) in hippocampal cholinergic nerve terminals. The study showed that the uptake of extracellular acetate was saturable having an apparent Michaelis constant (K_m) of 22mM.

Comeau and Wentzel (1986), Mino (1987), and Smolders (1994), all have proposed an active transport mechanism for acetate in their EBPR models. It also has been proposed that the energy requirements for active transport are obtained through the hydrolysis of ATP. Therefore, a certain amount of P-release would be required for active transport of acetate in each model. However, if passive diffusion occurs, no P release would be associated with the acetate transport process and the model predictions would be in error. As has been presented, acetate transport is an integral part of the anaerobic stoichiometry of the EBPR process. In addition, the study of the transport mechanism may provide essential information for temperature research, because, if the transport of acetate is passive, the rate of acetate diffusion will be heavily influenced by solution temperature. Then the reduced acetate uptake in EBPR studies at low temperatures may simply be related to the limitation of acetate transport at cold temperatures. Further investigation of the SCVFA transport mechanism(s) is needed to further EBPR model development and temperature research.

METHODS AND MATERIALS

A lab scale University of Cape Town (UCT) configuration EBPR system containing two anaerobic (2L each), two anoxic (2L each) and three aerobic (3.5 L each) reactors in series and fed with synthetic wastewater was operated at 20°C for more than 6 months. The system schematic is illustrated by Figure 5. Synthetic feed was prepared daily to contain 420 mg/L acetate, and sufficient yeast extract to increase the COD concentration to 500 mg/L. Other chemicals and concentrations in the wastewater were (NH₄)₂SO₄ 40 mgN/L, K₂HPO₄ 25 to 80 mgP/L, 125 mg/L alkalinity, 210 mg/L MgSO₄, 44.4 mg/L CaCl₂, 1.11 mg/L FeCl₃, 0.66 mg/L MnCl₂·6H₂O, 0.44 mg/L ZnSO₄·7H₂O, 0.14 mg/L CuSO₄·5H₂O, 0.14 mg/L CoCl₂·6H₂O, 0.05 mg/L KI, 0.12 mg/L H₃BO₄, and 0.05 mg/L EDTA in accordance with Punratanasin (1997) and Kisoglu et al. (2000). The system was housed in a constant temperature room maintained at 20 ±0.5°C and operated at a constant SRT of 10 days. Steady state data collected included concentrations of SCOD, acetate, MLSS, MLVSS, NO₃⁻-N, NO₂⁻-N, NH₄⁺-N, and PO₄⁻³-P. The cation and anion analyses of filtered samples were performed using a DIONEX Ion Chromatograph. SCOD, MLSS and MLVSS were analyzed as outlined in APHA (1995). The temperature was then dropped to 5°C in two day intermediate steps over a week's time. The intermediate temperatures were 18, 15 and 10°C. Both system performance and cellular membrane fluidity changes were determined at each temperature, and the determinations were continued to define acclimated conditions at 5°C.

It was reported by deMendoza and Cronan (1983) that homeoviscous adaptation was completed within 15 seconds by the protozoan, *Tetrahymena pyriformis*, when the temperature was dropped from 42 to 24°C. Even though mixed liquor temperature changes are gradual in wastewater treatment plants, it would be useful to know how quickly adaptation occurs. To determine the quickness of homeoviscous adaptation, sludge acclimated to 20°C was exposed to 5°C by providing instant cooling and was kept at 5°C for 24 hours. Samples are taken 0, 1, 2, 4, 8 and 24 hours after exposure to the lower temperature. Fatty acid composition of the cells was determined during the short

term temperature exposure study and compared to those observed following long term temperature exposure. Paired t-tests as outlined by Zar (1999) were performed for comparison of the fatty acid compositions under short and long temperature exposures. An alpha value of 0.10 was used for determination of t critical values.

Membrane fatty acid composition was determined according to the direct transesterification method developed by Lepage and Roy (1986). This method is as follows: A 0.2 mL aliquot of an activated sludge sample from the aerobic reactor was precisely measured and poured into a glass tube. Due to the absence of tridecanoic acid in bacterial cells, an internal standard consisting of 1 mg to 10 mg of tridecanoic acid (C13: 0), was dissolved in 50 mL of methanol-toluene 4:1 (v/v), and 4 mL of this solvent mixture was added to the activated sludge samples. A small magnetic bar placed into each tube provided stirring while 0.4 mL of acetyl chloride was slowly added to each tube. The tubes were tightly closed with teflon-lined caps and subjected to methanolysis at 100°C for 1 hr. The tubes were weighted before and after heating as a check for leakage. After the tubes cooled in water, 10 mL of 6% K₂CO₃ solution was slowly added to neutralize the mixture. Then the tubes were shaken and centrifuged, and an aliquot of the upper phase was injected into a GC. Before injection, the samples were dried by introducing argon gas, and then dissolved in 1 mL of hexane.

VFA standards purchased from Sigma were used during GC analysis as external standards. These standards are tetradecanoic acid (C14: 0), pentadecanoic acid (C15:0), hexadecanoic acid (C16:0), octadecanoic acid (C18:0), cis-9-hexadecanoic acid (C16: 1), cis-octadecanoic acid (C18: 1), cis,cis-9,12 hexadecanoic acid (C16: 2) and cis,cis 9,12 octadecanoic acid (C18: 2). A VG 7070 organic mass spectrometer coupled with a gas chromatograph, located in the biochemistry department of Virginia Tech, was used throughout the GC/MS runs.

