DEVELOPMENT OF MICROBIAL FATTY ACID ANALYSIS AS A MONITORING TOOL FOR BIOLOGICAL WASTEWATER TREATMENT SYSTEMS

by

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Abstract

The development of culture-independent methods for the identification of microbial community has opened a new era of study into real microbial populations and their dynamics. Although many attempts have been made to link such dynamics to system performance, few have been successful.

In this research, a microbial fatty acid analysis technique was developed and evaluated for the detection of changes in microbial community populations associated with system performance. For the evaluation, three phases of experiments were executed. In Chapter 2, operating parameters such as pH, organic loading and chlorine addition, were varied in two identical laboratory-scale conventional activated sludge systems. Daily and cumulative similarity indices based on microbial fatty acid analysis were used to express the stability of microbial community populations in the systems. It was found that microbial compositions changed daily even under constant operating conditions and that the rate of change increased under dynamic operating conditions. The analyses of microbial fatty acids (MFA) also conveyed additional information: if they are routinely executed as a monitoring tool for biological wastewater treatment systems, MFA analyses could be used for the calculation of biomass concentrations in a wastewater treatment system. The total fatty acid concentrations were estimated to be about 6.1% of the biomass concentration, measured as mixed liquor volatile suspended solids concentrations in this research.

In Chapter 3, the new monitoring technique developed in Chapter 2, was applied to bioreactors with real municipal wastewater. The experiments demonstrated that the MFA analysis technique was also applicable to the reactors fed with real municipal wastewater. From the results, it was found that changes in operating factors such as pH, DO, chlorine addition impacted more significantly on the microbial community population and system performance in real municipal wastewater and the monitoring method developed was also applicable to a system subject to more dynamic operating conditions, suggesting that this technique could be used for pilot-scale or full-scale wastewater treatment monitoring.

The MFA monitoring technique was also evaluated using a lab-scale simplified University of Cape Town (UCT) reactor, to determine whether the technique was applicable for
detecting a trend in a microbial population of an EBPR process (phosphorus-accumulating organism (PAO) dominant microbial population) toward a glycogen-accumulating organism (GAO) dominant microbial community structure. In order to convert a PAO-dominant microbial population to a GAO-dominant structure, several methods were tried: namely, low phosphate to carbon ratio (P/C) feed, high-temperature operating conditions (25°C and 30°C), and a mixed glucose and acetate feed. Among the three trials, only the glucose and acetate feed effectively converted the EBPR system to a GAO-dominant structure. The MFA technique clearly showed the transition from a PAO-dominant microbial population to a GAO-dominant one, through an increase in shift rates of microbial community structure. Similarity indices (daily and cumulative) based on microbial fatty acid analysis were used to estimate the shift rates of microbial community structures in the system. The experimental results demonstrated that the new monitoring technique could be a useful tool for preventing EBPR deterioration by monitoring the change in microbial population from PAO dominance to GAO dominance in EBPR.

These experimental results showed that the MFA analysis technique with a simple similarity index calculation could be a useful tool to monitor changes in microbial community populations, which might be applied to advanced biological wastewater treatment system design and operation.
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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ARDRA</td>
<td>Amplified ribosomal DNA restriction analysis</td>
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<tr>
<td>BNR</td>
<td>Biological nutrient removal</td>
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<tr>
<td>BPR</td>
<td>Biological phosphorus removal</td>
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<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DO</td>
<td>Dissolved oxygen</td>
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<td>EBPR</td>
<td>Enhanced biological phosphate removal</td>
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<td>ED pathway</td>
<td>Embden-Doudoroff pathway</td>
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<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>GAC</td>
<td>Granular-activated carbon</td>
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<td>GAO</td>
<td>Glycogen accumulating organisms</td>
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<td>GC/MS</td>
<td>Gas spectrometer/mass spectrometer</td>
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<td>HRT</td>
<td>Hydraulic retention time</td>
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<td>MFA</td>
<td>Microbial fatty acid</td>
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<td>MK</td>
<td>Menaquinones</td>
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<td>MLSS</td>
<td>Mixed liquor suspended solids</td>
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<td>MLVSS</td>
<td>Mixed liquor volatile suspended solids</td>
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<td>NaAc</td>
<td>Sodium acetate</td>
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<tr>
<td>pA</td>
<td>Pico Ampere</td>
</tr>
<tr>
<td>PAO</td>
<td>Phosphorus accumulating organisms</td>
</tr>
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<td>PCA</td>
<td>Principal component analysis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PHA</td>
<td>Polyhydroxyalkanoate</td>
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<td>PHB</td>
<td>Poly-beta-hydroxybutyrate</td>
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<td>PLFA</td>
<td>Phospholipid fatty acid analysis</td>
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<td>Q</td>
<td>Ubiquinones</td>
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<td>RFLP</td>
<td>Restricted fragment length polymorphism</td>
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<td>RISA</td>
<td>Ribosomal intergenic spacer analysis</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SBR</td>
<td>Sequencing batch reactor</td>
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<td>SRT</td>
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<td>Suspended solids</td>
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<td>TKN</td>
<td>Total Kjeldahl nitrogen</td>
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<tr>
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<td>Total phosphorus</td>
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<td>Total suspended solids</td>
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Chapter 1
Dissertation Objective and Overview

1.1 Introduction

Although the concept of wastewater treatment using micro-organisms began more than 100 years ago, even the most modern biological wastewater treatment processes are still designed and operated without serious consideration of the microbial ecology (Werker, 1998). In general, simple lab-scale tests or mathematical models are applied for the design and operation of the wastewater treatment systems (Porter and Pickup, 2000). However, the parameters that have been developed from the so-called “black box” approach might be of limited value for application to full-scale treatment systems, because the systems’ microbial populations may be different based on culture history and growth conditions created by the system configuration and operating conditions. Without understanding the microbial ecology in a biological wastewater treatment system, it may be difficult to overcome current problems that are often faced when running a system, such as bulking, foaming, rising sludge, and phosphorus removal breakdown. These problems are likely associated with the microbial populations. Although these phenomena seemingly occur without any “early warning” to operators, the change from desirable microbial populations to undesirable microbial populations may begin earlier than the problems are detected.

During the last few decades, many efforts have been made to develop tools for studying microbial populations in an ecosystem. The efforts have been mainly focused on
developing culture-independent methods, because traditional culture methods can only identify a low percentage (0.1 – 10%) of the bacteria that have been seen by microscopic techniques (Head et al., 1998). The culture-independent methods use nucleotide sequences of micro-organisms (deoxyribonucleic acid (DNA)/ribonucleic acid (RNA)), such as fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and ribosomal intergenic spacer analysis (RISA). From these culture-independent techniques, many remarkable results have been reported that were not achievable with traditional plate-counting methods (Amann et al., 1998). FISH has been used to track population changes of nitrifying or phosphorus accumulation bacteria (Onuki et al., 2000; Nielsen et al., 1998; Luxmy et al., 2000). DGGE and RISA can be used to monitor community structures and can track single or multiple strains of bacteria by DNA/RNA sequencing and probing. Along with these molecular techniques, several chemo-taxonomic methods, such as respiratory quinone and microbial fatty acid analysis, have been successfully applied to characterizing bacterial community structures (Hiraishi et al., 1998). (Details about culture-independent methods can be found in the introduction section of Chapter 2).

However, in spite of the developments of molecular or chemo-taxonomic methods for microbial ecology, research on the relationship between microbial community structures and process performance is still in the early stages (Amann et al., 1998).

From a practical point of view (for system operation), the knowledge about the detailed
microbiology may still be a hard subject to understand. For example, the current research information that β-proteobacteria are predominant in an effective wastewater treatment system may not be useful to operating engineers. They may need to know how to keep the bacterial populations using the information in their systems. Therefore, the gaps between scientific information and practical requirements need to be bridged.

From the literature, it has been shown that changes in operating factors of the system such as HRT, temperature, DO, and pH affect its microbial populations, which may influence system performance (Wang and Park, 1998; Princic et al., 1998; Fernandez et al., 1999). The monitoring of the microbial population in a system may detect and prevent system problems in advance, which is a good starting point for more efficient design and control of activated sludge systems. Therefore, the development of monitoring tools for microbial community structures can be a beneficial endeavour.

1.2 Dissertation Objective and Overview

As discussed above, the development of monitoring tools may be one way to bridge the gap between information on microbial community composition and its application to system design and operation. Through a review of the literature, the microbial fatty acid (MFA) analysis technique using whole cell fatty acids was selected as a potential tool for the current research (refer to Section of 2.1 in Chapter 2).
Therefore, the hypothesis of this dissertation is:

"MFA analysis may be used as a monitoring tool for correlating microbial community structures and system performance."

The experiments for this dissertation were designed to test this hypothesis. The objective of each chapter was developed from a question on how the hypothesis could be tested.

The objective of Chapter 2 was the establishment of basic tools and relationships between operating conditions and microbial community structures. Two identical lab-scale conventional activated sludge systems with synthetic wastewater (sodium acetate as a main carbon source) were prepared in the Chapter 2 experiment. A tool to express the changes in microbial community structures was selected (similarity indices – daily and cumulative) to determine its feasibility. Further, because the use of microbial fatty acid profiles with a simple similarity index is not a conventional approach and the literature does not report any relationship between a similarity index and microbial population changes, the relationship between the two factors was established using model microbial community systems. With the selected tools as described above, it was determined whether or not the changes in microbial community structures could be applied to monitor changes in microbial community structures associated with changes in operating factors, such as substrate composition and strength, pH, and toxic material loading. Also, it was determined whether or not the concentration of microbial fatty acid could be used as an estimator of biomass.
The experimental results in Chapter 2 demonstrated a new concept that can link the monitoring of microbial community structure with biological wastewater treatment system performance. Further experiments of this type necessitated a change from a synthetic feed based on acetate, to raw municipal wastewater. It would be a valuable accomplishment to compare the results acquired from bioreactors fed with synthetic wastewater to reactors fed with real municipal wastewater from a pilot plant, since the many interesting results from bioreactors with synthetic wastewater have shown limited applicability to waste treatment systems that are subjected to more dynamic operating conditions. Therefore, the main objective of Chapter 3 was to determine whether the monitoring method using MFA analysis would be applicable to the more dynamic microbial community structures such as might be expected in pilot-scale or full-scale systems treating real municipal wastewater. Although the reactors used in this experiment were not pilot-scale or full-scale systems, real municipal wastewater was assumed to provide a more dynamic environment for the microbial population because the strength and composition of the raw wastewater, which were shown to impact on microbial community structure in the previous experiment, were continuously changing.

In Chapter 4, the applicability of the MFA technique as a monitoring tool was extended to an enhanced biological phosphorus removal (EBPR) system. A literature reported that the MFA profiles of PAO (phosphorus accumulating organism)-dominant and GAO (glycogen accumulating organism)-dominant communities were significantly different (Liu et al., 2000; Lee et al., 2002), was the motivation to determine the applicability of the MFA
technique for EBPR systems. The transition from a PAO-dominant microbial community structure to a GAO-dominant one necessitates changes in MFA profiles of biomass, resulting in an increased shift rate of the microbial community structure. The main objective in Chapter 4 was the answer to the following question: "Is analysis of microbial fatty acids a useful monitoring tool for detecting the transition of an EBPR process from a desirable PAO-dominant community structure to a detrimental GAO-dominant structure?"

To answer the research question, a lab-scale simplified UCT process was used for the EBPR operation. In order to convert a PAO-dominant community structure to a GAO-dominant one, three well-known methods (according to the literature) were employed. These were a low phosphate to carbon ratio (1.5:120) feed, high temperature (30°C) application, and a glucose and acetate substrate mixture. To calculate the shift rate of the microbial community structure, daily and cumulative similarity indices were also employed, as described in Chapters 2 and 3.

In Chapter 5, a brief summary of the research results, research contributions and future work has been provided in Chapter 6.
1.3 References


Chapter 1: Dissertation Objectives and Overview


Chapter 2

Development of Monitoring Tool Based on Microbial Fatty Acid (MFA) Analysis Using Lab-scale Bioreactors Fed with Synthetic Wastewater

Abstract

Estimating the stability of microbial community populations may be useful in advanced biological wastewater treatment system design and operation. In this research, a monitoring method using fatty acid profiles was evaluated for the detection of changes in microbial community populations. For the evaluation, operating parameters such as pH, organic loading and chlorine addition, were varied in two identical laboratory-scale conventional activated sludge systems. A similarity index based on microbial fatty acid analysis was used to express the stability of microbial community populations in the systems. The experimental results showed that microbial compositions changed daily even under constant operating conditions and that the rate of change increased under dynamic operating conditions. Substrate changes brought about a relatively large change in a microbial community population, eventually resulting in a very different microbial community. After only 7 days following a substrate change in a lab-scale bioreactor, the biomass exhibited only 45% similarity to the original population. The analyses of microbial fatty acids (MFA) also conveyed additional information; if they are routinely executed as a monitoring tool for biological wastewater treatment systems, MFA analyses could be used for the calculation of biomass concentrations in a wastewater treatment system. The total fatty acid concentrations were estimated to be about 6.1% of the biomass concentration, measured as mixed liquor volatile suspended solids concentrations in this research. These experimental results showed that the MFA analysis technique with a simple similarity index calculation could be a useful tool to monitor changes in microbial community populations, which might be applied to advanced biological wastewater treatment system design and operation.
2.1 Introduction

Biological wastewater treatment systems are complex entities composed of large numbers of micro-organisms such as bacteria, archaea, and microeucaryotes (including fungi, algae, and protozoa) in community populations that are influenced by environmental factors (Werker and Hall, 2001; Porter and Pickup, 2000). However, the design and operation of wastewater treatment systems is usually based on simple lab-scale tests or mathematical models that consider these microbial communities to be a single or pseudo-species (Werker, 1998). The kinetic and design parameters that result from this approach are potentially limited in usefulness because the composition of the microbial community depends on the growth conditions created by the system configuration and the operating conditions (Werker and Hall, 2001). For more efficient design and control of activated sludge systems, it is necessary to understand the relationships between system performance and the microbial community population. If system problems could be detected and prevented by advance monitoring of microbial community stability, it would be a good starting point for system optimization. Therefore, the development of monitoring tools for microbial community populations can be beneficial.

2.1.1 Understanding of Microbial Community Population

The study of microbial community populations in microbial ecology is a rapidly growing research field, catalyzed by the development of culture-independent techniques.
Conventionally, the study of population dynamics has used microscopic observations, respirometers and traditional culture-dependent techniques such as plate counting methods (Amann et al., 1998). However, these techniques have some drawbacks. Microscopic methods are useful for the detection of filamentous organisms that can be indicators of sludge bulking and foaming, and of higher organisms such as protozoa and metazoa that can be indicators of sludge status. It should be noted, however, that morphology of all bacteria is a quite unstable character and subsequently is not a good foundation for a sound identification (Amann et al., 1998). For example, although several treatment plants seemingly exhibited the same filamentous organisms using the microscopic method, some exhibited bulking problems, while others did not (Kanagawa et al., 2000).

Respirometric methods have been applied in the detection of toxic materials in the influents to wastewater treatment systems. Moreover, significant effort has been expended on the online assessment of toxic wastewaters (Geenens and Thoeye, 1998; Chan et al., 1999). Since these methods detect only O₂ or CO₂ or CH₄ concentrations associated with biological activity, the techniques cannot detect problems caused by changes in microbial community composition, that may be associated with bulking, foaming and poor settling.

Recently, the rapid advancement of molecular ecological methodologies has allowed the field of microbial community dynamics to take a closer look at microbial community composition. Through the use of culture-independent molecular or chemo-taxonomic techniques, new insights into the composition of uncultivated microbial communities have
been gained (MacNaughton et al., 1999). Various methods of assessing microbial diversity have been developed. Many chemo-taxonomic and molecular methods are now available. These methods include: whole-cell microbial fatty acid (MFA) analysis (Werker, 1998); phospholipid fatty acid analysis (PLFA) (Webster et al., 1997; Haack et al., 1994); respiratory quinones analysis (Hu et al., 1999; Hiraishi et al., 1998; Nozawa et al., 1998); amplified ribosomal DNA restriction analysis (ARDRA) (Liu et al., 1997; Roberts et al., 1998); denaturing gradient gel electrophoresis (DGGE) (MacNaughton et al., 1999; Zhang and Fang, 2000); ribosomal intergenic spacer analysis (Yu and Mohn, 2001; Garcia-Martinez et al., 1999; Fisher and Tripplet, 1999); and length heterogeneity polymerase chain reaction (LH-PCR) analysis (Suzuki et al., 1998).

The techniques have both strengths and weaknesses and detailed understanding of the techniques would be useful in the selection of the best technique for the present research. In the following section, the principles and applications of the each technique will be introduced in more detail.

2.1.2 Introduction of the Techniques that are Available for Monitoring Use

2.1.2.1 Microbial Fatty Acid Analysis

Lipid analysis has been developed by microbial ecologists to measure microbial community biomass, population and activity under in situ conditions (Vestal and White, 1989). Phospholipids are essential membrane components of living cells. Since different groups of
micro-organisms synthesize a variety of phospholipid fatty acids (PLFA) through various biochemical pathways, PLFA are effective taxonomic markers (Werker, 1998). While quantitative variations in the relative abundance of fatty acids in a given profile have been observed with changes in the composition of the growth medium and with the age of the culture, the characteristic patterns remain distinctive for many groups. Thus, pure bacterial cultures grown under controlled growth conditions display unique whole cell fatty acid profiles that can be used to differentiate even closely related organisms (Eerola and Lehtonen, 1988). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community, as certain fatty acids, such as trans and cyclopropyl PLFA, provide indications of environmental stress (White et al., 1998).

The PLFA method has been successfully applied to the identification of the microbial community compositions in soil (Kelly et al., 1999; Baath et al., 1995; Frostegard et al., 1997; Kelly and Tate, 1998; Griffiths et al., 1999; Wagner-Dobler et al., 1998), in wastewater treatment systems (Moll and Summers, 1999; Werker and Hall, 1999; Wang and Park, 1998; Princic et al., 1998), and in biofilter systems (Lipski et al., 1992; Webster et al., 1997). Webster et al., (1997) investigated how the declining pH associated with sulfide oxidation affected microbial growth, microbial density, micro-organism stress and microbial community composition by using phospholipid fatty acid analyses. In their experiments, compost and granular-activated carbon (GAC) biofilters were applied to low concentrations of hydrogen sulfide and volatile organic compounds. Figure 2-1 shows the change of microbial community population with changes in time. From the results, it can be
seen that micro-organisms demonstrated increases in microbial densities, varying degrees of environmental stress, and domination by gram-negative bacteria. However, the declining pH had little effect itself on system efficiency.

The effect of wastewater composition on microbial populations in biological P removal processes was studied through a cellular PLFA analysis technique (Wang and Park, 1998). In this research, bench scale sequencing batch reactors (SBRs) were fed with glucose- and acetate-containing synthetic wastewaters to evaluate microbial population dynamics and types of phosphorus-accumulating organisms (PAOs). The resulting fatty acid profiles showed that the microbial population changed over time depending on the type of substrate fed, although initially the microbial populations were very similar. In addition, the glucose-fed SBR maintained the same fatty acid profile before and after biological phosphorus removal (BPR) occurred, while the acetate-fed SBR exhibited a different fatty acid profile. A cluster analysis of the experimental results illustrated how the microbial community
populations were influenced by the substrate fed over time (Figure 2-2).

