A thermal adaptation of bacteria to cold temperatures in an enhanced biological phosphorus removal 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 regarding the effect of temperature on kinetic reaction rates, there are contradictory results in the literature regarding the effect of temperature on EBPR system performance. Early investigators (Sell, Ekama *et al*., Daigger *et al*.) reported better performance with lower temperatures, but others have reported partial or complete loss of EBPR functions at low temperatures (McClintock *et al*., Brdjanovic *et al*., Beatons *et al*.). One speculation is that deterioration in the EBPR system performance at cold temperatures can be attributed to rigid-like behavior of the cell membranes. 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. 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 suggested 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 (low SRT, excessive electron acceptors, low anaerobic detention time, non-acclimated sludge, etc.).

Keywords Activated sludge; cold temperatures; EBPR; homeoviscous adaptation

Introduction

The membranes of microbial cells do not simply define the boundaries of the cell and delineate its compartments, but also serve to control specific functions such as regulating movement of substances into and out of the cells and there compartments (Becker *et al*., 1996). 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 number of *cis* double bonds which makes them unsaturated (Pringle and Chapman, 1981). The heterogenity of the fatty acid structure confers unique thermodynamic properties for cells such as setting the transition temperature. The transition temperature is a critical temperature below which the membrane is in solid state and above which the membrane is in fluid state. 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). However, the resulting bends lower Van der Waals interaction and thereby lower the transition temperature (Voet and Voet, 1995).

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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. More commonly, adaptation to temperature involves alteration in the extent of unsaturation rather than in the chain lengths. Even though the mechanism of homeoviscous adaptation is not fully understood yet, 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).

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 Souza (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. Satoh 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, 16 carbon-number with one double bond) in the membrane phospholipids. 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. Trigari *et al*. (1992) investigated homeoviscous adaptation phenomena in warm and cold adapted sea bass. The total lipids of liver mitochondria and microsomes indicated no increase in unsaturation response to the cold, indicating that homeoviscous adaptation did not occur. Fodor (1997) investigated lipid compositions of two symbiotic photosynthetic bacteria, *Xenorhabdus nematophilus* and *Photorhadbus 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. This suggested that these particular bacterial species were unable to make 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 was reported in several studies under downward temperature shifts (McClintock *et al*., 1991; Mamais and Jenkins, 1992; Brdjanovic *et al*., 1997). Such deterioration may be related to the inability of bacteria to make homeoviscous adaptation. However, no study has been performed to investigate this paradoxial case in activated sludge systems. In this study, the membrane fluidity of EBPR sludge enriched with phosphorus accumulating organisms (PAOs) is investigated at 20, 18,15,10 and 5°C to understand whether phosphate-accumulating organisms (PAOs) lose their ability to take-up substrate at low temperatures or they can adapt.

Methods and materials

A lab scale University of Cape Town (UCT) system containing two anaerobic (2 L each), two anoxic (2 L each) and three aerobic (3.5 L each) reactors in series and fed with synthetic

wastewater, was operated at 20°C for more than 6 months. The plant schematic is illustrated by Figure 1. Synthetic feed was prepared daily with acetate and supplemental yeast extract to contain 450 mg/L COD, $(NH4)_2SO_4$ 40 mgN/L, K_2HPO_4 25 to 80 mg/L, 125 mg/L alkalinity, 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 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 includes SCOD, acetate, MLSS, MLVSS, NO_3^--N, NO_2^--N, NH_4^+-N, PO_4^{-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 steps 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. Both system performance and cellular membrane fluidity changes were determined at each temperature, and the determinations were continued to define acclimated conditions.

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 acclimized 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, 6, 12 and 24 hours after exposure to the lower temperature. Fatty acid composition of the cells were determined during the short term temperature exposure study and compared to those observed during the long term temperature exposure.

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), 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. 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% K_2CO_3 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 spectrometer furnished with a mass spectrophotometer, located in the biochemistry department of Virginia Tech, was used throughout the GC/MS runs.

Results and discussion

The results of membrane fatty acids analysis 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 2. As shown by the figure, the unsaturation to saturation ratio increased as the temperature