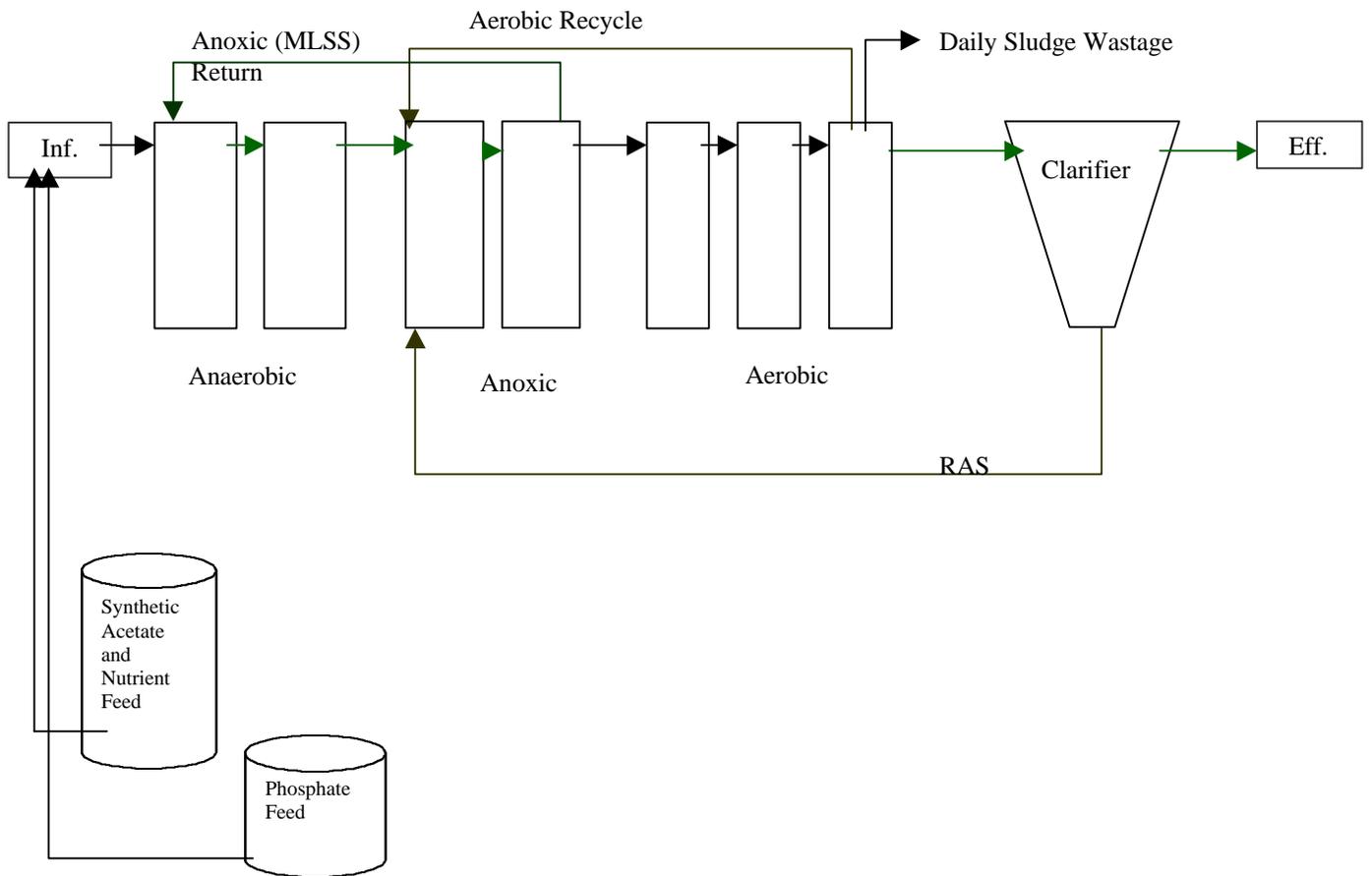


Figure 5. Flow schematic of the lab-scale UCT system.

Determination of transport mechanism for acetate and propionate in EBPR sludge:

During simple passive diffusion, the rate of solute entry is relatively slow and does not approach a maximum even at very high concentrations of solute, but is proportional to the concentration gradient (White, 1995). The solute concentration of 10 mM or higher is usually recommended when this relationship is investigated (Voet and Voet, 1995). The distinction between simple passive diffusion and other transport mechanisms (facilitated and active transport) can easily be made because the relationship between substrate uptake rate and substrate concentration is linear in simple passive diffusion whereas it is hyperbolic in facilitated and active transport. Acetate concentration ranges of 0 to 38

mM and 0 to 21 mM were used in batch test 1 and 2, respectively. Propionate concentrations of 0, 250, 500, 750 and 1250 mg/L (0 to 17 mM) were added to EBPR activated sludge in propionate batch test. The activated sludge was obtained from the last anoxic section of System 1, which did not contain any readily biodegradable substrate (e.g. acetate, propionate). The activated sludge was equally divided into 5 volumetric flasks and different amount of substrates (acetate or propionate) was added into each flask. The last flask (flask 5) served as a control and no substrate addition was made. The uptake rates of acetate and propionate were then determined through a series of anaerobic batch tests. Each batch test was performed at 20°C in volumetric flasks with an O₂ free environment. Acetate and propionate samples were taken every 15 minutes to determine the maximum uptake rate of acetate and propionate. Acetate and propionate concentrations were measured using a Dionex Ion chromatograph. For the uptake rate calculations, the substrate utilized was divided by biomass concentration (as MLVSS), thereby normalizing the substrate uptake rates. The solution pH was 7.45, 7.58 and 7.32 at the beginning of the acetate batch test 1, acetate batch test 2 and propionate batch test experiment, respectively.

RESULTS and DISCUSSION

The results of the membrane fatty acids analyses of samples taken from the aerobic stage of the EBPR system at 20, 18, 15, 10 and 5°C are given in Table 1. The relationship between temperature and the fatty acid unsaturated/saturated ratio is illustrated in Figure 6. As shown by the figure, the unsaturated to saturated ratio increased as the temperature decreased, indicating that the bacterial community was capable of homeoviscous adaptation. The results of the study indicate that the membrane fatty acids were composed mainly of C16:1 (33.8%), C16:0 (27.5%) and C18:1 (21.2%), and the unsaturated to saturated fatty acid ratio was 1.31 at 20°C. The unsaturated to saturated fatty acid ratios were 1.40, 1.55, and 2.31 at 18, 15, and 10°C, respectively (Table 1). The composition of the major fatty acids was C16:1 (49.8%), C16:0 (13.2%), C18:1 (25.3%) and the unsaturated to saturated ratio was 3.61 at 5°C. It appears that increased unsaturated fatty acid content (especially cis-9-hexadecanoic acid) at low temperatures is

the mechanism used by EBPR activated sludge to accomplish homeoviscous adaptation. However, in the absence of membrane viscosity measurements, these results alone do not conclusively prove that membrane fluidity increased or stayed constant as temperature was lowered. Due to the complexity of such determinations and their low accuracy for mixed cultures, no attempt was made to determine the membrane viscosity of the samples at different temperatures. Despite the lack of this information, complete acetate uptake (Figure 7) and very good P removal observed at 5°C indicate that the membrane of the EBPR bacterial community was in a fluid rather than a gelled state. Note, however, that the removal of acetate in the second anaerobic reactor was considerably slower at 5°C than at the other temperatures.

The t-test results showed that at an α value of 0.10 there was no significant difference between the membrane composition between short and long-term (steady state) temperature exposures as long as an 8-hour adjustment period was provided. In reality, such large changes in mixed liquor temperatures take several hours to occur. Therefore, from a practical wastewater treatment standpoint, the results of this study have shown that the kinetics of homeoviscous adaptation is not a critical issue to consider.