The fatty acid analysis showed that the microbial population in the glucose-fed SBR was significantly different in terms of cellular fatty acid profile, from that produced in the acetate-fed SBR.

Shifts in nitrifying community population and function in response to different feed ammonium concentrations (0.03, 0.5, 1.0, and 3.0 g of N/liter), pH values (pH 6.0, 7.0, and 8.2) and oxygen concentrations (1, 7, and 21% of aeration gas mixture) were studied in experimental reactors that had been inoculated with nitrifying bacteria from a wastewater treatment plant (Princic et al., 1998). In this study, the authors assessed the changes in nitrifying community population: (1) by performing an amplified ribosomal deoxyribonucleic acid (rDNA) restriction analysis (ARDRA) of PCR products obtained with ammonia oxidizer-specific rDNA primers; (2) by phylogenetic probing; (3) by small-
subunit (SSU) rDNA sequencing; and, (4) by performing a cellular fatty acid analysis (detailed introductions of the molecular techniques follow later in this section). According to the results, digestion of ammonia-oxidizer SSU rDNA with five restriction enzymes showed that a high ammonium level resulted in a great community compositional change, which was reversible once the ammonium concentration was returned to its original level. However, the changes in community population brought about by the two pH extremes were irreversible. Also sequence analysis revealed that the highest ammonium environment stimulated growth of a nitrifier strain that exhibited 92.6% similarity in a partial SSU rRNA sequence to its nearest relative, *Nitrosomonas eutropha* C-91, although the PCR product did not hybridize with a general phylogenetic probe for ammonia-oxidizers belonging to the beta subgroup of the class *Proteobacteria*.

A principal component analysis of fatty acid methyl ester data detected changes from the starter culture in all communities under the new selective conditions. However, after the standard conditions were restored, all communities returned to the original fatty acid profiles (Figure 2-3). This conflicting result was explained with the hypothesis that the FAME result reflected all microbial communities and that the heterotrophs in the system were predominant compared to the ammonia-oxidizing bacteria. In this study, it was found that the FAME analysis appeared to be much more sensitive than ARDRA for revealing community shifts. Shifts were detected under different pH and nitrogen level conditions by the former method, whereas the latter method barely detected shifts caused by pH differences (Princic et al., 1998).
2.1.2.2 Analysis of Respiratory Quinones

Quinones exist in almost all organisms and play an important role as an electron transporter in the respiratory chain (Voet and Voet, 1990). There are two major groups of quinones: ubiquinones (1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinones) and menaquinones (1-isoprenyl-2-methyl-naphthoquinones), which are abbreviated as Q and MK, respectively (Hu et al., 1999). According to the number and position of the isoprenoid unit, there are many different kinds of Q and MK that can be present and each bacterium generally has one dominant type of quinone. So the quinone profile, defined as the mole fractional content of each quinone species in a mixed population, can be used as an index to
represent the population of a bacterial community (Hiraishi, 1988; Suzuki et al., 1998). Respiratory quinones have been used as biomarkers in order to study bacterial community populations in: (1) activated sludge reactors used for enhanced biological phosphorus removal (EBPR) (Hiraishi et al., 1998); (2) a submerged aerobic biofilter (Hu et al., 1999); and, (3) a bioreactor system developed for the treatment of chromate wastewater (Nozawa et al., 1998). Through the experiments, quinone profiles were generated to identify microbial compositional changes; and, using a new diversity index suggested in the published report, the changes could be determined quantitatively (Hu et al., 1999).

Hiraishi et al., (1998) used respiratory quinones as biomarkers to study bacterial community populations in activated sludge reactors used for EBPR. They compared the quinone profiles of EBPR sludges and standard sludges, of natural sewage and synthetic sewage, and of full-scale and laboratory-scale systems. The differences in MK/Q ratios were much larger when they compared different wastewater sludges (i.e. real sewage and synthetic sewage) than when they compared sludges from EBPR with standard processes or full-scale with laboratory-scale systems. Also, a numerical cluster analysis of the profiles showed that the sludges tested fell into two major clusters, independent of the operational mode and scale of the reactors and the phosphate accumulation: one cluster included all real sewage sludges, and the other consisted of all synthetic sewage sludges. These data also suggested that Q-8-containing species belonging to the class *Proteobacteria* (i.e. species belonging to the beta subclass) were the major constituents of the bacterial populations in the EBPR sludge, as well as in the standard activated sludge (Hiraishi et al., 1998).
2.1.2.3 Molecular Methods using rRNA and rDNA

Since rRNA molecules are comprised of highly conserved sequence domains interspersed with more variable regions (Head et al., 1998), these methods are usually based on the study of small-subunit rRNAs (ribosomal ribonucleic acids) and the respective genes. In general, essential rRNA domains are conserved across all phylogenetic domains, thus "universal" tracts of sequences can be identified. In addition, it is also possible to identify sequence motifs of increasing phylogenetic resolution. For example "signature" sequences for archaea, bacteria, and eukarya have been recognized, as well as short stretches of sequence characteristic of many bacterial divisions and subdivisions (alpha-, beta-, delta-, gamma-Proteobacteria, high %G+C Gram-positive bacteria, and the Flavobacterium-Cytophaga division). Species- and subspecies-specific sequences have also been identified (Head et al., 1998).

(1) Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a method by which fragments of DNA of the same length but of different sequence can be resolved electrophoretically (MacNaughton et al., 1999). This method has been applied to the analysis of 16S rRNA genes from environmental samples and allows the separation of a heterogeneous mixture of PCR-amplified genes on a polyacrylamide gel. Individual bands may be excised, reamplified and sequenced, or challenged with a range of oligonucleotide probes, to give an indication of the composition and diversity of the microbial community (Zhang and Fang,
Chapter 2: Development of Monitoring Tool Based on Microbial Fatty Acid (MFA) Analysis Using Lab-scale Bioreactors

2000). DGGE is relatively rapid to perform and many samples can be run simultaneously. The methods, therefore, are particularly useful for time series analysis and the study of population dynamics.

Recently, Zhang and Fang (2000) developed a new technique for detecting the DGGE band patterns using a digitized image analyzer. The digitized image analyzer detected individual bands precisely. The technique was successfully applied to compare the microbial communities in a biofilm and in suspended sludge obtained from the same system. In that study, biofilm samples and suspended growth samples were collected at the same time for cluster analysis on the basis of their PCR-DGGE patterns. A cluster analysis of the type shown in Figure 2-4 indicates similarity in DGGE patterns through location in the cluster diagram. When sample results are located on adjacent or nearby branches, a high degree of similarity is implied. Sample results located on distal branches suggest much lower degrees of similarity. In the Zhang and Fang (2000) study, the cluster trees indicated that the biofilm microbial population and the suspended microbial population diverged with time, even though both populations originated from same seed population (Figure 2-4). This result was similar to those reported by Acinas et al., (1999) and Werker (1998), in that microbial differences among the biofilm communities were less than those among suspended growth communities, indicating that the biofilm communities are more stable and less susceptible to changes in the conditions of the bulk solution.
Figure 2-4. Cluster analysis trees of the microbial communities. BC: biofilm communities, SC: suspended growth communities, MS: microbial seed. Numbers mean elapsed time (days) (adapted from Zhang and Fang, 2000).

(2) Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis of 16S rDNA, otherwise known as Amplified rDNA Restriction Analysis (ARDRA), has been used for several years as a method for rapid comparison of rDNA (Roberts et al., 1998). In this technique, rDNAs are obtained by PCR amplification using universal primers and the product is digested with restriction enzymes with 4-bp recognition sites. The typical analysis of restriction digests for isolates or clones is performed on relatively low-resolution agarose gels. For community analysis, the potentially large number of fragments can be resolved by using polyacrylamide gels to produce a community-specific pattern (Roberts et al., 1998). This method has been made more rapid and sensitive by using primers labeled with a fluorescent dye (Liu et al., 1997). When the preparation is analyzed with an automated DNA sequencer, the sizes of terminal
restriction fragment (T-RF) can be determined, and the amount can be quantified. The method can be used with DNA from complex microbial communities, which is called terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997).

The ability of T-RFLP analysis to distinguish phylogenetic groups of bacteria was theoretically evaluated through a computer simulation by Liu et al. (1997). Four different pairs of domain-specific or universal PCR primers were selected to anneal to as many 16S rDNA sequences in the Ribosomal Database Project (RDP) database as possible. Through this simulation, it was proved that computer-simulated analysis of T-RFLP for 1,002 eubacterial sequences showed that, with proper selection of PCR primers and restriction enzymes, 686 sequences could be PCR-amplified and classified into 233 unique terminal restriction fragment lengths or "ribotypes". This means polymorphisms among T-RF lengths could be used to characterize microbial communities.

(3) **Length Heterogeneity Polymerase Chain Reaction (LH-PCR)**

LH-PCR is similar to T-RFLP. In both methods, the proportion of PCR amplicons originating from different genes are estimated from the fluorescence emission of labeled PCR primers. However, instead of identifying PCR amplicons based on restriction endonuclease sites, the discrimination in LH-PCR is based on natural variation in the length of small subunit (SSU) rDNAs (Suzuki et al., 1998).

The main advantages of LH-PCR are that it surveys relative gene frequencies within
complex mixtures of DNA, is reproducible, requires small sample sizes, and can be performed with many samples simultaneously. The main technical problem is the accuracy of peak detection, especially when longer domains are used (Suzuki et al., 1998).

(4) Ribosomal Intergenic Spacer Analysis (RISA)

Although 16S rDNA has been the most commonly used target in many studies, many times 16S rDNA sequences are not divergent enough to distinguish closely related bacteria. (e.g. species of the same genus and some strains of bacteria with considerably different physiologies have been reported to have identical 16S rDNA genes) (Yu and Mohn, 1999). Recently, the 16S-23S rDNA intergenic spacer (RIS) has been studied to investigate microbial communities (Fisher and Triplett, 1999; Garcia-Martinez et al., 1999). The 16S-23S intergenic region displays significant heterogeneity in both length and nucleotide sequence. Therefore, both types of variation can be used to distinguish bacterial strains and even closely related bacterial species (Fisher and Triplett, 1999).

In RISA, which exploits the length heterogeneity of the intergenic spacer, the PCR product (a mixture of fragments contributed by community members) is electrophoresed in a polyacrylamide gel, and the DNA is visualized usually by silver staining. The resulting complex banding pattern provides a community-specific profile and each DNA band corresponds to at least one organism in the original assemblage (Fisher and Triplett, 1999).
Fluorescent in situ Hybridization (FISH)

The FISH method was first developed in the late 1980's for studies in microbial ecology. In recent years, the technique has been used successfully to analyze many ecosystems (Bond et al., 1999). This technique uses labeled rRNA-directed oligonucleotide probes to determine bacterial community population. At one time, *Acinetobacter* had been thought to be the major component in EBPR activated sludge when studied using culture-dependent techniques. However, a FISH technique indicated that bacteria of the beta-subclass of the *Proteobacteria* and high mol % G+C Gram positive bacteria were abundant in EBPR activated sludge (Wagner et al., 1996; Bond et al., 1999).

Recently, Nielsen et al. (1999) suggested that the combination of microautoradiography (MAR) and FISH provides a strong tool to characterize and enumerate functionally important groups of micro-organisms in activated sludge systems. MAR is a method that can be used to study the micro-scale distribution of a radio-labeled compound that typically appears in the investigated cell or biological population as a result of adsorption of a tracer or by an uptake of a labeled substrate (Nielsen et al., 1999).

The Nielsen et al. study provided a good explanation of why a certain control strategy for a bulking problem does not always work in a particular treatment plant, even though the causative filamentous organisms seemed to be type 021N, as identified by traditional morphological analysis methods. According to the results from the MAR technique, the type 021N found in all tested plants was able to take up both acetate and glucose. However,
for ethanol, glycine, leucine and oleic acid, a pronounced difference in the uptake pattern was found among filaments from different treatment systems. Thus, a group of filaments, which was lumped together by the name type 021N and assumed to possess a similar physiology, was shown to contain bacteria that belonged to different phylogenetic groups and exhibited different physiologies (Nielsen et al., 1999).

In addition to these methods, several techniques such as Thermal Gradient Gel Electrophoresis (TGGE) analysis (Eichner et al., 1999; Sakano and Kerkhof, 1998; Webster et al., 1996), small-subunit rDNA (rRNA) analysis (Godon et al., 1997; Sekiguchi et al., 1998), Randomly Amplified Polymorphic DNA (RAPD) (Franklin et al., 1999), comparison of whole cell protein profiles after SDS-PAGE (Ehlers and Cloete, 1999) and G+C composition analysis (Griffiths et al., 1999) are available to identify microbial community populations and their changes.

2.1.3. Microbial Fatty Acid Analysis for Community Population Monitoring

DNA(RNA)-based molecular techniques are generally very informative, in that they can monitor specific bacteria and identify them by DNA/RNA sequencing or probing. However, molecular techniques are still expensive, time-consuming and labour-intensive for monitoring purposes. A monitoring technique needs to be simple, quick and inexpensive.

The microbial fatty acid (MFA) analysis technique using whole cell fatty acids is gaining attention because it offers several advantages in addition to those mentioned in the
introduction above (Werker and Hall, 2001; Webster et al., 1998). The first advantage is that the MFA technique is relatively simple and quick, which means that many samples can be processed at the same time. Extraction and analysis of fatty acids take only a few hours (refer to Methods and Materials section). The second advantage is that it can group or classify bacteria according to their specific marker fatty acids. Although this technique cannot track individual bacteria, sometimes trophic-level taxonomic monitoring is possible because prokaryotes, eucaryotes and fungi have distinctive fatty acid compositions (Griffiths et al., 1999). The third advantage is that micro-organisms with unique fatty acids can be exclusively monitored. Recent research showed that monitoring of *Nocardia* levels in activated sludge systems was possible through the analysis of OH-C19 concentrations. *Nocardia* contains this particular fatty acid as a signature fatty acid that can be easily tracked (Cha et al., 1999). The final advantage of this technique is that it can be applied to the quantification of mixed culture biomass (Werker and Hall, 2000). However, there have been controversial results reported regarding the direct relationship between total fatty acid concentration and total biomass, although the total fatty acid concentrations are related to the total biomass. This is mainly due to the fact that each bacterium contains a different amount of total fatty acids (Kates, 1964; Tunlid and White, 1992). Recently, it was shown that there is proportionality between mixed culture biomass during batch growth and the total concentration of fatty acids, which demonstrates the possibility that the MFA technique can be used for quantifying mixed culture biomass. Werker and Hall (2000) reported that total MFA concentrations extracted from batch-growth bioreactors exhibited good correlations to parameters such as the biomass dry weight and protein, which have
traditionally been used for the quantification of biomass.

2.1.4 Research Objective and Specific Issues

The main objective at this stage of the research was the development of a MFA analysis technique, selected through the literature review, as a useful monitoring tool for the correlation of microbial community populations and system performance. The literature review showed the possibility that the monitoring of microbial community population may be beneficial for advanced biological wastewater treatment systems and that MFA analysis has many suitable advantages for the purpose of monitoring. However, the utilization of this tool for the purpose of monitoring necessitated that four specific issues be explored.

1. It was necessary to determine whether the weighted stack similarity method was appropriate and simple enough to interpret the MFA analysis results, so that the information would be useful to operators and engineers.

2. The use of microbial fatty acid profiles with a simple similarity index is not a conventional approach; and, the literature did not report any relationship between a similarity index and microbial population changes. Therefore, it was necessary to establish the relationship between the two factors.

3. The literature showed that changes in operating conditions and system configurations in biological systems affected the stability of microbial
community populations and system performance. However, most of the experimental observations were focused only on steady state or batch test results. Therefore, it was necessary to determine whether the changes in microbial community populations could be applied to monitoring a continuously changing bioreactor.

4. As stated in the introduction section, it has been controversial to use a concentration of microbial fatty acid as an estimator of biomass. In this experiment, the relationship was repeatedly examined to test the feasibility of the total fatty acid concentration as an alternative to mixed liquor volatile suspended solid measurement.
2.2 Methods and Materials

2.2.1 Control and Operation of the Lab-scale Bioreactors

Two identical conventional activated sludge (CAS) systems (CAS-1, CAS-2) with 3-liter bioreactors were set up in a constant temperature chamber maintained at 20 ± 1°C. The biomass used was introduced into the reactors from a UCT (University of Cape Town) pilot plant that was treating municipal wastewater at the University of British Columbia, Vancouver, Canada. The lab-scale reactors were continuously mixed with mechanical stirrers. Dissolved oxygen (DO) levels were controlled between 3.0 ± 1.0 mg/L manually by an air pressure regulator and a flow meter. The DO concentration was measured by DO probes (YSI Model number 5739), which were connected to DO meters, YSI 54A (YSI incorporated, Yellow Springs, Ohio). The pH levels were maintained by a pH controller (Cole-Palmer Instruments, Chicago, Illinois) within the range of 6.5 - 6.8 using 0.2 N NaOH. The hydraulic retention time (HRT) was 12 hours and the sludge retention time (SRT) was 10 days. Table 2-1 shows the experimental operating conditions and the associated variation. These operating conditions were maintained for the entire experimental period, except for short-term experimental runs designed to assess how operational changes affected the microbial community populations. While one reactor was subjected to variations in operating conditions, the other reactor was operated as a control reactor. CAS-1 was subjected to pH and substrate loading variations and CAS-2 was subjected to chlorine and substrate loading shocks. The increases in substrate loading were applied by increasing the amount of sodium acetate in the feed. The pH shocks from 6.5 to
Chapter 2: Development of Monitoring Tool Based on Microbial Fatty Acid (MFA) Analysis Using Lab-scale Bioreactors

8.5 were implemented by addition of 0.2 N NaOH using the pH controller. For each chlorine spiking to the system (CAS-2), the needed volume of a hypochlorite stock solution (50,000 mg/L as Cl₂) was quickly added to the bioreactor with a pipette. The stock and resulting chlorine concentrations were measured according to Standard Methods (APHA et al., 1995).

Table 2-1. Lab-scale bioreactor operating conditions for steady state and operational changes.

<table>
<thead>
<tr>
<th>Items</th>
<th>Temp (°C)</th>
<th>DO (mg/L)</th>
<th>SRT (day)</th>
<th>HRT (day)</th>
<th>pH</th>
<th>Substrate loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>20</td>
<td>2-4</td>
<td>10</td>
<td>0.5</td>
<td>6.5</td>
<td>1,100 mg/L·d</td>
</tr>
<tr>
<td>Changes</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>5.2-8.5</td>
<td>1,430 mg/L·d</td>
</tr>
</tbody>
</table>

Note: Substrate loading = influent BOD (mg/L)/HRT (day)
NC: No change.