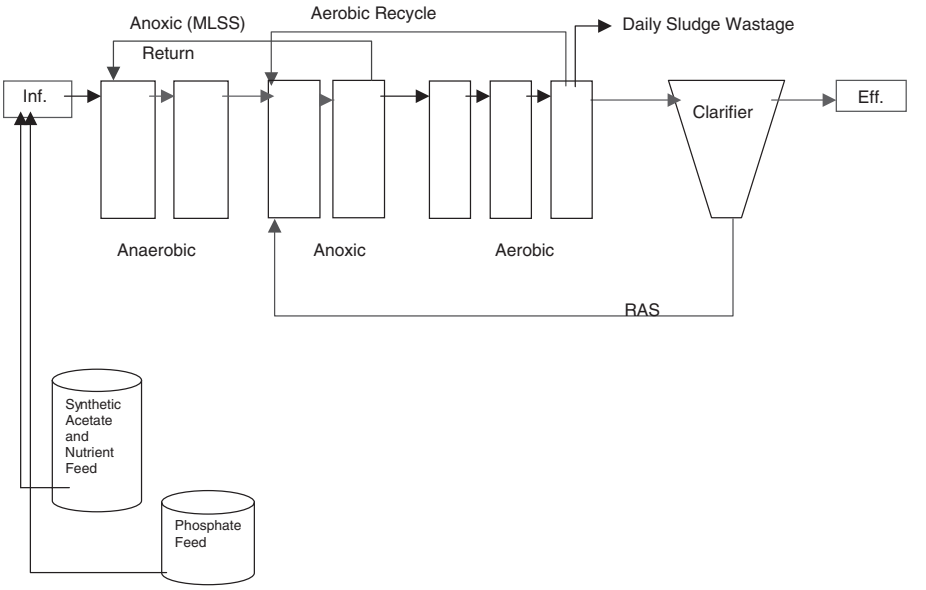

Figure 1 Flow schematic of the lab-scale UCT system

decreased. 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, 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 responsible for achieving homeoviscous adaptation in activated sludge. However, in the absence of membrane viscosity measurements, these results alone do prove that membrane fluidity increased or stayed constant as temperature was lowered. Due to the complexity of such determinations and low accuracy in mixed cultures, no attempt was made to determine the membrane viscosity of samples at different temperatures. Despite the lack of this information, complete acetate uptake and very good P removal observed at 5°C suggest the conclusion that the membrane of the EBPR bacterial community was in a fluid rather than a gelled state.

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
three 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
penta decanoic	C15:(0)	1.50±0.24	1.69±0.36	1.66±0.29	2.41±0.27	2.50±0.28
cis,*cis* -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

* three 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

The *t*-test result at an α value of 0.05 showed that there is no significance difference between short and long-term temperature exposures (steady state) as long as a 2 hour adjustment period was provided ($t_{calc} < 2.26$). In reality, such significant changes in mixed liquor temperature 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.

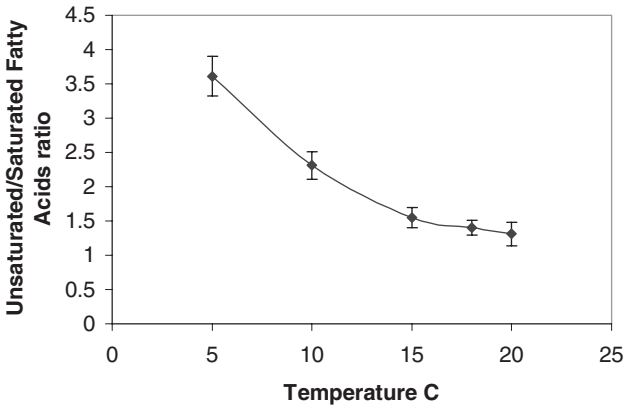

Figure 2 Changes in the fatty acid unsaturated/saturated ratio vs. temperature

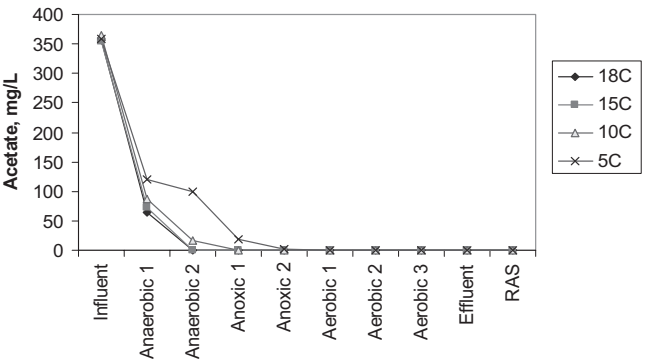

Figure 3 The uptake of acetate throughout the reactors of the EBPR system

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						Steady
	0 hr	1 hr	2 hrs	4 hrs	8 hrs	24 hrs	State
C16:(2)	0.99	1.02	1.53	1.48	1.33	1.29	1.63
C16:(1)	34.8	39.5	43.0	46.7	48.1	51.0	49.8
C18:(2)	0.86	1.23	1.31	1.69	1.29	0.98	1.00
C18:(1)	22.8	22.8	25.2	24.9	26.3	26.1	25.9
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
$t_{calculated}$	2.83	2.89	2.19	2.21	1.38	1.53	

$t_{critical} = 2.26$ at $\alpha = 0.05$

Conclusions

- The EBPR bacterial community can achieve homeoviscous adaptation at cold temperatures simply by increasing the unsaturated fatty acid ratio in the side chains of the fatty acids in the cellular membrane.
- It is likely that the decreased EBPR efficiency reported at cold temperatures reported in the literature occurred because of the application of unsuitable operational conditions (electron acceptor recycle, low SRT, low anaerobic detention time, non-acclimated sludge, etc.), rather than from cellular membrane changes or enzymatic inhibition.

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