Table 1. Percent abundance of membrane fatty acids at different temperatures

Fatty acids		Percent abundance of fatty acids				
IUPAC name	# of Carbon	20°C	18°C	15°C	10°C	5°C
dodecanoic	C12(0)	1.94±0.42	2.97±0.34	1.18±0.11	1.0±0.52	0.83±0.31
tri- decanoic	C13(0)*	2.08±0.23	2.24±0.41	2.17±0.16	1.46±0.27	0.84±0.12
n-tetradecanoic	C14:(0)	2.53±1.05	3.78±0.19	2.57±0.07	1.8±0.39	0.97±0.23
pentadecanoic	C15:(0)	1.50±0.24	1.69±0.36	1.66±0.29	2.41±0.27	2.50±0.28
cis,ci- -9,12 hexadecanoic	C16:(2)	0.65±0.35	1.35±0.13	1.18±0.09	0.67±0.38	1.63±0.48
cis-9-hexadecanoic	C16:(1)	33.8±4.14	33.3±1.74	34.7±1.83	40.5±5.86	49.8±2.36
n-hexadecanoic	C16:(0)	27.5±3.65	25.4±0.97	24.9±0.69	20.2±1.57	13.2±1.59
cis,cis-9,12 octadecadienoic	C18:(2)	0.92±0.38	1.08±0.20	0.99±0.8	2.00±0.05	1.00±0.08
cis-9-octadecanoic	C18:(1)	21.2±2.65	22.5±0.69	23.9±0.76	26.6±1.66	25.9±1.96
n-octadecanoic	C18:(0)	7.89±2.4	4.30±0.29	4.9±0.36	3.06±0.41	3.31±0.69

*tri-decanoic acid was used as an internal standard.

Numbers in parentheses refers to number of double bond(s) in fatty acid carbon chain.

Numbers after each ± sign are standard deviation of the mean.

N= 6,2,2,2 and 4 at 20,18,15,10 and 5°C

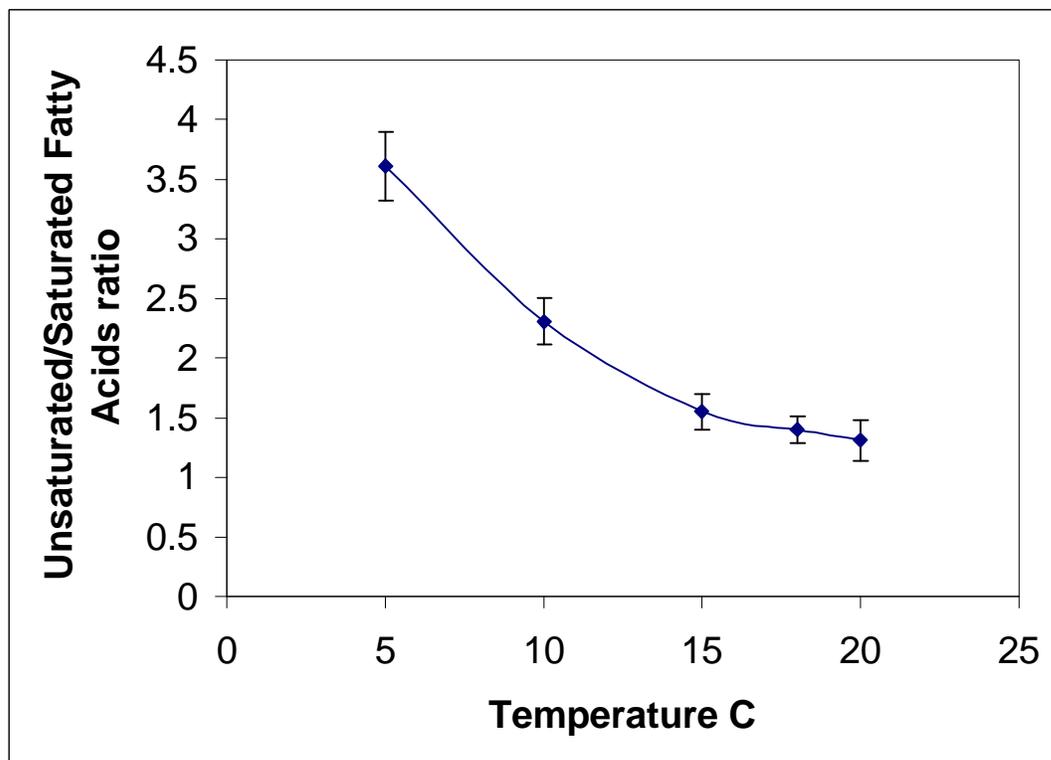


Figure 6. Changes in the fatty acid unsaturated/saturated ratio vs. temperature

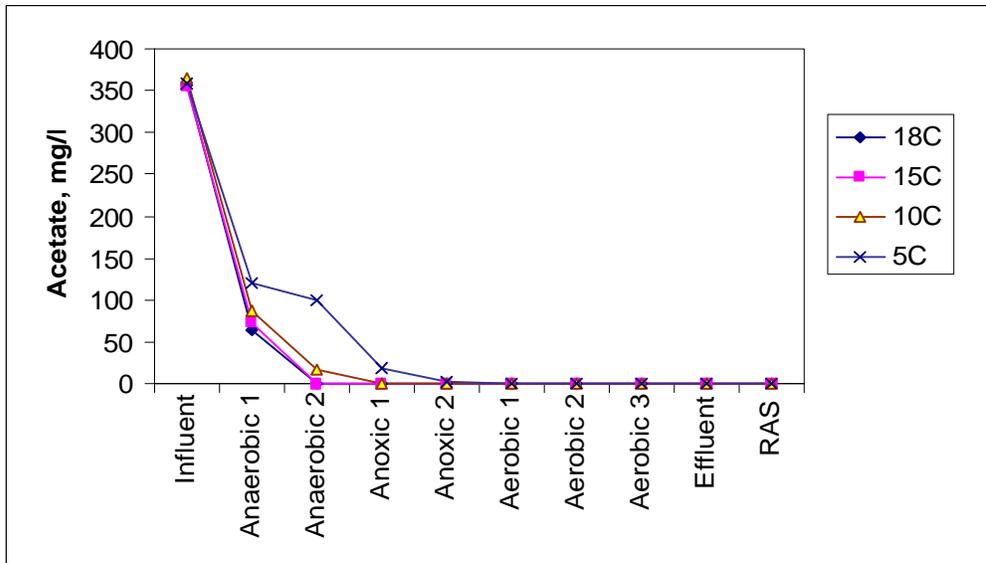


Figure 7. The uptake of acetate throughout the reactors of the EBPR system.

Table 2. Changes in unsaturated fatty acid composition when EBPR sludge taken from 20°C was exposed to 5°C for 24 hours.