2.2.2 Synthetic Raw Wastewater

The synthetic wastewater used for the experiment consisted of sodium acetate as a carbon source, ammonium chloride as a nitrogen source, and potassium phosphate as a phosphorus source. In addition, peptone and yeast extract were provided with some minerals such as Mg, Ca, and Fe. The detailed composition of the raw wastewater is presented in Table 2-2. The concentration of the raw wastewater was 180 ± 20 mg/L of total organic carbon (TOC), which was equivalent to 510 ± 70 mg/L of BOD and 840 ± 45 mg/L of COD.
Table 2-2. Composition of synthetic wastewater

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical formula</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>CH₃COONa·3H₂O</td>
<td>471</td>
</tr>
<tr>
<td>Peptone</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂·2H₂O</td>
<td>32</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄</td>
<td>214</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>(NH₄)₂SO₄</td>
<td>40</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>KH₂PO₄</td>
<td>20</td>
</tr>
<tr>
<td>monobasic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>FeSO₄</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Fifteen liters of synthetic wastewater were prepared daily from a 10 X stock solution. The feed tank was also cleaned daily using diluted bleach solution and tap water to prevent microbial growth.

2.2.3 Analysis

2.2.3.1 Total Organic Carbon (TOC) Measurement

The performance of the reactors was measured by carbon removal based on TOC removal.
Samples were filtered through Whatman #4 filters. The TOC concentrations were measured using a Shimadzu 5000 TOC analyzer equipped with a non-dispersive infrared (NDIR) detector (Columbia, Maryland). Zero air at 150 mL/min was applied as the carrier gas flow. Acidification and sparging pre-treatment (1.0 N hydrochloric acid, pH between 2.0 to 3.0. CO$_2$-free N$_2$ gas sparging for 5 minutes to remove inorganic carbon CO$_2$ carbonate-bicarbonate species) was practiced and the reaction tube was operated at 640°C for total carbon (TC).

2.2.3.2 Suspended solids (MLSS/MLVSS)

Activated sludge mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were determined by the total suspended solids and volatile suspended solids procedure described in the standard methods (APHA et al., 1995). A known volume of mixed liquor sample from the reactors was filtered by glass fiber filter paper (Whatman® 934-AH) and dried at 105°C overnight in the oven (VWR Scientific® 1350 FM Forced Air Oven) to determine the MLSS. Samples were then ignited at 550°C for 30 minutes in a muffle furnace (ThermoLyne® 30400 Furnace or LindBerg® Furnace) to determine the MLVSS.

2.2.3.3 Microbial Fatty Acid Analysis

Microbial fatty acid analysis was executed according to the method described in the
dissertation by Werker (1998) with few modifications. Mixed liquor samples of 5-10 mL were drawn, transferred to 15-mL glass tubes with Teflon screw caps and centrifuged (3000 x g for 20 min). After discarding the supernatant, the pellets were stored at -20°C pending extraction. Prior to extraction, 3 mL of distilled water and 1 mL of 1 N NaOH in 50% HPLC grade methanol, spiked with O-methylpodocarpic acid (O-MPCA), was added to each thawed sample. Samples were then mixed well using a vortex mixer. The sealed sample tubes were incubated at 90°C for 30 minutes to saponify the cellular phospholipid fatty acids. One mL of 1 N H₂SO₄ was used to acidify the sample and to reduce the aqueous solubility of fatty acids prior to extraction. Samples were extracted with 2 mL methyl-tert-butyl ether (MTBE). Cellular debris suspended in the solvent phase was forced to the aqueous-solvent interface by centrifugation (3000 x g for 20 minutes). For each extraction, the solvent was transferred directly to 2 mL gas chromatography (GC) vials and dried under vacuum. After drying, a 100 μL aliquot of MTBE spiked with heneicosanoic acid methyl ester (HCA-ME) and tricosanoic acid (TCA) was added to each vial. HCA-ME was the internal standard and TCA the control for methylation. Methylation was accomplished by dispensing and vortexing, in the dried GC vials, 400 μl of chilled (0-4°C) MTBE and HPLC grade methanol (80:20) containing excess dissolved diazomethane. Diazomethane was dissolved into the chilled MTBE methanol mixture by using a nitrogen gas flow to purge the diazomethane from a reaction vessel into the solvent. The reaction vessel held 10 mL of MTBE, 5 mL of HPLC grade methanol into which approximately 0.5 g of diazald (N-methyl-N-nitroso-p-toluenesulfonamide) was dissolved and then 5 mL of 10 N KOH was added to start the diazomethane generation. Diazomethane dissolved in MTBE in this
manner would remain potent for weeks when stored in the freezer in 25 mL vials sealed with Teflon-lined caps.

Extracted microbial fatty acids were identified and measured by GC/MS (HP6890 Series with a model 5973 selective detector, DB5 column of 30 m having a 0.32 mm ID and 0.25 mm film thickness) with 1 μl splitless injection. The inlet and detector temperature were 250°C and 300°C, respectively. The temperature program was 4 minutes at 130°C followed by a ramp of 2°C/min to 250°C and a post-run temperature of 290°C for 5 minutes. A helium carrier gas was used with a column head pressure of 10 psi providing a total flow of 30 mL/min. Fatty acids are designated in the form X:Y, where X is the total number of carbon atoms and Y is the number of double bonds present. If double bonds are present, the location is designated ωZc or t, where Z indicates the number of carbon atoms from the terminal methyl group (ω) at the end of the molecule and c or t represent cis or trans geometry, respectively. In addition, i (iso branching), a (anteiso branching), and the location of hydroxy (OH), cyclopropane (cyc) or methyl (Me) groups may also be noted. All chromatography peaks were positively identified with pure standards, equivalent chain length (ECL) calibration using fatty acid standards (Supelco 37 component FAME mix, 26 component BAME mix, and pure fatty acid standards such as C18:1ω7c, C18:1ω7t, C18:1ω9c, C18:1 ω9t) and subsequent GC/MS confirmation (Werker, 1998).

ECL expresses the relative elution position of any peak with respect to the closest saturated fatty acid. Gas chromatograms of a standard mixture containing a homologous series of
saturated fatty acid methyl esters are used to equate retention time to an equivalent chain length. ECL for a peak, x, is calculated by interpolation of the retention times (t) of the two neighboring saturated fatty acids (n: 0 and (n+1):0)

\[ ECL_x = \frac{t_x - t_n}{t_{n+1} - t_n} + n \]

A family plot of carbon length (n) versus relative peak position (ECLx-n) for the FAMEs in the standard mixture serves as an identification template. Since members of a homologous series of fatty acids tend to be linear on the family plot, probable identification of FAME peaks is made from their ECL and interpolation on the family plot. Increased confidence in identification of a fatty acid peak can be made by a confirmatory result using the same analysis with a different column of by GC with mass spectrometry (MS) on selected samples.

2.2.3.4 Ribosomal Intergenic Spacer Analysis (RISA)

(1) Chromosomal DNA Extraction from Sludge Samples

The RISA was executed according to the method developed by Yu and Mohn (1999) and all materials were kindly provided by Prof. Mohn lab (Department of Microbiology & Immunology, UBC, B.C, Canada). Aliquots of 1 to 2 mL of sludge in 2-mL screw-cap tubes were centrifuged at 16,000 x g at 4°C for 5 minutes; and, the supernatants were removed with aspiration. Then, 0.8 mL of extraction buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 3 % SDS) and 2 g of silica beads (0.1 mm in diameter) were added to the tubes. To break
the cell walls, a bead beater (BioSpec Products, Inc., Okla.) was used for 2.5 minutes at 5,000 rpm, after which the samples were cooled on ice for 1 minute. A bead beating was then done once more for 2.5 minutes. The tube was centrifuged at 16,000 x g for 5 minutes at 4°C; and, the supernatant was collected into a fresh 1.7 mL tube, and put on ice (first lysate). Another 0.8 mL of extraction buffer was added to the cell debris and the bead beating procedure was repeated once more (second lysate). A volume of 10 M ammonium acetate was added to both the lysates to give a final concentration of 2 M, and the mixture was put on ice for 5 min to precipitate impurities. The sample was centrifuged at 4°C at 16,000 x g for 10 min, and the supernatant was collected into a fresh 2-mL tube. A 1/10 volume of 3 M ammonium acetate and one volume of isopropanol were added to the sample, and well mixed by vortexing. After sitting on ice for 30 minutes, the sample was centrifuged for 15 minutes. The supernatant was removed using aspiration. The pellet was rinsed with 70% ethanol, centrifuged for 5 minutes and then dried for 2 minutes under vacuum. 200 µL of TE (10 mM Tris, 1mM EDTA) buffer was added to each tube to dissolve the nucleic acid pellet. A 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol were added to precipitate nucleic acid again. After 30 minutes on ice, the sample was centrifuged for 15 minutes. The supernatant was removed and the pellet was dried for 2 minutes. Finally, the extracted nucleic acid was dissolved in 100 µL of TE buffer and stored at -20°C.

(2) PCR Amplification of the rDNA-RIS Fragment

A DNA fragment containing approximately 600 bp (3' end) of the 16S rDNA, RIS and 190
bp (5' end) of 23S rDNA was amplified using the universal bacterial primers S926f and L189r (forward primer - 5'-CTYAAAKGAATTGACGG-3' and reverse primer - 5'-TACTGAGATGYTTMARTTC-3', respectively). A 50 μl total volume PCR mixture contained 5 μl 10X PCR buffer (Tris-HCl, pH 8.7, KCl, (NH₃)₂SO₄, 15 mM MgCl₂), 672 μg of bovine serum albumin (BSA) per mL, 100 μM of each deoxynucleoside triphosphate, 1.0 μM of each primer and 1.25 U of Taq DNA polymerase (Life Technologies, MD). Template DNA from the extracted samples ranged from 165 to 392 ng per PCR reaction. The PCR reaction, processed in a MiniCycler™ (MJ Research), started with a simplified hot start at 90°C, an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of a 30-second denaturation step at 94°C, a 30-second annealing step at 47°C, and a 2-minute extension step at 72°C. The whole PCR reaction ended with a 5-minute extension step at 72°C. A negative control, containing no template DNA, was run in parallel with the samples. To confirm desired PCR reaction results, 5 μl of the rDNA-RIS fragment amplicons were separated on a 2% agarose gel, stained with ethidium bromide and observed by UV illumination. The remainder of the PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, CA) and eluted in 30 μl of EB buffer (10mM Tris-Cl, pH 8.5). A 10 μl aliquot of the purified PCR product was separated on a 3.5% polyacrylamide (38:1) gel, stained with GelStar (BioWhittaker Molecular Applications, Rockland, ME) and observed by UV illumination. rDNA-RIS LP banding patterns were analyzed using Alphalmager 1200® (Alpha Innotech, San Leandro, California). Individual bands were detected by the alphaEase program (Version 4) of the Alphalmager. The relative mobility of each band was calculated using the 800-bp band of the 100-bp molecular size marker as the
Chapter 2: Development of Monitoring Tool Based on Microbial Fatty Acid (MFA) Analysis Using Lab-scale Bioreactors

The density and percent relative abundance of each band was calculated using the 1D-multi program of AlphaEase. For band matching, a maximum tolerance of 1% was chosen. Community percent similarity was calculated from pair-wise comparisons of the RIS-LP banding patterns. Community percent similarity was calculated as the sum of shared relative abundance of all matching bands. The shared relative abundance of each matching band was defined as the lower of the two percent relative abundances. Subtraction of the community percent similarity from 100% yielded the community dissimilarities, which were then used to create dissimilarity matrices. Dendrograms (UPGMA) were generated from the above dissimilarity matrices using the neighbor-joining program in the Phylip package (Version 4.0).

2.2.4 Estimating Changes in Microbial Community Populations

In order to show how a similarity index indicating changes in MFA profiles could represent changes in microbial community populations, five different bacteria (A, B, C, D and E) were isolated from the lab-scale bioreactor CAS-1 according to colony colors and morphologies on Luria-Bertani (LB, trypton: 10 g/L, NaCl: 10 g/L, Yeast extract: 5 g/L) plates and cultured for 2 days with 100 mL LB broth medium at 20°C in a shaking incubator. Ten mL of each culture medium was taken from the vials and centrifuged for fatty acid analysis as described in section 2.2.3.3. The concentrations of bacteria were calculated based on the MLSS method. The different fatty acid profiles of each isolate confirmed that they were different bacteria (Figure 2-5): fatty acid profiles have proven useful in taxonomic classification of bacteria and fungi (Werker and Hall, 2000).
Figure 2-5. Fatty acid profiles of each bacterium used for model microbial community populations. The scale on the Y-axis is pA (Pico Ampere)
From each culture vial, 2 mL of well-mixed culture medium were transferred to a 15 mL glass tube for a final total volume of 10 mL, resulting in bacterial composition of 10.0:20.1:19.8:25.0:25.2 (based on each percent concentration out of 100%) of A:B:C:D:E (Table 2-3).

<table>
<thead>
<tr>
<th>Bacterial concentration (mg/mL)</th>
<th>Volume added (mL)</th>
<th>Bacterial Mass added (mg)</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.7</td>
<td>2.00</td>
<td>1.3</td>
</tr>
<tr>
<td>B</td>
<td>1.3</td>
<td>2.00</td>
<td>2.6</td>
</tr>
<tr>
<td>C</td>
<td>1.3</td>
<td>2.00</td>
<td>2.6</td>
</tr>
<tr>
<td>D</td>
<td>1.6</td>
<td>2.00</td>
<td>3.2</td>
</tr>
<tr>
<td>E</td>
<td>1.6</td>
<td>2.00</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Each culture vial contained different concentrations of biomass because of the different growth yield. Changes in microbial community populations of each model system were made by decreasing the addition of each bacterial isolate in turn. For example, a 9.5% change in composition was made by decreasing the addition volume of isolate A into sample 1 by half (from 2 mL to 1 mL) (Table 2-4). In this manner, each sample vial contained different final volumes ranging from 6.2 mL to 9.6 mL and the resulting changes in bacterial composition of each sample relative to the reference are presented in Table 2-5. The samples were subjected to centrifugation after composition. Therefore, it was not necessary to add buffer to make 10 mL samples. Also, the total concentrations of each
sample were not adjusted because the similarity calculation was based on percent concentrations of each fatty acid out of total MFA concentration. The entire experiment was duplicated.

Table 2-4. Example calculations of change in bacterial composition for model community of sample 1.

<table>
<thead>
<tr>
<th></th>
<th>Bacterial concentration in isolate sample (mg/mL)</th>
<th>Volume added (mL)</th>
<th>Bacterial mass added for sample 1 (mg/mL)</th>
<th>Bacterial composition in sample 1 (%)</th>
<th>Difference between reference sample and sample 1 in composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.7</td>
<td>1.00</td>
<td>0.7</td>
<td>5.3</td>
<td>4.8</td>
</tr>
<tr>
<td>B</td>
<td>1.3</td>
<td>2.00</td>
<td>2.6</td>
<td>21.1</td>
<td>1.1</td>
</tr>
<tr>
<td>C</td>
<td>1.3</td>
<td>2.00</td>
<td>2.6</td>
<td>20.8</td>
<td>1.0</td>
</tr>
<tr>
<td>D</td>
<td>1.6</td>
<td>2.00</td>
<td>3.6</td>
<td>26.3</td>
<td>1.3</td>
</tr>
<tr>
<td>E</td>
<td>1.6</td>
<td>2.00</td>
<td>3.2</td>
<td>26.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>9</td>
<td>12.7</td>
<td>100.0</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Table 2-5. Bacterial composition in each sample. The changes in composition represent the differences in % concentrations compared to control.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Volume added (mL)</th>
<th>Changes in volume (mL)</th>
<th>Changes in compositions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Reference</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
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</tr>
<tr>
<td>2</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>0.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>4</td>
<td>2.00</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>2.00</td>
<td>1.50</td>
</tr>
<tr>
<td>7</td>
<td>2.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>8</td>
<td>2.00</td>
<td>2.00</td>
<td>1.00</td>
</tr>
<tr>
<td>9</td>
<td>2.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>10</td>
<td>2.00</td>
<td>2.00</td>
<td>1.50</td>
</tr>
<tr>
<td>11</td>
<td>0.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>12</td>
<td>0.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

2.2.5 Numerical Analysis

2.2.5.1 Similarity Index (SI) and Dissimilarity Index (DI)

The weighted stack method was used for the calculation of similarity between two microbial community populations, based on microbial fatty acid analysis. For the monitoring of population shifts in community populations over time, a daily similarity between present-day and previous-day samples was calculated every day. Using the daily
similarity index, shift rates of microbial community population in a system can be calculated as following

\[
\text{Shift rate (per day)} = \frac{(100 - \text{daily similarity index})}{\text{day}}. \quad (1)
\]

For example, a daily similarity index of 94.5% on April 4th indicated 94.5% similarity between the two fatty acid profiles from the samples taken on April 3rd and April 4th (shift rate is 5.5/day). The weighted stack method of calculation is given by:

\[
\sum_{i=1}^{n} \frac{X_{ia} + X_{ib}}{2} \frac{\text{Min}(X_{ia}, X_{ib})}{X_{ib}'X_{ia}'}
\]

where, \( \text{Min} \) indicates minimum, and \( X_i \) is the mole % of \( i^{th} \) fatty acid, the subscripts \( a, b \) represent the two different compared profiles samples. A dissimilarity index was simply calculated by:

\[
\text{Dissimilarity index (\%)} = 100 - \text{similarity index (\%)} \quad (3)
\]

To estimate the shift of microbial community populations due to changes in operation for a certain period of time, a cumulative similarity index was calculated. Cumulative SI was calculated using a single fatty acid profile as a reference. The reference fatty acid profile was generally from a sample collected two or three days before an operating variation was imposed. Once the reference fatty acid profile was determined, the fatty acid profiles
thereafter were compared to the reference using the cumulative SI.

The score for each fatty acid was the amount of that fatty acid present in the sample, expressed as a percentage of the total fatty acids in the sample. In this study, only the fatty acids that contributed at least 0.5% to the total fatty acid concentration were considered for similarity calculations.

The fatty acid profiles of ten samples of the same biomass were analyzed at the same time and the similarities were estimated in order to calculate the background noise of the MFA analysis, using a student T-test with a 95% confidence interval.

2.2.5.2 Principal Component Analysis (PCA)

In order to assess each microbial community population, principal component analysis (PCA), a multivariate statistical technique, was performed using MFA profiles expressed as mole percent. The PCA technique has been successfully applied to samples that have a high number of variables by summarizing multidimensional data into a few dimensions called principal components. The principal components represent a linear combination of the variables. The first principal component accounts for as much variation in the data as possible and each succeeding principal component accounts for as much of the remaining variation as possible that is unaccounted for by the preceding principal components.
Microbial community populations were represented as variable fatty acid concentrations, and each fatty acid concentration was considered to be a variable. The score for each fatty acid was the amount of that fatty acid present in the sample, expressed as a percentage of the total fatty acids. In this study, only the fatty acids that contributed at least 0.5% to the total fatty acid concentration were considered for PCA. PCA was completed using SAS/STAT, version 6.02 (SAS Institute, Cary, N.C), by following the PRINCOMP procedure.
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2.3 Results and Discussion

The main objective of this study was the determination of whether the analysis of microbial fatty acids is a useful monitoring tool to detect and prevent system problems associated with undesirable or perturbed microbial populations, caused by improper operation such as shock loadings, sudden changes in feed materials, and pH and chlorine addition. To develop the MFA analysis technique as a monitoring tool, a simple similarity index (weighted stack method) and PCA were employed to quantitatively analyze changes in microbial populations. Twelve model microbial community systems were constructed to confirm that the similarity index could track changes in microbial population proportionally, according to the magnitude of the change. Lastly, the ability of total MFA concentration to estimate biomass concentration was evaluated.