	Percent Unsaturated Fatty Acids						5°C Steady State
	0 hr	1 hr	2 hrs	4 hrs	8 hrs	24 hrs	
C16:(2)	0.99	1.02	1.53	1.48	1.33	1.29	1.63 \pm 0.48
C16:(1)	34.8	39.5	43.0	46.7	48.1	51.0	49.8 \pm 2.36
C18:(2)	0.86	1.23	1.31	1.69	1.29	0.98	1.00 \pm 0.08
C18:(1)	22.8	22.8	25.2	24.9	26.3	26.1	25.9 \pm 1.96
total %							
unsaturation	59.5	64.6	71.0	74.8	77.0	79.4	78.3
unsaturation ratio	1.47	1.82	2.45	2.96	3.35	3.85	3.61 \pm 0.30
$t_{\text{calculated}}$	2.83	2.89	2.19	2.21	1.38	1.53	
$t_{\text{critical}}=2.13$ at $\alpha=0.10$							

Substrate transport mechanism for acetate:

The anaerobic acetate utilization rates determined through two anaerobic batch tests are given in Figures 8 and 9. While flask number 1 through 4 in each batch test was received increased amount acetate, flask 5 served as control. Mixed liquor volatile suspended solid (MLVSS) and acetate concentrations at t=0 is given in table 3.

Table 3. Acetate and MLVSS values at t=0.

Flask number	Batch test 1		Batch test 2	
	Acetate mg/L	MLVSS mg/L	Acetate mg/L	MLVSS mg/L
1	225	2152	361	2204
2	525	2141	742	2210
3	786	2139	1320	2149
4	1290	2120	2240	2154
5 (control)	0	2160	0	2208

The maximum acetate uptake rates were observed within the first 45 minutes of each batch test. Each slope value was then divided by the corresponding MLVSS values to determine normalized acetate uptake rates (mgAc/gVSS-min). The relationship between the acetate uptake rates and the acetate concentrations are given in Figure 10 and 11. According to Figure 10 and 11, acetate transport does not obey simple passive diffusion because a linear relationship was not observed for the entire acetate concentration range of 0 to 2240 mg/L (0 to 38 mM). Baronofsky et al. (1984) and Hume et al. (1992) suggested that acetate uptake follows passive diffusion between 0-150 mM and 10-50mM, respectively. An apparent saturation curve with low Km values was also reported byCarroll (1996) who investigated the uptake of labeled acetate by nerve terminals in the acetate range of 0.1 to 100OM. Baranofsky and coworkers (1984) applied much lower pH values as low as 5 was during determination of the transport type of acetate. It should be remembered that, at pH value of 5, 33% of the acetate is in protonated or uncharged form (acetic acid) whereas at pH 7, only 0.5% of the acetate is in uncharged form. Small

uncharged molecules like acetic acid can cross membrane freely via simple passive diffusion (Becker et al. 1996). Therefore their results cover the transport of small uncharged acetate molecules. In this study, the saturation curves indicate that acetate transport is not simple passive diffusion but is either facilitated or active transport. The second evidence to support this conclusion was obtained based upon the batch tests performed at 5 °C (Erdal and Randall 2002 c). In the first batch test, bacterial cells with enriched and depleted poly-P pool utilized acetate anaerobically as shown in Figure 12. It is clear that very negligible acetate was used (62 mg/L vs. 360 mg/L) when poly-P pool of biomass was depleted. If acetate transport were a general leakage (passive diffusion), the same amount of acetate would be taken up in both cases since the same biomass was splitted into two flasks and pH values were identical (7.62). This test clearly showed that acetate uptake does not obey the simple passive diffusion in neutral pH range. The second batch test shown in Figure 13 was performed at 20°C with depleted and enriched biomass conditions. The second batch test showed that bacterial cells took up significant amount of acetate under both conditions but lower amount of acetate was taken up when poly-P reserve of biomass was depleted. In this case, the main energy reserve was glycogen. This result suggests that ATP directly or indirectly involves during acetate transport since bacterial cells at 5°C with depleted poly-P storage only transported a small amount of acetate. Due to slow glycolysis process (Z.K. Erdal, 2002) at 5°C, a limited amount of ATP was generated and therefore very limited ATP was incorporated with transport mechanisms. Batch test 1 performed at 20oC also showed that ATP production with Poly-P cleavage over shadowed that formed through glycolysis. Therefore, it is obvious that ATP plays a key role during acetate transport even though acetate transport is not against its concentration gradient. ATP may directly involve or catalyze symport , antiport or uniporters. These findings favor the metabolic model developed by Smolders and coworkers (1994) who suggested ATP involvement during active transport of acetate molecules into the cells. They also support the assumption for the active acetate transport in the existing models (e.g. Comeau-Wentzel, 1986; Mino model 1987).