2.3.1 Overall Reactor Performance

Two identical conventional activated sludge systems (CAS-1 and CAS-2) were operated for 100 days in order to investigate the effect of variable operating factors on microbial community populations and system performance. One reactor was operated with stable operating conditions, while the other one was subjected to operating variations.

Figure 2-6 shows the overall changes of daily SI and carbon removal during the entire experimental period. Figures 2-6 (A) and 2-6 (B) present daily SIs and carbon removal efficiencies for CAS-1 and CAS-2, respectively. Functional stability with respect to TOC
reduction was reached relatively quickly after changes in operating conditions, such as pH and substrate loading variations, and 4 mg/L of chlorine spiking (Figure 2-6). This result may be attributed to the fact that the carbon source used was easy for most bacteria to utilize. Therefore, even if some bacterial species may have experienced negative impacts from pH changes and toxic material loading, other bacteria that were resistant to the effects might easily take up the carbon source. During the entire experimental period, fresh biomass was introduced three times (on day 1, day 49 and day 78) to observe the reproducibility of assessments of microbial community population associated with substrate changes (Figure 2-6).
Figure 2-6. Similarity index and carbon removal efficiency for CAS-1 and CAS-2.
2.3.2 Statistical Evaluation of MFA analysis

For most analytical procedures, replicate samples don’t show 100% reproducibility, because experimental procedures are subject to errors during sampling and analysis. Therefore, it is important to determine the type and range of errors that an experimental procedure may have. Since replicate samples do not produce the same MFA profile from GC-measurement as the originals, this section was prepared in order to be aware of the possible errors that may occur during MFA analysis. It is important, therefore, to calculate the error range of the MFA analysis procedure before the main experiment started.

A T-test was completed with a 95% confidence interval, using ten replicate biomass samples from CAS-1 at day 1. The average similarity of the ten samples, prepared from the same microbial community, was 97.9%, and the standard deviation was 0.99%. The T-test results (one-tail test) indicated that two microbial community populations exhibiting more than 97.3% similarity could be considered to be essentially identical for the whole analytical process. Table 2-6 represented the similarity result and statistical values calculated.
Table 2-6. Similarity index and statistical values calculated for experimental errors (n=10)

<table>
<thead>
<tr>
<th>Sample #</th>
<th>1-2</th>
<th>2-3</th>
<th>3-4</th>
<th>4-5</th>
<th>5-6</th>
<th>6-7</th>
<th>7-8</th>
<th>8-9</th>
<th>9-10</th>
<th>10-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98.5</td>
<td>99.2</td>
<td>96.5</td>
<td>98.1</td>
<td>97.6</td>
<td>97.4</td>
<td>99.2</td>
<td>96.5</td>
<td>97.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average</th>
<th>S.D</th>
<th>D.F</th>
<th>C.I</th>
<th>S.N</th>
</tr>
</thead>
<tbody>
<tr>
<td>97.7</td>
<td>0.99</td>
<td>9</td>
<td>95%</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: S.D: Standard deviation
D.F: Degree of Freedom,
C.I: Confidence level,
S.N: Sample Number
Sample # 1-2 represents the similarity between sample 1 and sample 2.

2.3.3 Estimation of the Change in Microbial Community Populations

Since the use of microbial fatty acid profiles with a similarity index is not a conventional approach (specific issue 2 in section 2.1.5), Experiments were designed so that a relationship could be established between the similarity index and microbial population changes. The MFA analysis technique has been used to discriminate between microbial community populations (Werker, 1998). However, little information is available regarding the relationship between the estimated similarities using a similarity index and real population differences.

In the present experiment, five different bacteria were isolated from CAS-1 according to colony colors and morphologies. The microbial fatty acid profiles of each bacterium
isolated confirmed that they were all different species of bacteria as shown in Figure 2-5. A reference and twelve model microbial community populations were prepared using the bacteria. With the model systems, the changes in microbial populations were manipulated in various ways as described in Section 2.2.4 and to investigate the relationship, the similarities of the compared model microbial populations to the reference population were calculated.

The dissimilarities (100% minus the similarity value) calculated from the model systems showed a good relationship ($R^2=0.8194$) with the designed compositional changes in simulated microbial community population, up to 31.5% (Figure 2-7).

Figure 2-7. Dissimilarity (100% minus observed similarity) changes versus differences in community populations in model systems. R2 value was calculated by MS-Excel (MS-Office, Version. 2000).
Using equation (4) that was acquired from the graph through linear regression, the magnitude of the changes in microbial composition could be roughly estimated by using the similarity index.

\[ Y = 0.8397 \times X + 6.0617 \]  

(4)

where, X represents the dissimilarity index value between two microbial fatty acid profiles, and Y represents actual differences in composition between two microbial populations.

For example, if the similarity between two microbial community populations is 90% (10% dissimilarity), there may be about 15.1% difference between the compositions of the two microbial populations. However, it should be noted that the results may not be generalized due to the very limited microbial populations used in the model microbial communities. If different micro-organisms had been used, the results may have differed. The importance of the experiment was that it was confirmed that the magnitudes of the population differences could be proportionally detected by the MFA analysis. In the experiment using the microbial model systems, the replicate reference populations showed an average of 97.4% similarity, consistent with the T-test result.
2.3.4 Monitoring Changes in Microbial Community Population

2.3.4.1 Biomass Transfer from the Pilot Plant to the Lab Reactors

One of the important factors that may affect microbial community population in a biological wastewater treatment system may be the substrate composition of the untreated wastewater. Recent ecological studies have shown that microbial populations change according to the substrate characteristics, such as the organic material populations and/or C:N:P ratios (Princic et al., 1998; Liu et al., 1997). Wastewater treatment system configurations are also thought to affect microbial community populations (Hiraishi et al., 1998). However, little information regarding the impact of different system configurations on microbial community populations has been published.

The transfer of biomass from the pilot plant to seed the lab-scale bioreactors provided a good opportunity to study the changes of the microbial population in the lab-scale system, in response to a change in substrate and treatment system configuration (Table 2-7). As expected, dramatic similarity changes were observed after new biomass was transferred from the pilot plant to the lab-scale bioreactors. The similarities between the original biomass from the pilot plant and the biomass assessed after one day in the new lab-scale operating environment, averaged only 68% (on day 1, 42, and 78) (Figure 2-8).
Figure 2-8. The average daily SI and cumulative SI and carbon removal efficiency during the transition after biomass transfer from the pilot plant to lab scale CASs (on day 1, 42, and 78)

Table 2-7. Process conditions of lab-scale CAS reactor and pilot-scale UCT

<table>
<thead>
<tr>
<th>Lab-scale CAS Process</th>
<th>Pilot-scale UCT Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO (mg/L) (in aerobic tank)</td>
<td>2.5</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
<tr>
<td>SRT (day)</td>
<td>10</td>
</tr>
<tr>
<td>HRT (day)</td>
<td>0.5</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>20</td>
</tr>
<tr>
<td>MLSS (mg/L)</td>
<td>1,500</td>
</tr>
</tbody>
</table>
These large changes in microbial community population were attributed to the mixed effects of different substrate characteristics and system configurations between the pilot plant and the lab-scale bioreactors. As a result, a fundamental question was raised. The question was: which one of the two factors (feed composition and process configuration) was the most responsible for the observed changes in microbial population?

The following experiment was completed to determine which factor, feed composition or process configuration, was most responsible for the observed changes in microbial population during the transition state.

On day 79, new biomass from the pilot plant was transferred to the two CAS systems. Instead of sewage wastewater, the synthetic wastewater was fed to CAS-1. CAS-2 was subjected to real sewage from the pilot plant for two more days to minimize the effects of substrate changes on the microbial population.

The similarity index for CAS-2 exhibited over 92% similarity during the first two days. On the other hand, the similarity index for CAS-1 immediately decreased to 68%. After two days, the raw wastewater for CAS-2 was changed to the synthetic wastewater, and the similarity dropped to below 75% (Figure 2-9).
Figure 2-9. The daily similarity index changes of CAS-1 and CAS-2 following biomass transfer.

These findings indicate that the main cause of the observed MFA compositional change was the change in substrate composition, rather than the change in system configuration, despite the significantly different HRT, SRT, pH, DO and temperature conditions in the pilot- and lab-scale systems. These results showed that lab-scale experiments using synthetic wastewater might have limited value since different substrate compositions will result in different microbial community populations. A full-size treatment plant would have a microbial composition that is dissimilar to that in lab-scale bioreactors, unless real wastewater is used for designing and modeling of the real wastewater treatment.

Another interesting finding was that a microbial community reached over 95% daily
similarity within 5-6 days at an SRT of 10 days, and the stability lasted as long as constant operating conditions were maintained (Figure 2-9). This may be due to the relatively simple and readily biodegradable substrate composition of the synthetic wastewater used for the experiment.

Two different experimental strategies were designed to determine whether the large observed changes in fatty acid profiles resulted from real changes in the microbial populations or whether they were influenced by the fatty acid composition of the untreated wastewaters. The first was the analysis of the microbial populations using ribosomal intergenic spacer analysis (RISA), which is one of the molecular techniques widely used for analysis of microbial community populations. The technique is not affected by the fatty acid composition of the samples analyzed. Samples for RISA were taken from CAS-1 on day 0, day 2 and day 4. Within a short time, the banding patterns of each sample were quite different. Furthermore, the major change in banding pattern occurred between day 0 and day 2, according to the similarity comparison using the Phylip package (version 4) (Figure 2-10). This RISA result confirmed that the observed changes in fatty acid profiles were associated with changes in the microbial community compositions and were not caused by the changes in fatty acid composition in the untreated wastewater that was fed to the systems.
The other strategy was the changing of the synthetic wastewater carbon source from an acetate-base to a glucose-base (same concentration as mg/L) to see if the change in feed substrate produced similar changes in microbial population as the change from real sewage to acetate-based synthetic wastewater. Since the two feeds were the same except for the population of the carbon substrates, the fatty acid compositions of the two feeds were also the same (Table 2-2). For this experiment, one more CAS system (3 L) with an internal...
settling system was prepared, and its schematic diagram is presented in Figure 2-11. Operating conditions, including HRT, SRT, dissolved oxygen concentration, temperature and pH, were also kept the same, as described in Table 2-1.

![Schematic layout of the simplified lab scale conventional activated sludge system used](image)

Figure 2-11. Schematic layout of the simplified lab scale conventional activated sludge system used

The change from acetate to glucose in the feed also resulted in large changes in similarity for the first few days (Figure 2-12). The day after the feed was changed, the similarity index was 71%. The daily and cumulative similarity indices also showed very similar patterns to the indices of changes in wastewater from real sewage of the pilot plant to acetate-based synthetic feed. This result demonstrated that the different fatty acid profiles noted in this research could be attributed mainly to changes in the microbial populations, rather than to the different fatty acid compositions of the untreated wastewaters used for
According to the fatty acid peak analysis, the introduction of new biomass at three different times (day 0, day 42, and day 79) yielded a similar changing pattern in fatty acid profiles. For example, whenever new biomass was introduced to the lab-scale reactors, the mole percentages of C16:1ω7c and C18:1ω7c increased, and those of C16:1ω4t and C18:1ω4t decreased (Figure 2-13).
Figure 2-13. Observed fatty acid profiles of day 0 and day 8. Peak A, B, C and D represent C16:ω7c, C16:ω4t, C18:ω7c and C18:ω4t, respectively.

This finding was further confirmed by PCA analysis (Figure 2-14). Figure 2-14 shows that the microbial community populations that were transferred to the lab-scale bioreactors on days 0, 42 and 78 (Group 1) were much more similar in composition than those on days 8, 49 and 85 (Group 2) which had been maintained for 7 days in the lab-scale bioreactor. Based on the similarity calculation, the similarity of the microbial community populations within one group (Group 1 or Group 2) averaged 75%, while the similarity between these two groups was only 45%. This finding indicates that a microbial community maintained under relatively constant operating conditions retains its main characteristics for a long time. In this case, the main characteristics of the fatty acid profile of the pilot-scale UCT process...
(high percentages of C16:1ω4t and C18:1ω4t) were conserved for more than 2 months. However, only seven days after the same biomass was subjected to acetate-based synthetic acid, these typical characteristics disappeared. This can be attributed to the fact that the dominant bacterial population changed due to the change in operating factors. Similar experimental results have shown that short-term variability in feed composition affected the microbial community population of a pharmaceutical wastewater treatment system much more than did that of long-term consistent operation (LaPara et al., 2002).

Figure 2-14. PCA for the community populations of pilot plant biomass on day 1, 42, and 78 (Group 1) and lab scale CAS biomass 7 days after transfer from the pilot plant on day 8, 49 and 85 (Group 2). A: day 0, a: day 8, B: day 42, b: day 49, C: day 78, c: day 85.
2.3.4.2 Steady State

It is important to understand microbial community population behaviour under steady state operating conditions. Many research papers, dealing with microbial community populations have assumed that microbial community populations do not change unless changes occur in their surrounding conditions. Studies on the changes of microbial community populations in a very stable environment have largely been neglected. However, if microbial community populations are dynamic even under very stable operating conditions, it is important to know how much change may occur. Otherwise, it will not always be of significance to study population changes of a biological system with changes in some operating factors or surrounding conditions. For example, if 30% of the change noticed in a microbial population is due to an increase in temperature for 50 days, the observation would not be valid until it can be determined that the change in the microbial population was caused only by the change in the temperature and not by the natural dynamics of the microbial population. It is valuable, therefore, to gain information regarding the dynamics of microbial community composition under stable operating conditions. The experiments in this section were designed to generate such information.

Four or five days after the new biomass was introduced into the lab-scale reactors, the carbon removal efficiency and the daily similarity index were observed to be stable. The high stability in this microbial community was maintained as long as operating conditions were constant. The average daily similarity and the standard deviation at steady state were
calculated to be 95.6% and 1.2% respectively. This suggests that the microbial community populations were probably changing slowly under the very stable operating conditions, since the average daily similarity was only slightly less than the similarity value of 97.3% calculated from the ten identical samples prepared to estimate experimental errors in MFA analysis. Figure 2-15 represents typical daily similarity index, cumulative similarity index and carbon removal under steady-state operation.

The daily similarity index and carbon removal efficiency were seen to be quite stable. The cumulative similarity index showed that the similarity compared to the reference biomass continuously decreased at steady state, such that the cumulative similarity after ten days averaged around 80%. After ten days, the cumulative similarity index didn’t change much

Figure 2-15. Typical daily similarity index, cumulative similarity index and carbon removal efficiency under steady-state operation.
further. The two experimental findings observed in section of 2.3.4.1 and 2.3.4.2 – (1) microbial populations are changing even under very constant operating conditions, and (2) the characteristics of a microbial population in a system are retained for a certain period of time – appear to be contradictory. This phenomenon may be explained using an example. If there is a fish in a pot, it moves all the time. However, the boundary of the movement is defined by the pot size. Here, we may define the movement of the fish as a daily change in microbial community population, and the size of the pot as an imposed operating condition to a system. Like a fish in the pot, the microbial population may change all the time. However, its main characteristics are likely retained for a long time within a certain boundary.

It was also suggested from the experimental results that research dealing with microbial community populations shouldn’t neglect the natural dynamics of microbial community populations in a very stable environment.

2.3.4.3 Impact of a Chlorine Shock

The goal of wastewater treatment systems is to consistently produce high-quality effluent. However, if a harmful or toxic substance is introduced to wastewater treatment systems, the process can be upset. In the present experiment, it was investigated whether MFA monitoring technique could detect the effect of a toxic material on microbial community populations and system performance. In this experiment, chlorine was selected as the toxic
material, and the impact of several chlorine additions on microbial community population was assessed. A measured volume of chlorine stock solution was quickly added to the system (CAS-2) with a pipette, resulting in chlorine concentrations (as Cl₂) of 4 mg/L or 10 mg/L in the bioreactor solution. Chlorine additions into CAS-2 were executed on day 20, 21, 24 and 26 with four mg/L chlorine spiking, and on day 87 with ten mg/L chlorine spiking. CAS-1 was operated as a control reactor during the period.

Figure 2-16 shows the effects of chlorine addition on the microbial fatty acid profile of the biological community population and the removal efficiency of organic materials measured as TOC. The effects of four mg/L chlorine spiking were well detected by the monitoring technique based on microbial fatty acid analysis and similarity index. Although the carbon removal efficiency remained above 95%, the daily similarity index one day after the first chlorine spike (on day 20) decreased to 88%, and the second chlorine addition on day 21 decreased the daily similarity to 87%. The third and fourth chlorine additions also resulted in decreases in daily SI (92.5% and 90.5% for the third and fourth chlorine additions, respectively). Although no chlorine was added to the system from day 21 to day 23, the daily similarities remained lower than the average daily SI of the control reactor (CAS-1) during the same period (Figures 2-16 and 2-17). This increase in microbial population dynamics due to chlorine addition was demonstrated by the cumulative index as well. Only eight days after the first chlorine addition, the similarity between day 28 and day 18 was only 50%. The value was abnormally low compared to the cumulative SI of CAS-1, which was 83% (Figure 2-17).
Figure 2-16. The daily and cumulative SIs and carbon removal of CAS-2 associated with chlorine addition. Arrows indicate the days when 4 mg/L chlorine was added.

Figure 2-17. The daily and cumulative SIs and carbon removal of CAS-1 (control unit)
The daily SI and cumulative SI of the control reactor (CAS-1) were quite stable, which suggests that the perturbations of the microbial community population of CAS-2 can be purely attributed to the chlorine additions, because the same operating conditions were imposed on both reactors (CAS-1 and CAS-2) except for the chlorine additions to CAS-2.

The results were also analyzed by principal component analysis (PCA), which has been previously used for characterizing microbial community compositions (Princic et al., 1998). Using the fatty acid profiles of CAS-1 (control reactor) and CAS-2 (experimental) during the chlorine addition period, PCA showed a similar result (Figure 2-18) to the results of the analysis using similarity index shown above (Figure 2-16, Figure 2-17).

Figure 2-18. Principal component analysis (PCA) of MFA patterns after four mg/L chlorine addition to CAS-2. Thirteen points in the circle represent PCA of control reactor (CAS-1) during the same period. Bold numbers indicate the days when chlorine was added.
Since the distances between the points on the PCA graph represent the similarity or dissimilarity of their microbial fatty acid profiles analyzed (closer in distance, the more similar the microbial community populations), the microbial community composition of CAS-2 (scattered pattern with time) is seen to be much more dynamic during the period than that of CAS-1, which was grouped through principal component analysis.

A final spike of ten mg/L of chlorine on day 87 produced a detrimental effect on carbon removal efficiency and significant changes in the microbial community composition as well (Figure 2-19).

The next day, the carbon removal efficiency for the synthetic wastewater decreased to 92%, and the similarity decreased to 82%. Two days after the chlorine spiking, the carbon removal efficiency further decreased, although daily similarity increased. In spite of the quick recovery in carbon removal efficiency, chlorine spiking rendered the microbial community less stable (Figure 2-19). An average daily similarity index for the 5 days after the ten mg/L chlorine spiking was about 91.5%, which was lower than that of normal operation. On the other hand, the control reactor (CAS-1) showed stable daily and cumulative SIs, and carbon removal efficiency during the same period, which indicates that the fluctuation of the biomass of CAS-2 was caused by the ten mg/L chlorine addition (Figure 2-20).
Figure 2-19. Daily SI, cumulative SI and carbon removal efficiencies for CAS-2 (experimental reactor) during 10 ppm chlorine addition. The arrow indicates the time at which 10 ppm chlorine was spiked to CAS-2.