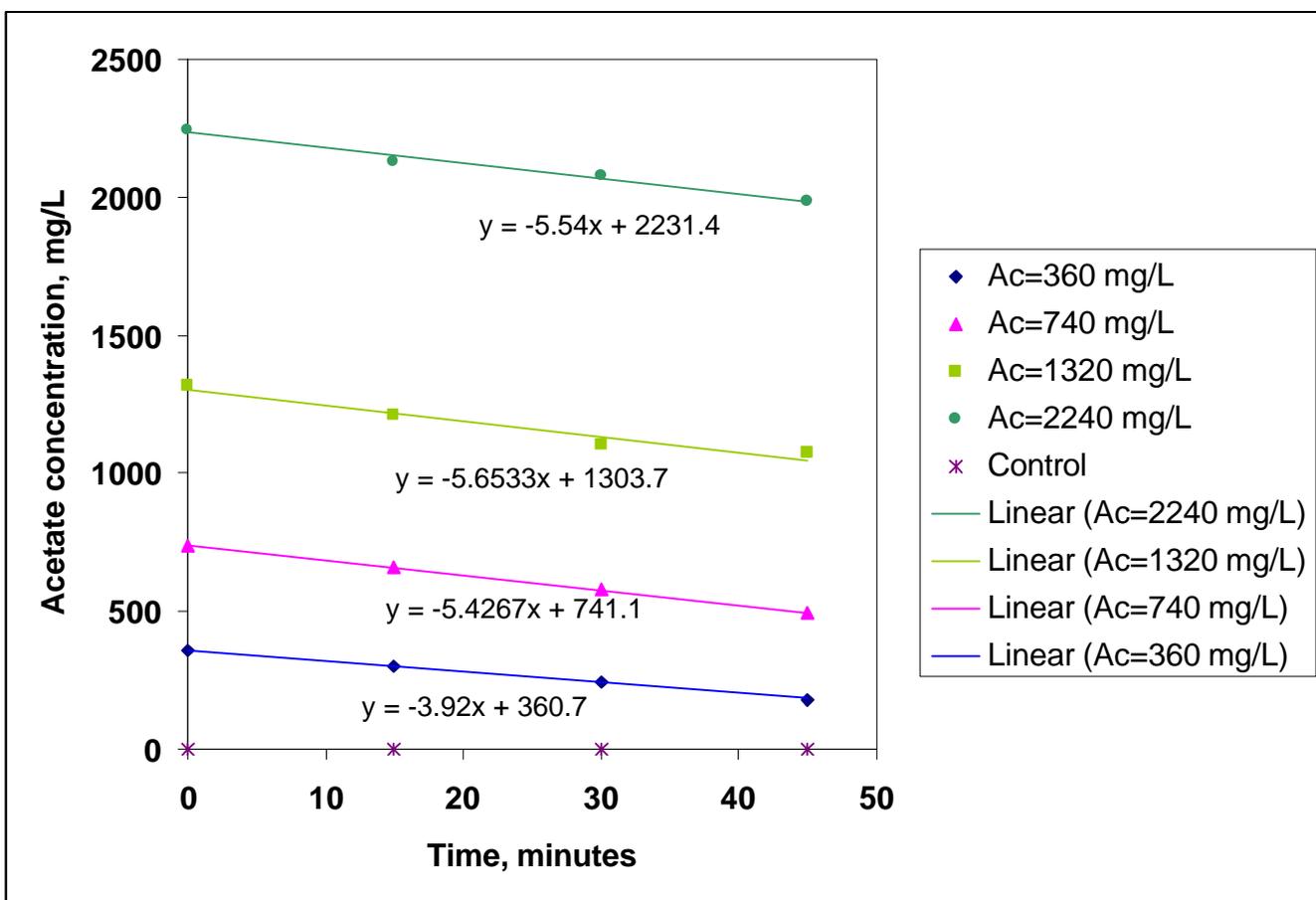


Figure 8. Acetate uptake rates (negative slopes values) obtained during anaerobic batch test 1.

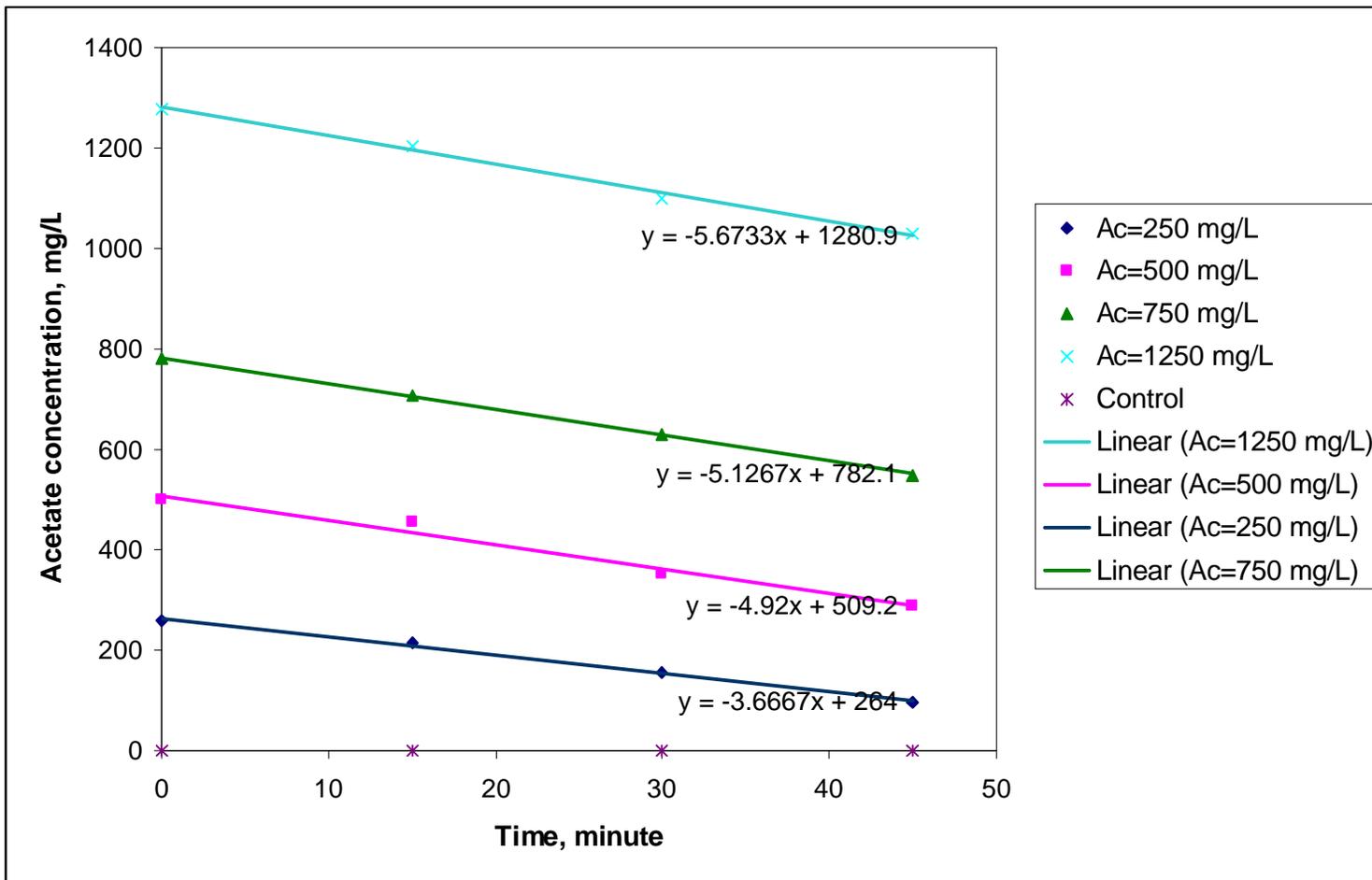


Figure 9. Acetate uptake rates (negative slope values) obtained during the second anaerobic batch test.