Figure 2-20. Daily SI, cumulative SI and carbon removal efficiencies for CAS-1 (control reactor) during the experimental period described in Figure 2-19.
These experimental results raised the fundamental question of whether the observed
changes in microbial fatty acid profiles could be due to a chemical reaction between MFA
and the chlorine added to the system, since chlorine is very reactive material. In order to
determine whether the changes in fatty acid profiles associated with the chlorine additions
originated from chemical reactions of chlorine with microbial fatty acids, or from changes
in the microbial population, four replicate biomass samples from CAS-1 were prepared and
divided into two groups at day 91. One group was subjected to the normal fatty acid
extraction procedure, and the other group was stored with 10 mg/L of chlorine overnight in
a refrigerator. The results showed that there were no statistically significant differences
between the two groups (similarity averaged 97.2%), which indicates that 10 mg/L of
chlorine did not chemically change the fatty acid compositions during the experiment
(Table 2-8).

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (Control)</th>
<th>Group 2 (10 mg/L Cl₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Similarity within group (%)</td>
<td>97.2</td>
<td>97.8</td>
</tr>
<tr>
<td>Similarity between groups (%)</td>
<td></td>
<td>97.2</td>
</tr>
</tbody>
</table>

The observed changes in fatty acid composition due to chlorine addition were, interestingly,
almost opposite to those associated with the transition state following biomass transfer from
the pilot plant to the lab scale bioreactor (section 2.3.4.1) For example, the mole
percentages of C16:1ω7c and C18:1ω7c (those fatty acids comprised almost 40% of total
fatty acids in the acetate-fed biomass) decreased, and those of C16:1ω4t and C18:1ω4t (those fatty acids were dominant in the UBC pilot-plant biomass) increased. These results suggest the possibility that the microbial species that proliferated due to the acetate feed in the lab-scale bioreactors were most affected by the addition of chlorine, resulting in a decrease in the population of that bacterial group.

2.3.4.4 Impact of a pH Shock

pH may be one of the most variable parameters in the operation of biological wastewater treatment systems. In order to investigate how changes in pH influence the microbial fatty acid profile of a microbial community, CAS-1 was operated without pH control for one week, between day 57 and day 63. During this period, the pH decreased slowly from 6.5 to 5.2. The daily similarity index fluctuated during the period over the range of 89 - 94%, and the average similarity index was 92.5% (Figure 2-21). The pH control was re-established at a set point of 6.5, and four days later, the set point was increased to 8.5. This pH increase resulted in an elevated shift rate of the microbial community population of CAS-1 (Figure 2-21). Although the daily similarity decreased to 89%, the carbon removal was stable. The cumulative similarity index demonstrated an increase in shift rate of the microbial population associated with the pH change. The similarity between the reference biomass and biomass on day 64 was 76%, while the biomass in the control reactor (CAS-2) on the same day showed 82% similarity to the reference biomass (Figure 2-22).
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Figure 2-21. The daily similarity index, cumulative similarity index and carbon removal efficiencies associated with pH variation in CAS-1 (experimental reactor).

Figure 2-22. The daily similarity index, cumulative similarity index and carbon removal efficiencies in CAS-2 (control reactor) during same period.

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After pH control was resumed, the microbial population began to return to that of the reference microbial population (between day 64 and day 68, Figure 2-21). This phenomenon was also found in the experiment by Princic et al. (1998). In their experiment, the same biomass was incubated at different pH levels of 6.0 and 8.2 for 72 days. Fifteen days after the differences in pH conditions were eliminated, the bacterial community populations returned to the original populations based on MFA analysis. These experimental results indicate that the main characteristics of the biomass might be largely determined by operating conditions and system configuration and the characteristics do not change significantly unless significant changes in operating or/and system configuration are imposed.

The observed response to the pH changes was unexpectedly small, even though the $\text{H}^+$ ion concentration became 100 times lower. The change was even smaller than that of the 4 mg/L chlorine addition. Most likely, this small change in microbial community was due to the fact that the range over which the pH was varied (6.5 - 8.5) was still a tolerable pH range for most of the micro-organisms, so that they could easily acclimate to the new environment. This finding confirmed the conventional knowledge that pH changes within the range of 6 - 8 are acceptable in biological wastewater treatment systems. However, for cases in which substrate population and/or solubility vary with pH value, subtle pH changes in a raw wastewater could affect microbial community populations and process performance, even within this range (Werker and Hall, 1998).
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The analysis of fatty acid peaks associated with the pH change showed that the predominant bacterial community with an acetate feed, represented by the main peak (C16:1ω7c), decreased due to the pH change. This was also observed during the chlorine addition experiments, which suggests that the operating conditions selected in this experiment were most favourable to the micro-organisms that have C16:1ω7c in a high percentage in the membrane.

2.3.4.5 Impact of a Substrate Loading Shock

Variations in substrate strength and composition were the main causes impacting the microbial community identified in several literature reports (LaPara et al., 2002; Stamper et al., 2003). Moreover, the variability in substrate composition and flow rate of influent is hard to control in many cases. Consequently, it is important to investigate the possible effects of the change in strength of incoming wastewater on microbial community population. In the present study, the effects on a microbial community could be monitored through MFA analysis.

In order to determine the influences of substrate loading on microbial fatty acid profile, a 30% increase in substrate load (from 1,100 mg/L·d to 1,430 mg/L·d) was applied to both CAS-1 and CAS-2 on day 37, and to CAS-2 only on day 53. The similarity monitoring results responded to the changes. On day 37, the daily similarity decreased from 95% to 85% and 91% to 80% for CAS-1 (Figure 2-23 (A)) and CAS-2 (Figure 2-23 (B)), respectively; and, on day 53 from 94% to 85% for CAS-2 (Figure 2-23 (B)).
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Carbon removal efficiency was not affected by the increase in substrate load (Figure 2-23 (A) and Figure 2-23 (B)).

Figure 2-23. Daily SI, cumulative SI, and carbon removal efficiencies associated with increase in organic loading for CAS-1 (A) and CAS-2 (B). Phase I represents normal organic loading (1100 mg/L·d) and phase II represents increased organic loading (1430 mg/L·d)
The stable carbon removal efficiency was thought to be due to the readily biodegradable organic source (mainly sodium acetate) in the synthetic wastewater. However, the sudden increase in substrate concentration of the feed resulted in system upsets. The two systems faced severe bulking problems, and the operation was stopped on day 41.

New biomass was transferred to the lab-scale CAS reactors on day 41. As described in the previous section (2.3.4.1), the transfer of new biomass brought about similar changes in daily and cumulative SI, and carbon removal efficiency.

It was found, through fatty acid peak analysis, that the main change observed in the MFA profiles was a sudden increase of two peaks; C16:1ω7c and C18:1ω7c. The changes in fatty acid composition were similar to those that occurred when biomass was originally transferred from the pilot plant to the lab-scale bioreactor (section 2.3.4.1). This finding may indicate that the bacterial group which was well adapted to acetate-based synthetic wastewater during the transition stage of this experiment, was more dominant in the reactor.

2.3.5 TMFA and MLVSS

Werker and Hall (2000) showed that TMFA could be used as a parameter for expressing biomass concentration for the purpose of estimating microbial growth kinetics associated with wastewater treatment during batch growth of biomass, although it has been known that microbial fatty acid compositions could change with environmental conditions and cell age.
(Haack et al., 1994). In the present study, the comparisons of total microbial fatty acid concentrations and biomass concentrations based on MLVSS measurements showed a good agreement with the results of Werker and Hall (2000). The biomass measured as VSS that was subject to relatively stable operating conditions, showed a good correlation ($R^2 = 0.9532$) with lipid content for various biomass concentrations from 1,000 to 2,500 mg/L (Figure 2-24).

This experimental observation could provide an additional advantage of MFA monitoring technique. If MFA analysis is already being routinely used as a monitoring tool for biological wastewater treatment systems, the similarity index monitoring technique may reduce the need to perform the traditional MLVSS measurement, Even though MFA analyses are more complex than traditional MLVSS measurements.

From the results, calculated lipid concentrations were estimated to be about 6.1% of the MLVSS concentrations in the samples collected.
Figure 2-24. Relationship between total microbial fatty acids and MLVSS.
2.4 Conclusions

The experimental results showed that a similarity calculation, based on microbial fatty acid analysis, was a good monitoring tool for the expression of microbial community stability and the correlation of the changes in community composition to process performance (specific issues 1 and 2 in section 2.1.4). The method could quantify changes in microbial composition by correlating the changes in composition with variations in operating parameters such as pH, substrate loading, substrate composition and a toxic material input. Also, this technique was easily interpreted by a simple calculation and graphs using a similarity index (specific issue 3).

It was shown that microbial communities were constantly changing, and that their shift rates increased under dynamic environmental conditions. If microbial fatty acid analyses are routinely executed as a monitoring tool, this method could also be used as a good estimator for the MLVSS concentration in a wastewater treatment system, minimizing the time and effort required for the conventional measurement of MLVSS (specific issue 4). However, the lab-scale bioreactors using synthetic wastewater (acetate-based) did not show enough information regarding system performance associated with the changes in operating conditions. It was postulated that the organic material used for the experiments was so simple, that its removal rate was not affected by the operating changes imposed.

This assumption partly motivated the experimental plan in Chapter 3, whereby two lab-scale bioreactors were fed with real sewage wastewater. The experiment using real sewage
wastewater would provide the possibility for this monitoring technique to be applicable to full-scale wastewater treatment systems.
2.5 References


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Chapter 3

Development of Monitoring Tool Based on Microbial Fatty Acid (MFA) Analysis Using Lab-scale Bioreactors Fed with Real Municipal Wastewater

Abstract

The development of culture-independent methods for the identification of microbial community has opened a new era of study into real microbial populations and their dynamics. Although many attempts have been made to link such dynamics to system performance, little information on this topic has been published. In the previous chapter, a new monitoring technique that can correlate microbial population dynamics to system performance was introduced by analyzing the fatty acid profiles of biomass, using two lab scale bioreactors fed with synthetic wastewater. The findings showed that fluctuations in operating conditions such as substrate loading, pH level and chlorine addition resulted in microbial population dynamics that may impact system performance. The MFA analysis was used as a monitoring technique for detecting increases in the rate of microbial population changes based on a simple similarity calculation. In this chapter, results are presented from experiments in which the MFA monitoring technique was applied to bioreactors with real municipal wastewater. The experiments demonstrated that the MFA analysis technique was also applicable to the reactors fed with real municipal wastewater. From the results, it was found that: (1) the microbial community that was subjected to real municipal wastewater responded in a similar fashion to that subjected to acetate-based synthetic wastewater; (2) changes in operating factors such as pH, DO, chlorine addition impacted more significantly on the microbial community and system performance in real municipal wastewater; and, (3) the monitoring method developed was also applicable to a system subject to more dynamic operating conditions, suggesting that that this technique could be used for pilot-scale or full-scale wastewater treatment monitoring.
Chapter 3: Development of Monitoring Tool Based on Microbial Fatty Acid Analysis using Lab-scale Bioreactors Fed with Real Municipal Wastewater

3.1 Introduction

Various biological treatment methods have been successfully applied to processes such as wastewater treatment, soil remediation, biofiltering and composting. Biotreatment methods are economical, and their by-products are usually less harmful than those generated by chemical treatments (Metcalf and Eddy, 1991). There is, however, one significant drawback to biological treatment methods: their efficiency is not always stable. This may be attributed to the lack of knowledge of complex microbial community populations in the systems (Nielsen et al., 1999).

A good understanding of microbiology in a biological system, beyond the so-called “black box approach”, has been thought to be essential in designing and operating treatment systems. The results from simple modeling, or testing that considers a complex microbial community population as a single or pseudo-species, may have a limited scope of application (Werker, 1998). Recently developed culture-independent methods using molecular techniques or chemo-taxonomic techniques have made it possible to gain detailed insight into microbial community populations (Yu and Mohn, 1999; Amann et al., 1998; Webster et al., 1997) (Refer to section 2.1 for detailed information about each method).

To date, there have been many experimental results that have shown that changes in feed materials, temperature, dissolved oxygen levels, system configuration, and nitrogen and phosphorus concentrations could change the microbial population in a biological treatment
process (Princic et al., 1999; Wang and Park, 1998; Webster et al., 1999; Kelly et al., 1999; Frostegard et al., 1997; Moll and Summers, 1999). However, the results have been focused on characterizing and enumerating the microbial population. There still remain substantial gaps in the knowledge about population changes and in the practical application of this knowledge to biological wastewater treatment systems.

Recently, more practical applications through direct monitoring of microbial community population in a wastewater treatment system have been developed. For example, Cha et al. (1999) showed that monitoring of Nocardia levels in activated sludge systems was possible through analysis of OH-C19 concentrations because Nocardia contains this particular fatty acid as a signature fatty acid that can be easily tracked.

Son and Hall (2003) demonstrated a new concept that can link the monitoring of microbial community populations with biological wastewater treatment system performance. In the experiment, it was shown that simple similarity calculations, based on microbial fatty acid analysis, provided a good monitoring tool for the estimation of microbial community composition stability and the correlation of the changes in community composition to process performance. The method could connect changes in microbial community composition by correlating the changes in composition to variations in operating parameters such as pH, substrate loading, substrate composition and a toxic material input.

Further experiments of this type necessitated change of the synthetic feed based on acetate,
to raw municipal wastewater, for two reasons. First, previous experimentation with lab-
scale bioreactors using synthetic wastewater (acetate-based) did not show a strong system
performance response associated with changes in operating conditions. It was assumed that
the organic material (sodium acetate) used for the experiment was so simple and
biodegradable that its removal rate was not significantly affected by operating changes.
(Refer to section 2.4 for detailed discussion.) Second, it would be a valuable challenge to
apply the results acquired from bioreactors fed with synthetic wastewater to reactors fed
with real municipal wastewater from a pilot plant. Many interesting results from bioreactors
with synthetic wastewater have shown limited applicability for waste treatment systems
that were subject to more dynamic operating conditions. One of the reasons may be that the
most experimental results from lab-scale experiments were acquired during steady state
operation, using synthetic feeds. As discussed in Chapter 2, actual microbial populations in
a full-scale system should be much more dynamic because many operating factors, such as
temperature and raw wastewater composition, may vary widely.

In this chapter, the main objective was to address the following question using two lab-
scale CAS systems fed with real municipal wastewater:

*Is the monitoring method using MFA analysis applicable to the more dynamic
microbial community populations that can be expected in pilot-scale or full-scale
systems treating real municipal wastewater?*
Although the reactors used in this experiment were not pilot-scale or full-scale systems, real municipal wastewater was assumed to provide a much more complex and dynamic environment for the microbial population in terms of wastewater strength and composition.

In the present experiments, the microbial population dynamics were monitored during a period of steady state operation to set up a base line for the similarity index measurements. Subsequently, the impact of changes in pH, DO, chlorine and substrate on the microbial communities were evaluated with respect to a control reactor that experienced stable conditions during the same period. As in Chapter 2, a simple similarity index calculation was employed to express the changes in the observed microbial community populations.
3.2 Methods and Materials

3.2.1 Control and Operation of the Lab-scale Bioreactors

Two identical conventional activated sludge (CAS) systems (CAS-1, CAS-2) with 3-liter bioreactors were set up in a constant temperature chamber maintained at 20 ± 1°C. The biomass used was transferred into the reactors from a UCT (University of Cape Town) pilot plant that was treating municipal wastewater at the University of British Columbia, Vancouver, Canada. Mixing of the lab-scale reactors was accomplished by using mechanical stirrers. Dissolved oxygen (DO) levels were manually controlled between 3.0 ± 1.0 mg/L by an air pressure regulator and a flow meter. The DO concentration was measured by DO probes (YSI Model number 5739), which were connected to DO meters, YSI 54A (YSI incorporated, Yellow Springs, Ohio). The pH levels were maintained within the range of 6.5 - 6.8 by a pH controller (Cole-Parmer Instruments, Chicago, Illinois) using 0.2 N NaOH. Most of time, the pH was maintained without any input of acid or alkaline solution. The hydraulic retention time (HRT) was 12 hours, and the sludge retention time (SRT) was 10 days. Table 3-1 shows the set-point experimental operating conditions and the associated variations. These operating conditions were maintained for the entire experimental period, except for short-term experimental runs designed to assess how operational changes affected microbial community populations. While one reactor was subjected to variations in operating conditions, the other reactor was operated as a control reactor. CAS-1 was subjected to the variations in pH and dissolved oxygen (DO) levels, and CAS-2 was subjected to toxic material inputs using a chlorine solution. CAS-2 was also
subjected to a change in substrate. The feed was changed from real municipal wastewater to synthetic wastewater based on sodium acetate. The pH shocks from 6.5 to 8.5 were implemented by addition of 0.2 N NaOH using a pH controller (Cole-Parmer Instruments, Chicago, Illinois). For each chlorine spike to the system (CAS-2), the needed volume of a hypochlorite stock solution (50,000 mg/L as Cl2) was quickly added to the bioreactor with a pipette. The chlorine concentrations were measured according to Standard Methods (APHA et al., 1995).

Table 3-1. Lab-scale bioreactor operating conditions for steady state and operational changes.

<table>
<thead>
<tr>
<th>Items</th>
<th>Temp (°C)</th>
<th>DO (mg/L)</th>
<th>SRT (day)</th>
<th>HRT (day)</th>
<th>pH</th>
<th>Substrate Loading</th>
</tr>
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<td>10</td>
<td>0.5</td>
<td>6.5</td>
<td>552 - 774 mg/L·d</td>
</tr>
<tr>
<td>Changes</td>
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<td>0.1</td>
<td>NC</td>
<td>NC</td>
<td>5.2-8.5</td>
<td>552 - 774 mg/L·d</td>
</tr>
</tbody>
</table>

Note: Substrate loading = influent TOC (mg/L)/HRT (day)
NC: No change.

3.2.2 Preparation of Municipal Wastewater and Synthetic Raw Wastewater

Fifteen liters of raw municipal wastewater were collected daily from the UBC wastewater treatment pilot plant. Seven liters of the raw wastewater were mixed with seven liters of the raw wastewater collected on the previous day and stored in a cold room. This was intended to prevent dramatic changes of constituents in the raw wastewater due to grab sample
collections. The total organic carbon (TOC) value of the mixed municipal wastewater used during the experimental period was measured every day and the ammonia and orthophosphate values of the wastewater were measured three times a week.

The synthetic wastewater used for the experiment consisted of sodium acetate as a carbon source, ammonium chloride as a nitrogen source, and potassium phosphate as a phosphorus source. In addition, peptone and yeast extract were provided with some minerals such as Mg, Ca, and Fe. The detailed composition of the raw wastewater is presented in Table 3-2. The concentration of the raw wastewater was 130 ± 20 mg/l of TOC.
Table 3-2. Composition of synthetic wastewater used.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>CH₃COONa·3H₂O</td>
<td>360</td>
</tr>
<tr>
<td>Peptone</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂·2H₂O</td>
<td>32</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO₄</td>
<td>214</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>(NH₄)₂SO₄</td>
<td>40</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>KH₂PO₄</td>
<td>20</td>
</tr>
<tr>
<td>monobasic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>FeSO₄</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Fifteen litres of synthetic wastewater were prepared every day from a 10 X stock solution. The feed tank was also cleaned every day using diluted bleach solution (0.5%) and tap water to prevent microbial contamination on the wall of the feed tank.

3.2.3 Analysis

Total Organic Carbon (TOC) measurement and suspended solids (MLSS/MLVSS) were measured as described in Chapter 2. Microbial fatty acid analysis and ribosomal intergenic spacer analysis were carried out as described in Chapter 2. For numerical analyses, such as similarity index and principal component analysis, refer to section 2.2.

3.2.3.1 Ammonia (NH₃ and NH₄⁺)

Real municipal wastewater samples were analyzed for ammonia using a LachatR Quikchem...
Automated Ion Analyzer. Samples were filtered through Whatman #4 filters and diluted as necessary. The pH was adjusted using sulphuric acid to a pH 3.0 and the samples were then stored at 4°C until analysis. Ammonia concentrations were determined using methods outlined in the Methods Manual for the Quikchem Automated Ion Analyzer (1987).

3.2.3.2 Orthophosphate (Ortho-P)

Real municipal wastewater samples were analyzed for orthophosphate using a Lachat™ Quikchem Automated Ion Analyzer. Samples were filtered through Whatman #4 filters, preserved with several drops of phenyl mercuric acetate, and stored at 4°C until analysis. Orthophosphate (Ortho-P) concentrations were determined using methods outlined in the Methods Manual for the Quikchem Automated Ion Analyzer (1987).
3.3 Results and Discussion

The previous experiments in Chapter 2 demonstrated the possibility that analysis of microbial fatty acids could be a useful monitoring tool for the detection and prevention of system problems associated with undesirable or damaged microbial populations. These experiments were completed using two identical CAS bioreactors fed with synthetic wastewater based on acetate as a major carbon source. The experimental results demonstrated that the microbial community responded to changes in operating conditions. Among the factors examined, changes in substrate loading and composition significantly affected the community.

The main objective of the experiments in Chapter 3 was the determination of the applicability of the MFA technique as a monitoring tool for a lab-scale reactor that was subjected to real municipal wastewater, rather than a simple synthetic feed (acetate-based). To compare the results of this experiment with the results from Chapter 2, similar changes in operating conditions were made to the lab-scale CAS bioreactors. As described in Chapter 2, a similarity index was employed to quantify the changes in the microbial community populations. Figure 3-1 represents the overall daily similarity index and carbon removal efficiency of the two CAS reactors (CAS-1 and CAS-2) during the entire experimental period. Unlike the results from Chapter 2, the carbon removal efficiency of the systems was significantly impacted by the changes in operating conditions, in parallel with the changes in microbial community populations.
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Figure 3-1. Overall daily similarity index and carbon removal efficiency for CAS-1 (A) and CAS-2 (B), respectively, during entire experimental period.
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The difference was attributed to the mixed substrate in the real municipal wastewater used for this experiment.

3.3.1 Municipal wastewater characteristics during experimental period

The soluble TOC value of the mixed municipal wastewater used during the whole experimental period, was measured every day and the ammonia and orthophosphate values of the wastewater were measured three times a week. Figure 3-2 shows the concentrations of TOC, ammonia and orthophosphate measured during the experimental period. The ranges of TOC, ammonia, and orthophosphate were 92-129 mg/L (as carbon), 18-25 mg/L (as nitrogen) and 3-7 mg/L (as phosphorus), respectively. From the measurements, it was concluded that the nitrogen and phosphorus contents were not limiting factors, which was generally anticipated for municipal wastewater. It was also assumed that other minerals necessary for the growth of bacteria were present in sufficient quantities. Therefore, no additional modification was made to the raw wastewater. For the 106 days of the experiment, the average TOC was 107, and the standard deviation was 11.4.

The fatty acid compositions of raw municipal wastewater used for the feed were analyzed because it might affect the microbial fatty acid profiles measured throughout the present experiment. Total fatty acid concentrations originating from the raw municipal wastewater during the period averaged 0.12 mg/L and the results are shown in Table 3-3.
Table 3-3. Concentration of total fatty acids originating from raw wastewater analyzed for 10 days from day 0 to day 10.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc.(mg/L)</td>
<td>0.16</td>
<td>0.13</td>
<td>0.12</td>
<td>0.08</td>
<td>0.11</td>
<td>0.07</td>
<td>0.12</td>
<td>0.15</td>
<td>0.13</td>
<td>0.14</td>
<td>0.12</td>
</tr>
</tbody>
</table>

The average total concentration of the fatty acid originating from the raw municipal wastewater was approximately 9% of total microbial fatty acid concentration from about 2,000 mg/L of micro-organisms as MLVSS.

Figure 3-2. Overall TOC, ammonia and orthophosphate concentrations during the experimental period
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Figure 3-3 shows the average mole percent and standard deviation of each fatty acid in the raw municipal wastewater used as calculated from the analysis of ten municipal wastewater samples (from day 0 to day 9). Most of the fatty acids in the wastewater were C16 and C18, or their derivatives. The mole percents of each fatty acid were relatively stable compared to the TOC variation of raw wastewater used.

This information implies that the variation of the raw wastewater fatty acid compositions is not the cause of the variation of the changes in microbial fatty acid profiles.

![Figure 3-3. Average mole percents and standard deviation of each fatty acid in municipal wastewater used during the experimental period. Lines in the bars indicate ± standard deviations (n=10).](image-url)
3.3.2 Similarity changes in microbial community population vs system performance
Associated with perturbations in operating factors

3.3.2.1 Biomass transfer from the pilot plant to the lab reactors

The configurations of wastewater treatment systems and substrate characteristics in raw wastewater may be important factors that affect microbial community population in a biological wastewater treatment system (LaPara et al., 2002; Stamper et al., 2003; Hiraishi et al., 1998). Different kinds or even different types of atomic bonding patterns in the same materials (for example, L- or R-forms of hydrocarbons) can affect the metabolic mechanisms of bacteria, resulting in different microbial populations in biological and natural systems (Werker and Hall, 1998; Liu et al., 1997; Princic et al., 1998). Wastewater treatment system configurations, including dissolved oxygen level, hydraulic retention time, sludge retention time and recycling rate, are also thought to affect microbial community populations (Wang and Park, 1998). The previous experiments in Chapter 2 showed that major impact factors were the changes in raw wastewater characteristics, rather than different treatment system configurations that resulted when biomass was transferred from the pilot-scale University of Cape Town (UCT) system to lab-scale CAS systems. The experiments described in this chapter further confirmed this finding. The day after new biomass was introduced into the lab-scale bioreactors from the pilot plant, the average similarity and carbon removal efficiency for both bioreactors were 92% and 85%, respectively (Figure 3-4). The similarity measured in this chapter during biomass transfer was much higher than that measured in section 2.3.4.1. That was attributed to the fact that
the raw wastewater used for the lab-scale reactor in the present experiment was the same municipal wastewater treated in the pilot-scale system from which the biomass was transformed.

Figure 3-4. Daily and cumulative similarity indices and carbon removal efficiency during the period of biomass transfer.

The carbon removal efficiency quickly reached an average value of 90% under steady state conditions. The small initial drop in removal efficiency was thought to be due to differences in the lab-scale and the pilot scale system configurations (Table 3-4). The 92% similarity value calculated after one day was a little below the average daily SI value (94%) under steady state operation in the present experiment, which will be explained in the next section.
This change in the microbial community population was unexpectedly small, considering the huge difference in two system configurations.

<table>
<thead>
<tr>
<th></th>
<th>Lab-scale CAS Process</th>
<th>Pilot-scale UCT Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO (mg/L) (in aerobic tank)</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>5.8</td>
</tr>
<tr>
<td>SRT (day)</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>HRT (day)</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>20</td>
<td>12 - 17</td>
</tr>
<tr>
<td>MLSS (mg/L)</td>
<td>1,500</td>
<td>3,400</td>
</tr>
</tbody>
</table>

Ribosomal intergenic spacer analysis (RISA) showed no noticeable change in the microbial population during the first four days (Figure 3-5). In contrast, as described in chapter 2, RISA showed a significant change in microbial banding pattern for the first four days, when biomass was transferred from the pilot plant to the lab-scale bioreactors that were subjected to synthetic feed (Figure 2-10). This result verified the finding that the substrate composition of the wastewater more strongly affects microbial community population than do other operating factors such as HRT, SRT, operating temperature and system configuration in a biological wastewater treatment system.

Similar results were observed in the experiment by Hiraishi et al. (1998). In their
experiment, they showed that the differences in microbial populations were much larger when they compared different wastewater sludges (i.e. raw municipal and synthetic municipal) than when they compared sludges from EBPR and standard processes or plantscale and laboratory-scale systems. Also a numerical cluster analysis of the profiles showed that the sludges tested fell into two major clusters: one included all raw municipal sludges, and the other consisted of all synthetic municipal sludges, independent of the operational mode and scale of the reactors, and the presence or absence of phosphate accumulation.

Figure 3-5. RISA banding patterns of day 0, day 2 and day 4. M represents marker and K indicates 1,000bp length of nucleotide base pair. Samples were taken from CAS-1.
Overall, it was concluded from the present experiment, along with the experiments obtained in Chapter 2, that a change in substrate composition gave rise to strong and acute impacts on microbial community population. Although the differences in wastewater treatment system configuration may affect microbial community populations, the effect would be slow and relatively small, within a short period of four days.

3.3.2.2 Steady state

As discussed in Chapter 2, it is important to know how daily and cumulative similarity indices change under steady state conditions, in order to compare the similarity indices brought about by abnormal operation. Unless we know this information, it will be difficult to judge, in the situation where the microbial community population is continuously changing even under very stable operating conditions, whether the similarity indices as a result of changes in pH or other factors are significant values or not.

Ten days after new biomass was introduced from the UBC pilot plant to the lab-scale CAS reactors, the daily SI started to show stable behaviour. Figure 3-6 presents average daily SI and cumulative SI data of CAS-1 and CAS-2 collected under steady state operation from day 11 through day 20 (CAS-1 and CAS-2 showed quite similar behaviour regarding the daily SI and cumulative SI). The average daily SI (93.9%) and standard deviation (1.3%) of the period were slightly lower than the average daily SI (95.6%) and the average standard deviation (1.2%) observed at steady state in Chapter 2. This result was attributed to the dynamic changes in substrate composition and the strength of the influent used for this
current experiment, compared to those of synthetic wastewater used in Chapter 2. Figure 3-7 presents TOC concentrations of influent raw wastewater and average carbon removal efficiency of CAS-1 and CAS-2, during the period of steady state operation (average carbon removal value and standard deviation based on TOC measurement were 90.5% and 0.7%, respectively). Although influent TOC concentrations fluctuated over a wider range (92 - 118 mg/L) than the TOC of the synthetic wastewater used in the previous chapter, the TOC removal efficiency was stable. This may indicate that the microbial population was well adapted to the wastewater characteristics.

Figure 3-6. Average daily SI and cumulative SI of CAS-1 and CAS-2 over time under steady state conditions.
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Figure 3-7. Average carbon removal efficiency of CAS-1 and CAS-2 and influent TOC changes over time under steady state conditions.

The average similarity values and standard deviations in the steady state period were calculated to be: 93.9% and 1.5% for CAS-1, and 93.7% and 1.5% for CAS-2, respectively. Since the average similarity was less than the similarity value of 96.3% calculated from the ten replicate samples prepared to estimate experimental errors in MFA analysis, these results suggest that the microbial community populations could still have been slowly changing under the stable operating conditions imposed. The cumulative similarity index showed that the similarity compared to the reference biomass (day 10) continuously decreased under steady state conditions, indicating continuous change in the microbial populations as well (Figure 3-6). The cumulative similarity ten days after the reference day
averaged around 72%. After ten days, the cumulative similarity index didn’t change much further, although the daily similarity index indicated some continuing changes in microbial populations, which is consistent with the research result mentioned above. Although a microbial population may change, it is likely to change within certain limits.

3.3.2.3 Impact of a pH Shock

In order to investigate the influence of a sudden pH increase on the microbial community populations in CAS-1, the pH was increased from 6.5 to 8.5 at day 21 for 7 days. This increase in pH gave rise to a significant change in the microbial community population of CAS-1. The next day, the daily similarity decreased to 84% in CAS-1 and this low daily similarity index persisted during the high pH condition period, whereas, the daily similarity index for the control unit (CAS-2) showed steady state characteristics during the same period (Figure 3-8). The similarity index also fluctuated following the pH change on day 27, which suggests that the microbial community population that had stabilized somewhat at pH 8.5, was affected again by the second pH change to 6.5.
The cumulative SI, as well as the daily SI for CAS-1, showed that the microbial community population in CAS-1 became very dynamic (unstable) after the change in pH. Only six days after the change, the cumulative SI indicated that the similarity to the reference biomass (day 18) was only about 53%, whereas the cumulative SI for the control unit was about 78% after the same period (Figure 3-9). Furthermore, the cumulative SI demonstrated that the response in CAS-2 was determined by pH conditions during the pH change period. According to the results of the cumulative SI graph for CAS-1, the second change of pH from 8.5 to 6.5 drove the microbial population back towards the original microbial composition that had been established at pH 6.5, before the increase in pH was
implemented (Figure 3-9). This indicates that pH may be an important factor in determining microbial community composition.

Figure 3-9. Cumulative similarity indices of CAS-1 and CAS-2 (the control) during the pH changes.

Figure 3-10 shows that carbon removal efficiency was strongly affected by the change in pH, which did not occur in the experiments using a synthetic wastewater feed in Chapter 2. During the same period, the control reactor (CAS-2) showed stable carbon removal efficiency. These results observed in CAS-1 were likely due to the complex organic materials in the municipal wastewater. Some materials change their metabolic characteristics according to the pH (e.g. ionized or tautomerized forms), which also affects
their degradability by micro-organisms (Werker and Hall, 1998). Therefore, it was assumed that some bacterial group used to treat the wastewater had been affected metabolically and/or physically by the increase in pH.

Carbon removal efficiency gradually recovered at pH 8.5. The seventh day after the change, the removal efficiency was 87%. Although it was still below the average of the previous period of steady state operation, it might indicate that pH 8.5 was not very inhibitory to the biological treatment.
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After the pH set-point was returned to 6.5 in CAS-1, the system performance was again negatively affected. Carbon removal efficiency one day after the change, dropped from 87% to 79%, and it took seven days to return to a normal removal efficiency. This finding confirms that pH is an important factor in maintaining microbial community composition and stable system performance.

3.3.2.4 Impact of Chlorine Shock

The impact of chlorine as a toxic material on microbial population and system performance was assessed using the MFA monitoring technique. On each of days 42, 43 and 44, a 2 mg/L chlorine spiking was made by adding 0.12 mL chlorine stock solution (50,000 mg/L as Cl₂) into CAS-2, to result in a final chlorine concentration of 2 mg/L (around 10:00 a.m.). For the first three days, these chlorine additions resulted in little decrease in the daily similarity index. The daily similarity indices decreased from 94% to 92%, 91%, and 90% for the first three days, respectively (Figure 3-11). It was also apparent that the 2 ppm chlorine additions did not cause any discernible change in carbon removal efficiency compared to the control reactor (CAS-1) (Figure 3-12).
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Figure 3-11. Daily similarity indices for CAS-2 and CAS-1 (the control unit) associated with chlorine spiking to CAS-2.

Figure 3-12. Carbon removal efficiency for CAS-2 and CAS-1 (the control unit) with TOC concentrations of influent during the same period as the chlorine spiking to CAS-2.
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On days 45, 46 and 47, additions to 4 mg/L chlorine were made to CAS-2 in the same manner each day. Figure 3-11 shows the effects of the chlorine spiking on microbial fatty acid profiles of the biological community population and the system performance of CAS-2, based on TOC removal (Figure 3-12), compared to the control unit (CAS-1) during the same period. The effect of the 4 mg/L chlorine spiking on the microbial community populations was detected by similarity index calculations. The similarity dropped to 87% from 90% during the period. The carbon removal efficiency also decreased to 84% from 91%.

The 8 mg/L chlorine spiking on days 49, 50 and 51 produced a detrimental effect on carbon removal efficiency and significant changes in the microbial fatty acid profiles of the microbial community population. The carbon removal efficiency for the wastewater decreased to 73% and the similarity index also decreased to 85%.

Cumulative similarity index observations demonstrated an increase in the shift rates of the microbial community population of CAS-2 associated with chlorine addition, compared to the control microbial community population of CAS-1 during the same period (Figure 3-13).
In the literature, it has been reported that microbial community is more sensitive than system performance to variations in operating conditions (Wang and Park, 1998; Princic et al., 1998; Fernandez et al., 2000; LaPara et al, 2002). Similar results were noted in the present experiment, through the stepped increase of chlorine additions. The daily similarity index (Figure 3-11) and cumulative similarity index (Figure 3-13) showed that the microbial community population was more affected than the system performance by a weak chlorine addition (2 mg/L). According to the carbon removal efficiency graph during the 2 mg/L chlorine spiking period (Figure 3-12), the system performance based on carbon removal efficiency did not seem to be affected. The results demonstrate a possibility that
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the monitoring technique based on microbial fatty acid analysis can be used to predict future system problems, with advanced detection of symptoms before the problems actually happen. Although a treatment system may not show any changes in its performance within a short period, more changes in microbial community population may have adverse effects on system performance in the long run.

After the chlorine addition was stopped at day 51, the microbial community population became stable relatively quickly (Figure 3-11). However, the cumulative similarity index showed that the community population affected by the chlorine spiking did not trend back to the original community population once the chlorine addition was stopped (Figure 3-13). This is a different result than that observed during the pH shock experiments (Figures 3-9). After chlorine addition was stopped, almost 10 days was required before carbon removal returned to the same steady state level as the control reactor (Figure 3-12). The response characteristics for microbial community composition and system performance were very different from the experimental results noted for the bioreactor fed with synthetic wastewater in Chapter 2. The previous experiment had shown that the carbon removal efficiency was not significantly affected even by 10 mg/L chlorine spiking and that the change in microbial composition associated with chlorine addition was reversible. The different results with real wastewater may be attributed to different exposure times of chlorine to biomass and to the different characteristics of influent substrate. Apparently, real municipal wastewater is harder for biomass to treat than acetate-based synthetic wastewater. Although there was a big population change associated with chlorine spiking, in the case of
synthetic wastewater, most bacteria could assimilate acetate easily, resulting in no difference in system performance. However, in the case of real municipal wastewater, if some of the bacterial groups that were mainly responsible for removing carbon sources in the wastewater were damaged by the chlorine spiking, system performance could be affected. The exposure time of the biomass to chlorine may be one reason that the microbial community composition did not return to the original microbial composition after chlorine spiking was stopped. In the present experiment, the chlorine spiking was executed over nine days in a row with different chlorine concentrations, instead of over four days as described in Chapter 2. It could be assumed that if some microbial species were damaged seriously by the longer exposure to chlorine, it would be more difficult for the communities to return to the norm that was established under steady state conditions. However, if the exposure time was not long enough to damage the population, the populations may have returned to normal. These results may be confirmed with the combination of some molecular technologies such as 16S rDNA/RNA sequencing with DGGE or RISA. The work is an interesting topic for future study but was not further addressed here.