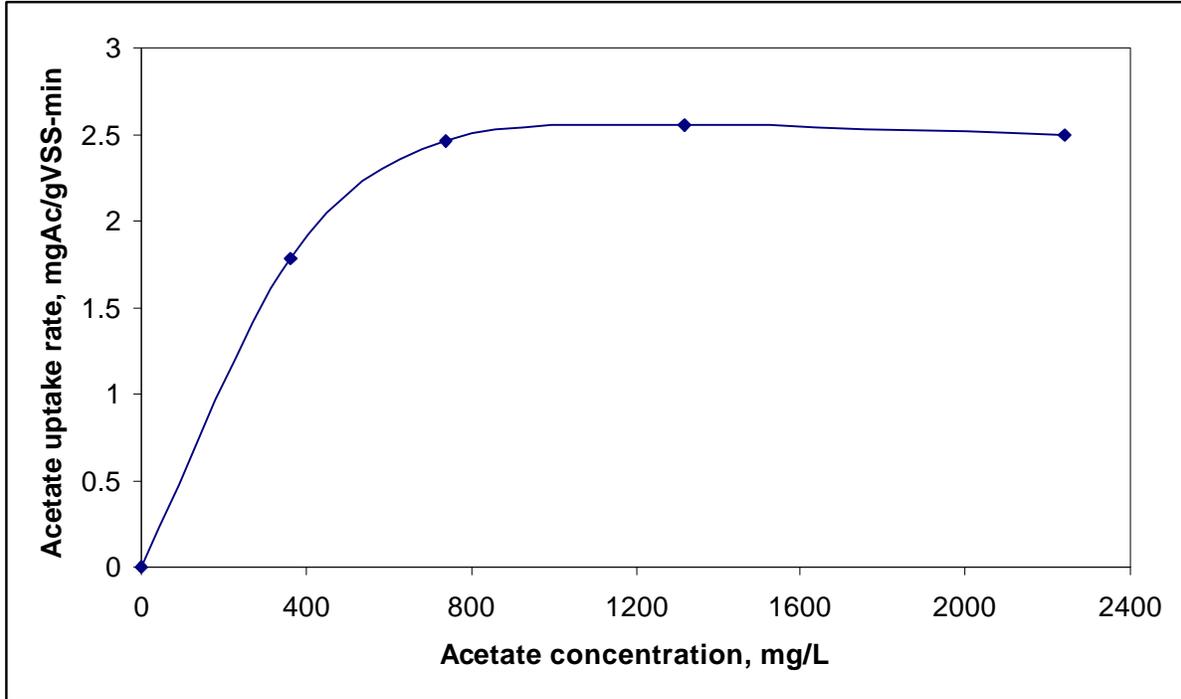


Figure 10. The anaerobic acetate uptake rate vs. acetate concentration through batch test 1 performed at 20°C.

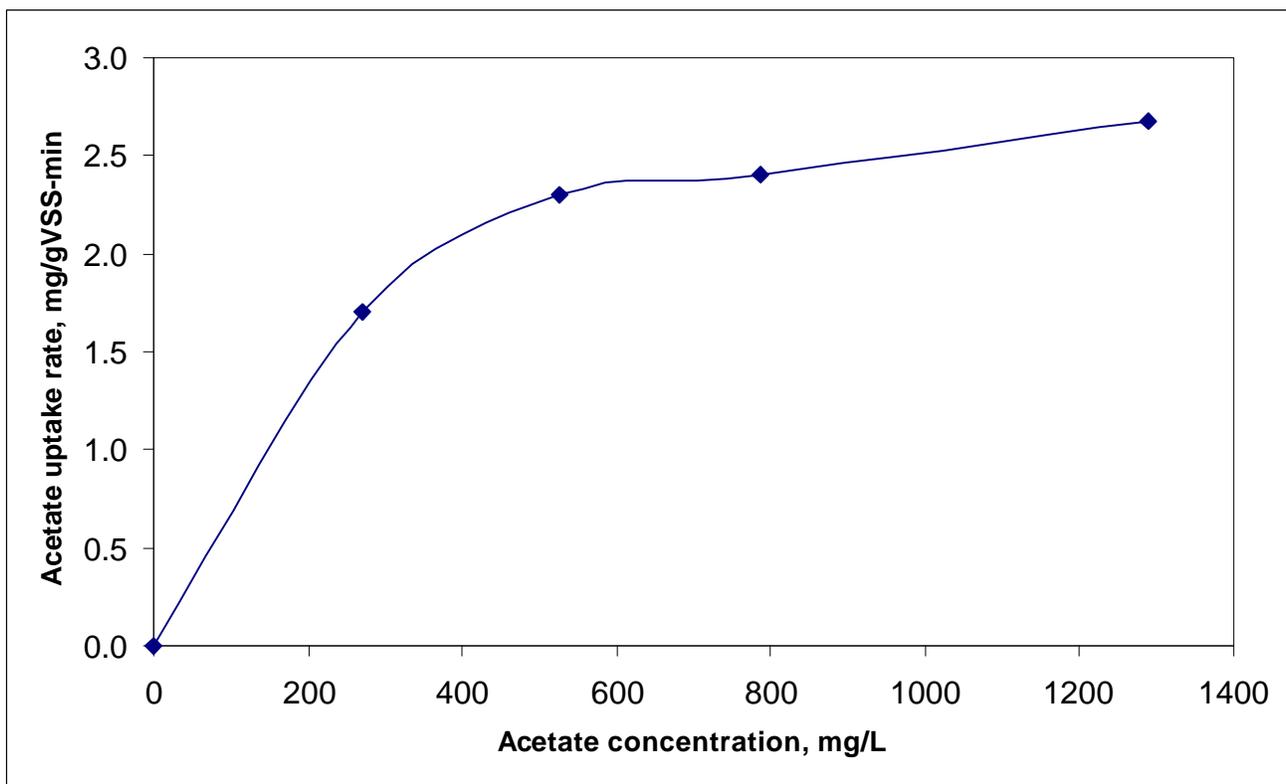


Figure 11. The anaerobic acetate uptake rate vs. acetate concentration through batch test 2 performed at 20°C.