3.3.2.5 Impact of Low Dissolved Oxygen Shock

In order to determine if the MFA monitoring technique is able to detect the influence that a decrease in DO concentration may have on the fatty acid profiles of an aerobic microbial community population, a very low level of DO ($\leq 0.1$ mg/L from 2.0 mg/L of normal DO concentration) was applied to CAS-1, by manual control, for 3 days from day 65 to day 67.
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The impact was significant, on both microbial community population (Figure 3-14) and system performance (Figure 3-15). After the change in DO set-point, the similarity index suddenly dropped from 93% to only 75% in just one day. System performance, based on TOC measurements, showed a very low removal efficiency of only 69%. The sudden drop in removal efficiency was apparently due to the change in DO, since the control bioreactor (CAS-2) showed a stable TOC removal efficiency and similarity index during the same period.

Figure 3-14. Daily similarity index changes associated with decrease in dissolved oxygen level in CAS-1, and those of the CAS-2 (the control unit) during the same period.
The results of the low DO operation on microbial community composition were unexpectedly significant. The biomass studied had been originally drawn from the UCT pilot plant at UBC, in which most of micro-organisms were thought to be facultative. RISA analysis and MFA analysis of the original biomass (day 0) and the biomass samples before (day 63) and after (day 67) the low DO experiment showed interesting results (Figure 3-16 and Figure 3-17). The similarities based on MFA analysis were 51.4% between day 0 and day 63, and 64.5% between day 63 and day 67. However, the similarities based on RISA analysis showed quite different results. The RISA similarities between day 0 and day 63, and between day 63 and day 67 were 62.3% and 92%, respectively.
Figure 3-16. RISA patterns of day 0, day 63 and day 67. K represents 1,000 base pair and M represents Marker.
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Figure 3-17. MFA Patterns of day 0, day 63 and day 67. X-axis: Retention Time, Y-axis: pA.
The importance of the results is two-fold. First, the microbial community population changed significantly during the first two months (although it was difficult to tell whether the community population was a facultative-dominant or aerobe-dominant population, which was beyond the scope of this experimentation). Second, MFA analysis showed large changes in MFA profiles before and after the decrease in DO, while RISA did not. The results observed may suggest an interesting possibility that the changes in MFA profiles associated with the sudden decrease in DO concentration were not wholly due to changes in microbial population, but perhaps due to membrane adaptation. However, it is still hard to distinguish the changes in microbial population and membrane adaptation through MFA analyses, because RISA itself has some limitations in indicating the changes in microbial populations due to uncertainty in bias by the polymerase chain reaction (PCR) (Suzuki et al., 1998). Therefore, the possibility proposed here should be confirmed by repeated experiments using model microbial populations, since a relationship between membrane adaptation and dissolved oxygen level has not been shown clearly in the literature.

The important finding derived from the experiment was that MFA analysis could detect a system perturbation that was not detected in RISA, suggesting an advantage of MFA analysis as a monitoring technique over molecular techniques, in addition to such advantages as simplicity, economy and rapid turnaround of results. However, the relationship between MFA analysis and RISA should be studied more through model microbial systems, as was done in Chapter 2.
The results of the cumulative similarity index show that the low DO concentration affected the fatty acid profiles of the microbial community. The cumulative similarity index dropped sharply after low DO was imposed on the system. When the DO set-point was returned to those of 2.0 mg/L, however, the similarity index returned to values that were comparable to those of the control reactor (CAS-2) (Figure 3-18). Since membrane adaptation may be a faster response than are changes in microbial composition (Werker, 1988), the results may support the assumption that the changes in fatty acid profiles associated with low DO were due to membrane adaptation rather than to real microbial population changes.
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3.3.2.6 Impact of Acetate Feed

Since the microbial community studied in Chapter 2 was affected most significantly by the change in feed composition, it was also of interest to determine whether the microbial community resident in the lab-scale bioreactor for more than two months would show similar changes in fatty acid profiles in response to a similar feed change. The feed of real municipal wastewater was changed to acetate-based synthetic wastewater for CAS-2 at day 80, and this was maintained for 9 days. As seen in Figure 3-19, a sudden change in the feed material resulted in a large change in the similarity index. The acetate feed made the microbial community very dynamic. The similarity index declined sharply over the first two days, and then gradually increased during the acetate feed period. The stabilized similarity index dropped again when municipal wastewater was reintroduced to the reactor at day 89. During the same period, the control unit (CAS-1) showed stable daily similarity indices. This indicates that the increased population dynamics were caused by the change in feed composition (Figure 3-19).
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One day after the feed change from real municipal wastewater to synthetic wastewater, the carbon removal efficiency fell to 80% from 90% (Figure 3-20). However, the system performance became stable very quickly within three days after the change, as the carbon removal efficiency returned to 97.2% four days later. This was likely due to the easily biodegradable characteristics of acetate in the raw wastewater as seen in Chapter 2. The stabilized carbon removal efficiency decreased again when municipal wastewater was reintroduced to the reactor on day 89. After the reintroduction of municipal wastewater into CAS-2, it took more than ten days for the carbon removal efficiency of CAS-2 to return to that of the control unit. This phenomenon was quite different from the previous result,
when the substrate material was changed from municipal wastewater to acetate-based synthetic wastewater and the carbon removal efficiency took little time to recover. It could be concluded that a biomass could quickly adjust to easily degradable carbon sources, which result in a short recovery time. On the other hand, a biomass generated with a readily degradable carbon source that encounters complex carbon materials would need more time to adjust to it. During the same period, the control unit (CAS-1) showed stable daily similarity indices. This indicates that the increased population dynamics were caused by the change in feed material (Figure 3-19).

![Carbon removal efficiency for CAS-2 and CAS-1 (the control unit) associated with feed change from real municipal wastewater to synthetic wastewater in CAS-2.](image)

Figure 3-20. Carbon removal efficiency for CAS-2 and CAS-1 (the control unit) associated with feed change from real municipal wastewater to synthetic wastewater in CAS-2.
The changes in microbial population associated with the feed composition changes were demonstrated by the cumulative similarity index (Figure 3-21). From the graphs, it can be seen that the acetate feed made the community population in CAS-2 much more dynamic than that in CAS-1, which received the same influent throughout the experiment. The microbial community population of the last day (day 89) of acetate feed showed only 43% similarity relative to day 76. However, after the municipal wastewater feed was resumed, the similarities increased again. The result confirmed the finding that the microbial community population was significantly affected by the influent characteristics of feed materials (Hiraishi et al., 1998; Son and Hall, 2003).

Figure 3-21. Cumulative similarity indices for CAS-2 and CAS-1 (the control unit) associated with feed change from real municipal wastewater to synthetic wastewater in CAS-2.
An interesting finding was that the changing patterns in fatty acid profiles associated with the changes in feed composition were quite similar to those observed during the transfer of biomass from the UBC pilot plant to the lab-scale reactors (or vice versa) in Chapter 2. Figure 3-22 shows the typical fatty acid patterns of biomass from municipal wastewater and acetate-based synthetic wastewater feeds. As described in Chapter 2, C16:ω7c (peak A in Figure 3-22) and C18:ω7c (E) increased in mole concentration (%) of the fatty acid profiles and C16:ω4t (B), iC18 (C), C18:ω10c (D) and C18:ω4t (F) decreased in mole concentration (%), when feed composition was changed from municipal to synthetic and vice versa.
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These results probably imply two possible conclusions. First, the microbial groups that became dominant when the feed was changed to acetate-based synthetic wastewater in this current experiment and in Chapter 2 may be the same species. Second, the feed of acetate may induce the synthesis of C16:ω7c and C18:ω7c. In the present experiments, it is hard to determine which assumption is correct. It may be possible for both factors to contribute to the phenomenon. This would be an interesting topic for future study.
Conclusions

The experimental results using the two CAS systems fed with real municipal wastewater showed that a similarity calculation based on microbial fatty acid analysis was applicable to the monitoring of the stability of biological wastewater treatment processes by estimating changes in microbial community composition based on MFA profiles.

The method could quantify changes in microbial community composition by correlating the changes in microbial fatty acid profiles of microbial populations with variations in operating parameters such as pH, DO, substrate composition and chlorine additions. The average values of daily SI and carbon removal efficiency during steady state operation were 93.9% and 91.2%, respectively. The values were lower than those observed during a previous experiment using bioreactors fed with a simple synthetic wastewater. The difference can be attributed to the complex organic materials in the real municipal wastewater used in the second group of experiments. However, the strength of the municipal wastewater used for the present experiment varied, although the similarity indices remained relatively stable. This fact indicates that the microbial population is more sensitive to changes in the composition of the substrate than to changes in the strength of the influent.

System performance was strongly influenced by the changes in operating factors in the Chapter 3 experiments, which are detected by the new monitoring tool based on the analysis of microbial fatty acid of biomass. In the experiments using synthetic wastewater
in Chapter 2, these results were not obtained. Apparently, the difference can be attributed to the fact that the wastewaters used for each experiment were different. The acetate-based synthetic wastewater could be easily treated by most bacterial groups established in the lab-scale reactors described in Chapter 2. Thus, bacterial groups that were sensitive to changes in operating factors were affected, while other groups that were relatively insensitive to the changes easily take up the responsibility of the affected bacteria. However, in the case of real municipal wastewater, the rather complex substrate composition may need more specialized microbial groups to treat the substrates in the wastewater. Therefore, if some species are affected by operating changes, it is more difficult for other bacterial groups to take up the role, resulting in reduced system performance.

The following are the major conclusions from the experimental results observed throughout the Chapter 3 experiments.

1. The microbial community with real municipal wastewater was more dynamic than the population that was developed with acetate-based synthetic wastewater. The reason was most likely due to the changes in substrate in real municipal wastewater. Since the real wastewater used for this experiment was mixed half and half with the wastewater of the previous day to prevent any dramatic changes in carbon materials, it was assumed that a full-scale wastewater treatment system may exhibit more significant population dynamics.
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2. Changes in operating factors, such as pH and chlorine input, had a significant impact on the microbial community population and system performance. The change associated with pH level was reversible, so the microbial population appeared to return to the original population after the change was removed. However, the change associated with prolonged chlorine additions was not readily reversible.

3. The MFA monitoring method also detected changes associated with a decrease in DO, which might be caused not by changes in microbial population but membrane adaptation as seen in the RISA analysis. It was postulated that both the changes in measured microbial populations and microbial membrane adaptation could contribute to the changes in observed microbial fatty acid profiles. This may not be a good characteristic for the study of population dynamics because membrane adaptation may confound the observation of population changes. However, from a monitoring point of view, membrane adaptation may be a reflection of changes in operating conditions, which may result in changes in system performance. Therefore, this feature may add one more advantage to the use of MFA analysis as a monitoring tool.

These experimental findings support the application of the monitoring method, developed in the previous experiments, to a system that was subject to more dynamic operating conditions. This indicates the potential for this technique to be used for pilot-scale or full-scale wastewater treatments.
3.5 References


Chapter 3: Development of Monitoring Tool Based on Microbial Fatty Acid Analysis using Lab-scale Bioreactors Fed with Real Municipal Wastewater


Metcalf and Eddy (1991) *Wastewater engineering: Treatment, Disposal, and Reuse.*, Chapman and Hall,


Chapter 3: Development of Monitoring Tool Based on Microbial Fatty Acid Analysis using Lab-scale Bioreactors Fed with Real Municipal Wastewater


Abstract

A proliferation of a particular type of bacteria, referred to as glycogen-accumulating organisms (GAOs), is believed to be related to the failure of enhanced biological phosphorus removal (EBPR) process. A recently developed monitoring technique using fatty acid analysis was evaluated using a lab-scale simplified University of Cape Town (UCT) reactor, to determine whether the technique was applicable for detecting a trend in a microbial population of an EBPR process toward a GAO-dominant microbial community composition. In order to convert a phosphorus-accumulating organism (PAO) dominant microbial population to a GAO-dominant composition, several methods were tried: namely, low phosphate to carbon ratio (P/C) feed, high-temperature operating conditions (25°C and 30°C), and a mixed glucose and acetate feed. Among the three trials, only the glucose and acetate feed effectively converted the EBPR system (PAO-dominant microbial population) to a GAO-dominant composition. The MFA technique clearly showed the transition from a PAO-dominant microbial population to a GAO-dominant one, through an increase in shift rates of microbial community composition. Similarity indices (daily and cumulative) based on microbial fatty acid analysis were used to estimate the shift rates of microbial community compositions in the system. The experimental results demonstrated that the new monitoring technique could be a useful tool for preventing EBPR deterioration by monitoring the change in microbial population from PAO dominance to GAO dominance in EBPR.
Chapter 4: New Application of Microbial Fatty Acid Analyses for the Monitoring of the Microbial Population Changes in Enhanced Biological Phosphorus Removal

4.1 Introduction

Since Barnard (1976) proposed the enhanced biological phosphorus removal process (EBPR), EBPR processes have gained a great deal of attention and research interest. In an EBPR system that circulates biomass through alternative anaerobic and aerobic cycles, food (mainly short-chain fatty acids) is introduced into the anaerobic phase, and the biomass releases orthophosphate into the bulk solution. In turn, the biomass accumulates the orthophosphate into polyphosphate during the aerobic phase. There have been many efforts to explain EBPR activities and so far, several empirical metabolic models have been introduced. The most popular models are the Mino model (Mino et al., 1986) and the Comeau-Wentzel model (Comeau et al., 1986; Wentzel et al., 1986).

4.1.1 Biochemical Models for EBPR Systems

The summary of typical EBPR phenomena in an EBPR system is that in the anaerobic zone, short-chain volatile fatty acids are assimilated rapidly and stored as poly-beta-hydroxyalkanoate (PHA). In this stage, intracellular poly-P is decomposed and excreted into the bulk solution. In the subsequent aerobic stage, the stored PHA content falls in parallel with an increase in poly-P inside the biomass. Therefore, the micro-organisms that can synthesize poly-P are better able to take up organic substrates in the anaerobic stage than other micro-organisms, by producing energy from the decomposition of poly-P into orthophosphates. Thus, they are better able to survive in the famine stage (aerobic) by using stored PHA as an energy source for growth and poly-P synthesis.
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Synthesis of PHA requires a source of reducing power, and the various models proposed differ in the source of reducing power. In the Mino model (Mino et al., 1985), the reducing power was derived from degradation of intracellular stored glycogen, whereas in the Comeau-Wentzel model (Comeau et al., 1986; Wentzel et al., 1986), partial oxidation of acetyl-CoA through the TCA cycle was assumed to produce the required reducing power. A C\textsuperscript{13}-labeled NMR technique showed that glycogen was used as a source of reducing power for PHA synthesis via the Entner Doudoroff (ED) pathway and the TCA cycle was also proposed as an additional source of reducing equivalents (Pereira et al., 1996). Figure 4-1 shows a summary of the major features of the biochemical models for EBPR presented by Pereira et al (1996).

To date, all models proposed are based on the study of the whole biomass rather than the study of pure phosphorus-accumulating organisms (PAO), because no single pure cultures of PAO have been isolated. Therefore, there have been few experimental biochemical data to validate any of these empirical models and even NMR data are unable to fully explain the behaviour of the different communities, since it is believed that different species of PAOs have different mechanisms of EBPR. Some of the differences in biochemical performance of biomass from the different studies may be attributed to the presence of microbial communities of quite different composition, developed under very similar conditions.
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4.1.2 Glycogen-accumulating Organisms (GAO)

Since the empirical discovery of the EBPR process, full-scale systems have been constructed and operated in many locations and much effort has been made to make the system more efficient and stable. However, in spite of extensive research of the last few decades, EBPR processes are still difficult to control and, sometimes, lose phosphate removal activities for unknown reasons. This may be attributed to the system’s complex

Figure 4-1. A Summary of the major features of the biochemical models for EBPR (adapted from Pereira et al., 1996).
There have been several reports that a particular type of micro-organism, which can assimilate organic substrates without P-release in an anaerobic zone, dominated in malfunctioning EBPR processes (Cech and Hartman, 1990 and 1993). Since these “G-bacteria”, which are now called glycogen-accumulating organisms (GAOs), were reported, EBPR activity has been explained as the competitive growth of two types of bacteria referred to as PAOs and GAOs (Mino et al., 1998). These two types of bacteria remove volatile fatty acids (VFAs) and store them as polyhydroxy alkanoates (PHAs) under anaerobic conditions. GAOs, however, accumulate glycogen under aerobic conditions as an internal energy source, instead of phosphorus. The proliferation of GAOs causes EBPR failure because excess phosphorus removal capacity is lost from the system (Cech and Hartman, 1990 and 1993; Satoh et al., 1994). Therefore, it is necessary to keep a PAO-dominant microbial community composition in the system for efficient EBPR. In order to do that, information about the different biochemistries of PAOs and GAOs may be essential. However, as discussed above, none of the biochemical models for PAOs and GAOs has been proven to be true, because no single pure cultures have been identified as predominant PAOs or GAOs, using culture-independent molecular techniques such as DGGE and FISH in the EBPR process (Mino et al., 1998).

There have been several empirical studies reporting the conditions leading to the proliferation of GAOs involving pH, sludge retention time (SRT), hydraulic retention time

Since Cech and Hartman (1990, 1993) reported a breakdown of EBPR due to domination of “G-bacteria” when an acetate and glucose mixture was fed to their system, this experimental finding has been confirmed by several subsequent papers (Randall et al., 1994; Satoh et al., 1994). Mino et al. (1994) proposed a metabolic model supporting the breakdown of EBPR from competition by G-bacteria.

Several other experimental results showed that a GAO-dominant microbial community and a PAO-dominant microbial community could be successfully constructed by controlling the phosphorus-to-carbon mass ratio (P/C ratio) in the feed (Liu et al., 1997; Liu et al., 2000). In those experiments, PAO-dominant and GAO-dominant microbial communities were achieved using three identical lab-scale sequencing batch reactor (SBR) systems and feeding each at different P/C ratios (for PAO-dominant composition – 12:100 and 20:100, for GAO-dominant composition – 2:100).

Recently, it has been also reported that an increase in temperature (from 20°C to 30°C) impacted an EBPR system (Wang and Park, 2002). In this study, using two identical lab-scale SBR systems, the system operated at 20°C showed typical EBPR activity (biological anaerobic P-release and aerobic P-uptake), while the other system, operated at 30°C, showed no detectable P-release during an anaerobic period and no phosphate removal in an
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aerobic period. Through batch tests, it was found that the two systems showed different anaerobic acetate uptake rates. The acetate uptake rates of PAO-dominant sludge decreased with the increase in temperature from 10°C to 30°C; but the uptake rates of GAO-dominant sludge increased with the elevation in temperature. Therefore, an operating temperature of 30°C gave the advantage of faster anaerobic acetate uptake to the GAO population, leading to the eventual failure of the EBPR process.

The results, however, have been controversial because definite conditions for the proliferation of GAOs have not yet been identified. Therefore, more microbiological and molecular biological studies are needed to establish optimal EBPR operation.