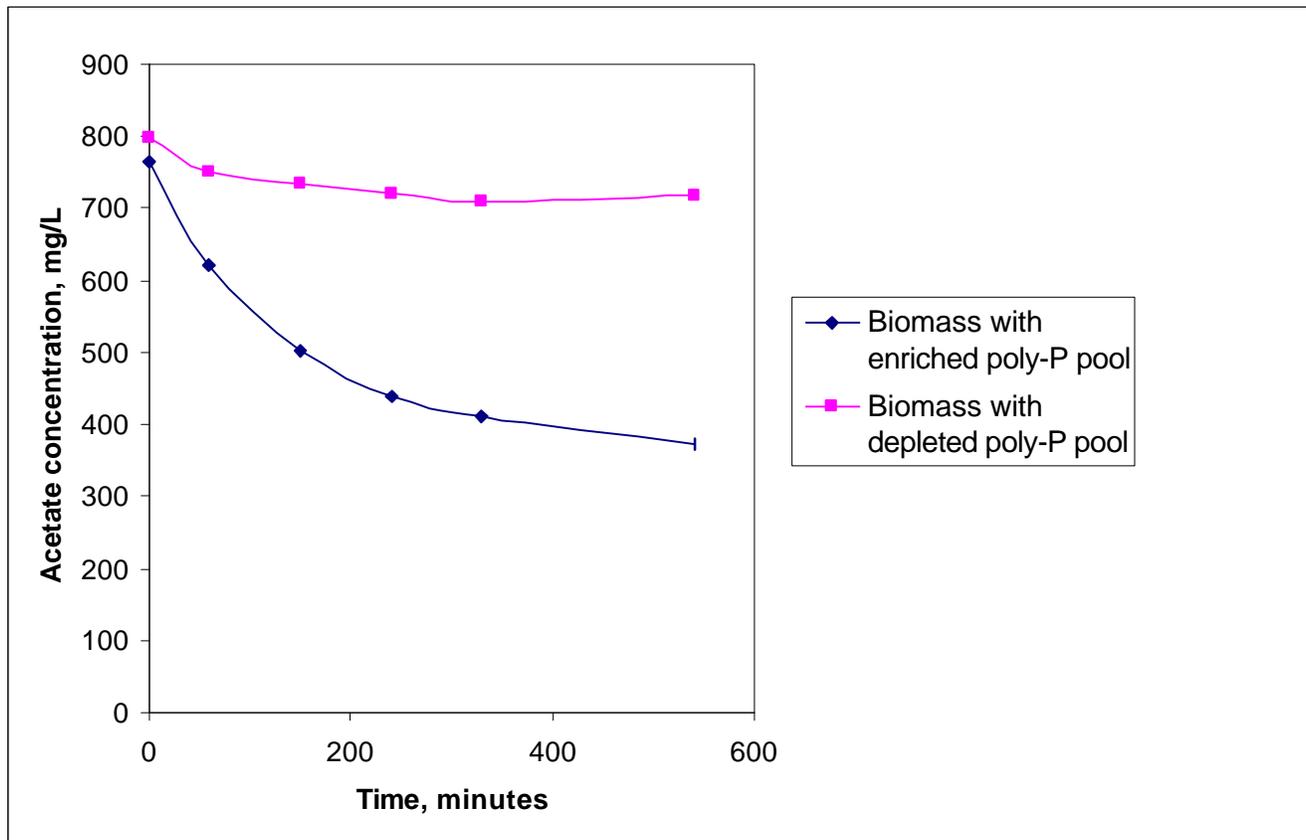


Figure 12. Acetate uptake through batch test 1 performed at 5°C.

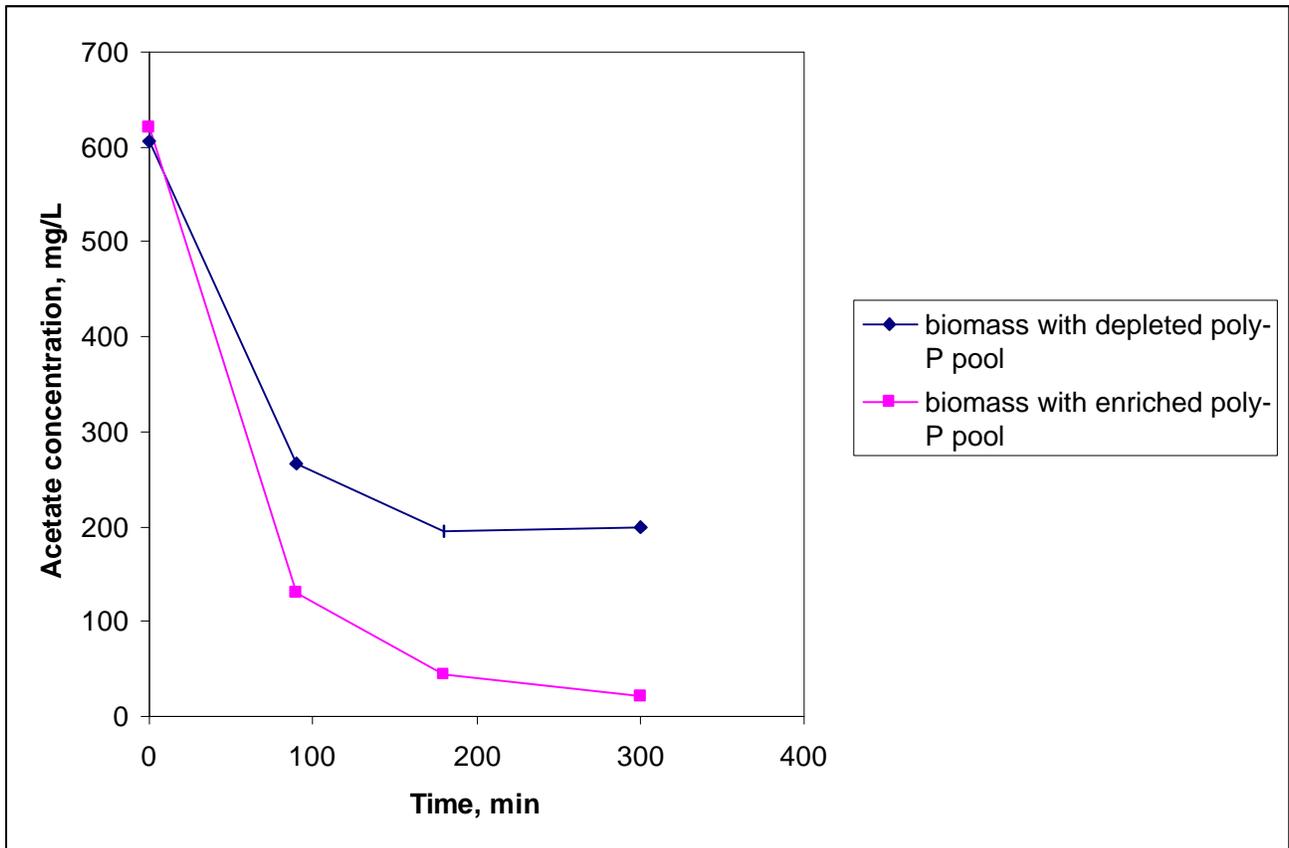


Figure 13. Acetate uptake through batch test 2 performed at 20°C.

Transport mechanism for propionate:

The relationship between anaerobic propionate uptake rate and propionate concentration at 20°C is given in Figure 14 and 15. Despite the application of a lower molar concentration range of propionate in the batch test (0 to 17 mM), no linear relationship was observed when propionate uptake rate was plotted against propionate concentration. Thus it is also concluded that propionate enters into EBPR bacterial cells via ATP dependent transport mechanisms (carrier mediated or active transport)

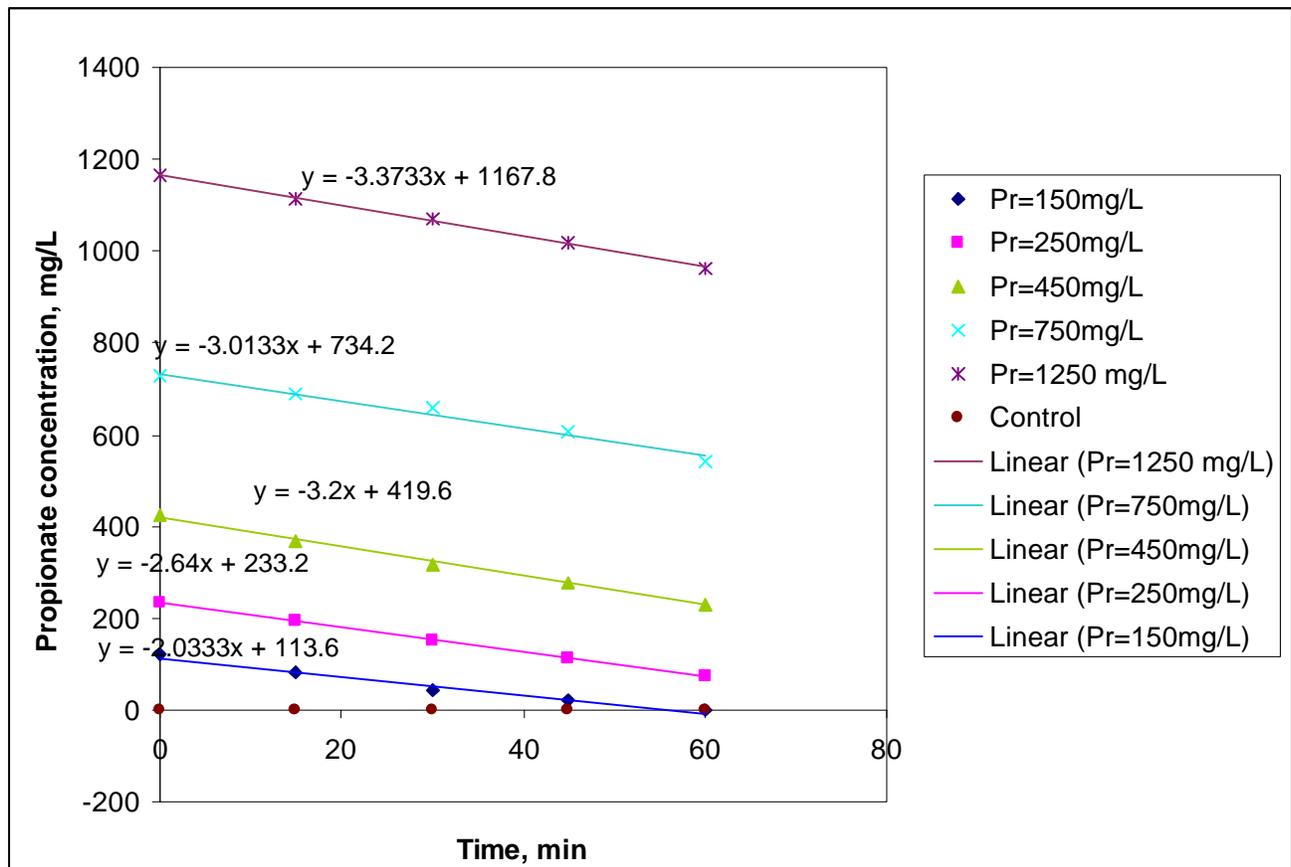


Figure 14. Propionate uptake rates (negative slope values) obtained during the anaerobic batch tests.

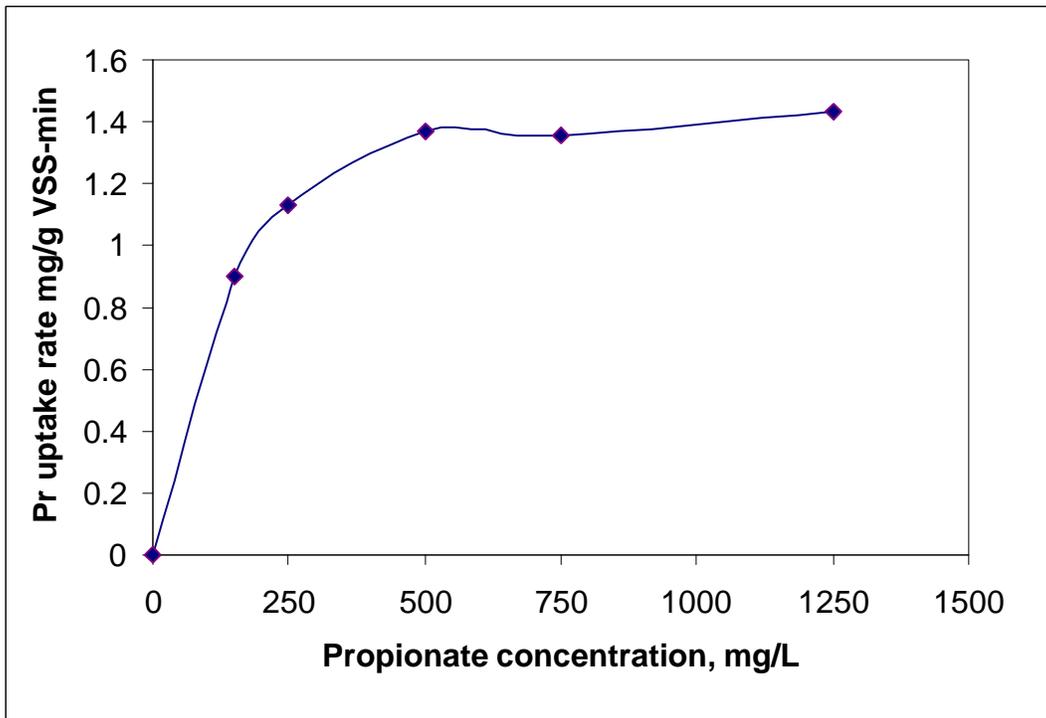


Figure 15. Anaerobic propionate uptake rate vs. propionate concentration in anoxic EBPR sludge at 20°C.

CONCLUSIONS

- The cellular membranes of EBPR bacteria do not become rigid at cold temperatures, Therefore EBPR bacterial community has able to make solute transport and several other functions (e.g. establishing electrochemical gradient) even at 5°C.
- The EBPR bacterial community accomplishes homeoviscous adaptation at cold temperatures by increasing the unsaturated to saturated fatty acid ratio in the side chains of the fatty acids in the cellular membrane.
- It is likely that the decreased EBPR efficiency at cold temperatures reported in the literature for acetate substrate occurred because of the application of unsuitable operationing conditions (excessive electron acceptor recycle, below critical SRT, low anaerobic detention time, use of non-acclimated sludge, enzyme denaturation, etc.), rather than from cellular membrane changes.
- Both acetate and propionate are transported across activated sludge bacterial cellular membranes by either carrier mediated transport or active transport, rather than by simple diffusion, because substrate uptake rates determined as a function of initial substrate concentration follow a saturation curve rather than a linear fit. Batch tests performed at 20 and 5°C strongly indicates that the acetate transport is ATP dependent process and its slow generation at cold temperature inhibits the transport process. Even though many active transport processes involve solute molecules to move against their concentration gradients, the symporter, antiporter and uniporter which, move solutes down to concentration gradient, needs energy (White, 1995).
- The apparent ATP requirement favors the metabolic model (Smolders et al. 1994) and the major EBPR models developed by Comeau-Wentzel (1986) and Mino and coworkers (1987).

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