4.1.3 The Research Objective

The other way to detect EBPR deterioration is by monitoring the microbial community composition. If a change in microbial community composition from PAO to GAO domination can be detected in advance, system failure may be prevented. A microbial fatty acid (MFA) analysis technique may be suitable for tracking EBPR microbial community compositions, as indicated in the previous chapters. This technique has been successfully applied to detecting shifts in microbial community compositions by using simple similarity index calculations and principal component analysis (PCA) (Son and Hall, 2003). The results showed that the calculation of shift rates (expressed as daily similarity index) in microbial populations was a good indicator of changes in process operating factors that may affect system performance.
Recently, it was reported that the MFA profiles of PAO-dominant and GAO-dominant community compositions were significantly different (Liu et al., 2000), which may explain the possibility that the transition from PAO-dominant microbial community composition to GAO-dominant one necessarily accompanies changes in the MFA profiles of biomass. This results in an increased shift rate of the microbial community composition. In Chapters 2 and 3, it was shown that the shift rates in microbial populations could be expressed by a simple daily similarity index \( \frac{100\% - \text{similarity index}}{\text{day}} \) based on MFA profile analysis and abnormal increases in this value were related to changes in process operating factors (Son and Hall, 2003).

In the experiments reported in the present chapter, the main objective was the answer to the following question.

\textit{Is analysis of microbial fatty acids a useful monitoring tool for detecting the transition of an EBPR process from a desirable PAO-dominant community composition to a detrimental GAO-dominant?}

To answer the research question, a lab-scale simplified University of Cape Town (UCT) process was used for the EBPR operation. In order to convert a PAO-dominant community composition to a GAO-dominant one, three well-known methods (according to the literature) were employed: low phosphate-to-carbon ratio (1.5:120) feed, high temperature
(30°C) application, and a glucose and acetate feed mixture. To calculate the shift rate of microbial community composition, daily and cumulative similarity indices were also employed, as described in the previous chapters.
4.2 Materials and Methods

4.2.1 EBPR Operation

A simplified lab-scale University of Cape Town (UCT) system that was composed of anaerobic, anoxic, and aerobic reactors (R1, R2 and R3, respectively) in series with internal mixed liquor recycles was set up for EBPR operation. Table 4-1 shows the design operating parameters and the physical configurations of the process; and, Figure 4-2 represents the schematic diagram of the simplified lab-scale UCT process.

Table 4-1. Design operating parameters and physical configurations of simplified lab-scale UCT process

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Wastewater Flow Rate (L/day)</td>
<td>12</td>
</tr>
<tr>
<td>Reactor Volumes (L)</td>
<td></td>
</tr>
<tr>
<td>Anaerobic (R1)</td>
<td>1</td>
</tr>
<tr>
<td>Anoxic (R2)</td>
<td>2</td>
</tr>
<tr>
<td>Aerobic (R3)</td>
<td>3</td>
</tr>
<tr>
<td>Secondary clarifier</td>
<td>1</td>
</tr>
<tr>
<td>Sludge Retention Time (Day)</td>
<td>15</td>
</tr>
<tr>
<td>Hydraulic Retention Time (Hr)</td>
<td>12</td>
</tr>
<tr>
<td>Recycle Flows (L/day)</td>
<td></td>
</tr>
<tr>
<td>Aerobic to Anoxic</td>
<td>12</td>
</tr>
<tr>
<td>Anoxic to Anaerobic</td>
<td>12</td>
</tr>
<tr>
<td>Dissolved Oxygen level (mg/L) in R3</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>
Figure 4-2. Schematic layout of lab-scale simplified UCT process. R1, R2, and R3 represent anaerobic, anoxic, and aerobic reactors, respectively; and C represents a clarifier.

The UCT system was operated in a temperature-control chamber maintained at 20 ± 1°C. The biomass was introduced into the system from a UCT pilot plant that was treating municipal wastewater at the University of British Columbia, Vancouver, Canada. R1 and R2 were continuously mixed with mechanical stirrers. The dissolved oxygen level in R3 was controlled manually at 4.0 ± 1.0 mg/L by an air pressure regulator and a flow meter. The DO concentration was measured by DO probes (YSI Model number 5739), connected to a DO meter, (YSI 54A, YSI Incorporated, Yellow Springs, Ohio). The pH was maintained within the range of 7.0 ± 0.3 in R2 by a pH controller (Cole-Parmer Instrument, Chicago, Illinois) using 0.1 N NaOH. The hydraulic retention time (HRT) was 12 hours, and the sludge retention time (SRT) was 15 days. These operating conditions were
maintained for the entire experimental period, except for experimental runs designed to convert a PAO-dominant microbial community composition to a GAO-dominant composition. During the experiment, sampling and analysis were completed as indicated in Table 4-2.

### Table 4-2. Sampling and analysis schedule.

<table>
<thead>
<tr>
<th>Source</th>
<th>Items</th>
<th>Method</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Wastewater</td>
<td>TOC</td>
<td>TOC analyzer (Shimadzu)</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>Influent flow rate</td>
<td>Direct measuring</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>Phosphorus</td>
<td>QuikChem ion analyzer (Lachat, 1987)</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>Ammonia, Nitrite and Nitrate</td>
<td>QuikChem ion analyzer (Lachat, 1987)</td>
<td>As needed</td>
</tr>
<tr>
<td>Bioreactors</td>
<td>MFA (R2)</td>
<td>As previously described in Chapter 2</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>pH (R2)</td>
<td>Probe</td>
<td>Controlled</td>
</tr>
<tr>
<td></td>
<td>DO (R3)</td>
<td>Probe</td>
<td>Controlled</td>
</tr>
<tr>
<td></td>
<td>Phosphorus (R1, R2, R3)</td>
<td>QuikChem ion analyzer (Lachat, 1987)</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>TOC (R1, R2, R3)</td>
<td>TOC analyzer (Shimadzu)</td>
<td>Daily</td>
</tr>
<tr>
<td></td>
<td>Neisser staining (R3)</td>
<td>Jenkins (1986)</td>
<td>As needed</td>
</tr>
</tbody>
</table>

### 4.2.2 Operating Conditions to Generate GAO-dominant Microbial Community Composition

Three known methods were employed to convert a PAO-dominant community composition to a GAO-dominant one. These were low P/C ratio (1.5:120) feed (Liu et al., 1996; Liu et al., 1997), high temperature (30°C) operation (Wang and Park, 2002), and a glucose and
acetate mixture in the raw wastewater (Cech and Hartman, 1990).

On day 52; the feed for the well established EBPR UCT process was changed from a high P/C ratio (20:120) to a low P/C ratio (1.5:120) for 58 days. The operating temperature was increased from 20°C to 25°C on day 120; and, the temperature was further increased to 30°C on day 125 for 15 days. The carbon source was changed from acetate to glucose by a stepped increase on day 175. The glucose concentration was increased from 0% to 80% (w/w) of total carbon in the feed (glucose:acetate ratio, 0:100 -> 20:80 -> 40:60 -> 60:40 -> 80:20)

### 4.2.3 Synthetic Raw Wastewater

The synthetic wastewater used for the experiment consisted of sodium acetate as a carbon source, ammonium sulfate as a nitrogen source, and potassium phosphate as a phosphorus source. In addition, peptone was provided with some minerals, such as Mg, Ca, and Fe. The concentrations of the raw wastewater were 120 ± 10 mg/L of total organic carbon (TOC), 10 ± 2 mg/L of ammonia and 20 ± 3 mg/L of phosphate. Details of the composition of the raw wastewater are presented in Table 4-3. The performance of the reactors was expressed by carbon and orthophosphate removal efficiencies, based on TOC and orthophosphate measurements. Fifteen liters of fresh synthetic wastewater were prepared daily from a 10X stock solution. The feed tank was also cleaned daily, using diluted bleach solution and tap water, in order to prevent microbial growth in the feed tank.
4.2.4 Analyses

Total organic carbon (TOC), suspended solids (SS), ammonia (NH$_3$ plus NH$_4^+$), and orthophosphate (ortho-P) concentrations were measured as described in Chapter 3. The analyses of microbial fatty acids were conducted as described in Chapter 2. Numerical analyses, such as similarity index (SI) and principal component analysis (PCA), were also performed as described in Chapter 2.

4.2.4.1 Biomass PHA analysis

The procedure for PHA analysis was adapted from the method developed by Comeau et al. (1988). For the analysis, activated sludge samples (10 to 15 mL) were centrifuged, and the sludge pellets were frozen and lyophilized (Lyophilizer: Multi-Dry, FTS systems Inc.). Two mL of acidified methanol (3% H$_2$SO$_4$), which contained benzoic acid as an internal
standard, were combined with about 20 mg of lyophilized samples; and, 2 mL of chloroform was added to the samples (DL-3-hydroxybutyric acid (HB) sodium salt dissolved in acidified methanol was used as the standard). The samples and the standards were heated at 100°C in Pyrex test tubes with Teflon-lined caps for 3.5 hours. Two mL of distilled water were then added to the vials and vigorously shaken. Then, 1.9 mL of the denser chloroform phase was transferred to another Pyrex tube containing 0.5 mL of distilled water. After 5 minutes of vigorous shaking, the samples were centrifuged, and the chloroform phase was transferred to a GC vial for GC measurement. Extracted PHA was identified by GC (HP6890 Series, DB5 column of 30 m having a 0.32 mm ID and 0.25 mm film thickness) with 1 μL splitless injection. The inlet and detector temperatures were 250°C and 300°C, respectively. The temperature program was 4 minutes at 130°C followed by a ramp of 2°C min⁻¹ to 250°C, and a post-run temperature of 290°C for 5 minutes. A helium carrier gas was used with a column head pressure of 10 psi providing a total flow of 30 mL/min. A biomass PHA concentration was calculated as a percentage value, compared to MLVSS of the biomass used (w/w).

4.2.4.2 Glycogen Analysis

Glycogen was analyzed according to Gerhardt (1981). Lyophilized sludge pellets were incubated at 100°C with 1 mL of 30% KOH for 3 hours with standard glucose samples. When cool, 3 mL of distilled water and 8 mL of ethanol were added to precipitate the glycogen. After centrifugation, the precipitate was washed with 8 mL of 60% ice-cold ethanol and dried in a vacuum desiccator. Five mL of cold anthrone reagent (prepared daily
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with 200 mg of anthrone to 5 mL of absolute ethanol, made up to 100 mL with 75%, vol/vol, H₂SO₄) were added to the vial. After that, the samples were heated in a boiling water bath for precisely 10 minutes and cooled in an ice-water bath. Using a spectrophotometer, the absorbance of samples at 625 nm was measured with standard glucose samples. A biomass glycogen concentration was calculated as a percentage value, compared to MLVSS of the biomass used (w/w).

4.2.4.3 Neisser Staining

For Neisser staining, two staining solutions (solution 1 and solution 2) were prepared and stored according to Jenkins et al. (1986).

For solution 1, two stock-solutions (solution A and solution B) were prepared separately as follows:

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>0.1 g Crystal violet (10% w/v in</td>
</tr>
<tr>
<td>Ethanol, 95%</td>
<td>3.3 mL 95% ethanol)</td>
</tr>
<tr>
<td>Acetic acid, glacial</td>
<td>5 mL Ethanol, 95%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 mL Distilled water</td>
</tr>
</tbody>
</table>

For the preparation of solution 1, 2 parts by volume of solution A with 1 part by volume of solution B were freshly mixed for each staining.
Solution 2 was made as follows:

Bismark Brown (1 % w/v aqueous)  33.3 mL
Distilled water  66.7 mL

For the procedure, thin smears of biomass on microscopic slides were prepared and dried thoroughly using air. Samples were stained for 30 seconds with solution 1 and rinsed for 1 second with water. After that, the samples were stained with solution 2 for 1 minute and rinsed well with water and blot dried. The samples were examined under oil immersion at 1000X magnification with direct illumination.

4.2.5 Batch Test to Calculate PAO Activities

Five hundred mL of mixed liquor were taken from the aerobic reactor to a 1.0 L beaker, to measure P-release activity. For ten minutes, the mixed liquor was slowly mixed with a magnetic stirrer, so that oxygen and nitrate levels would be reduced to zero. After that, 0.18 g of sodium acetate was added to the beaker. At the beginning of the batch experiment, a ten mL sample was taken from the beaker for TOC and ortho-P measurements. Subsequent samples were taken every 10 minutes for 70 minutes.

Under low P/C feed conditions, PAO activity was also estimated in a similar manner. Five hundred mL of mixed liquor were taken from the aerobic reactor to a 1.0 L beaker. For ten minutes, the mixed liquor was left in the beaker, while at least 2 mg/L of DO was maintained by aeration using an air stone. Potassium phosphate (dibasic, 60 mg) was added to the beaker. TOC and orthophosphate concentrations were measured at ten minute
intervals for 70 minutes. The TOC and orthophosphate concentrations of each sample were analyzed as described in Chapter 2.
4.3 Results and Discussion

4.3.1 The similarity among the biomass in anaerobic, anoxic and aerobic reactors of lab-scale UCT process

The simplified lab-scale UCT system included three bioreactors with internal recirculation; these were operated under anaerobic, anoxic and aerobic conditions. It was first a question of whether the microbial community compositions of each reactor were identical or significantly different in each reactor. If their MFA profiles were not found to be different, sampling and analysis of the process would be straightforward, because it would only be necessary to sample one reactor. Therefore, it was necessary to compare biomass MFA profiles from each reactor, to determine whether the biomass in each reactor contained different microbial populations resulting from the different operating conditions in each reactor, in terms of DO concentration, F/M ratio, and C:N:P composition.

In order to compare MFA profiles of biomass from each reactor (anaerobic, anoxic, and aerobic), biomass samples were taken six times from each reactor (days 1 through 6); and, the MFA profiles were compared. The microbial fatty acid profiles of all three reactors in the lab-scale UCT process on the same day were very similar (Table 4-4). Table 4-4 shows the average similarity index by comparing each reactor to the others, in turn. According to the results, the similarities between anaerobic and anoxic, anoxic and aerobic, and aerobic and anaerobic were high enough to be considered to be identical. This high similarity between each reactor can be attributed to the high recycling rates from the anoxic zone to the anaerobic zone and from the aerobic zone to the anoxic zone, although the
environmental conditions in each zone were quite different.

Since the biomass MFA profiles in all three zones were essentially identical, all subsequent samples for the present experiment were taken from the anoxic reactor only.

Table 4-4. The average similarity indices of the three reactors in the lab-scale UCT system (n=6, unit: %).

<table>
<thead>
<tr>
<th>%</th>
<th>Anaerobic</th>
<th>Anoxic</th>
<th>Aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>100</td>
<td>97.6</td>
<td>96.9</td>
</tr>
<tr>
<td>Anoxic</td>
<td>97.6</td>
<td>100</td>
<td>98.3</td>
</tr>
<tr>
<td>Aerobic</td>
<td>96.9</td>
<td>98.3</td>
<td>100</td>
</tr>
</tbody>
</table>

4.3.2 Establishment of EBPR system

4.3.2.1 Transition State

To achieve the objective of this experiment, first, it was necessary to establish a functioning EBPR system using a lab scale UCT process. A PAO-dominant microbial community composition in the process was successfully established using a high P/C ratio of feed (P/C ratio: 20/120) after one month from the process start-up. Figure 4-3 shows the daily and cumulative similarity indices for the 30-day start-up period.
The most dramatic change in microbial community composition took place during the first 5 days. The similarity between the first and the second day fatty acid profiles was only 69%. This low daily similarity gradually increased. Six days later, the similarity index reached 95%. The cumulative SI index showed that the microbial community composition in the lab-scale UCT bioreactor changed significantly during the 30 days after biomass transfer. These results were similar to the results shown in Chapter 2, confirming that a change in feed composition could result in a large impact on microbial populations.

Figure 4-4 shows the orthophosphate (P-) removal efficiency, and carbon (C-) removal efficiency during the 30-day start-up period. The carbon removal efficiency stabilized
quickly. Six days from the onset of the experiment, the system started to show very stable carbon removal efficiency, which may be attributed to the high biodegradability of sodium acetate, as discussed in Chapter 2.

Phosphorus removal, however, took a longer time to establish. For the first 20 days, no noticeable P-release was observed in the anaerobic zone, and no overall phosphorus removal was noted. This was not expected, since the original biomass was transferred from a pilot-plant UCT process. The seed sludge PAO population density may not have been large enough to accommodate the high phosphate concentration (20 mg/L) of synthetic wastewater, because the municipal wastewater treated by the UBC pilot plant contained relatively low level of orthophosphate (around 5 mg/L). Also, the EBPR activity of the
PAOs present in the seed sludge may have been low due to the very different substrate conditions, temperature, recycling rate and HRT in the lab-scale system.

On day 20, P-release started in the anaerobic reactor, and the P concentration in the reactor reached 38 mg/L by day 30 (Figure 4-5). With the increased anaerobic P-release, the overall phosphate removal efficiency also increased (Figure 4-3).

The fact that P removal developed after the MFA composition was stabilized may suggest that the EBPR function is inducible or regulated by some physiological or environmental factors. The literature is not clear on the question of whether the EBPR mechanism is inducible or not. However, it has been reported that there are at least 38 genes involved in phosphorus assimilation in *E-coli*, which are organized as one regulon (the *pho* regulon) and are, therefore, subject to a common molecular control (Schembri et al., 1995; Gavigan et al., 1999). In these reports, it was shown that transcription of the polyphosphate kinase gene, which polymerizes the terminal phosphate of ATP into poly-P, was induced by phosphate starvation in *Acinetobacter* sp. ADP1.
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Figure 4-5. Ortho-P concentrations in influent, anaerobic zone and effluent during the transition state.

However, the assumption that the EBPR activity may be inducible should be further researched. It may be possible that the MFA method cannot detect the change in microbial population to PAOs-dominant, unless the PAOs have very different fatty acid compositions from other bacteria in the reactor.

4.3.2.2 Steady State

After the transition or start-up period, the system reached a steady state condition. The system showed typical EBPR activities. Daily SI changes and carbon and phosphorus removal efficiencies were very stable during the steady state period (Figure 4-6). Cumulative SI showed that the similarity between the biomass of day 31 and day 52 was
83%. Biomass poly-P content in the aerobic zone averaged of 17% during the steady state period (Day 31 – Day 51). Carbon removal was always over 98%, and the daily SI averaged 96.5%.

Figure 4-6. Daily and cumulative SIs, and carbon and phosphorus removal efficiencies with time during the steady state.

On day 47, biomass from R3 (aerobic zone) was stained with Neisser staining solution, which stains polyphosphate inside the biomass. The microscopic image of the biomass stained with Neisser further demonstrated that most of the biomass during the steady state condition was composed of PAOs (Figure 4-7).
During the steady state period, weekly batch tests were completed in order to estimate the amount of P released per unit mass (1g) of acetate consumed. A typical result of these batch tests is shown in Figure 4-8. The mass of ortho-P released per unit mass of acetate consumed was determined from the graph, and the ratio was calculated to be 0.72 g P released/g acetate consumed. According to Smolders et al. (1994), the ratio for a well established PAO population was 0.6 g ortho-P released per 1g acetate consumed at pH 7. This present result confirmed that the lab-scale simplified UCT system used was enriched with PAOs.
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Figure 4-8. Typical anaerobic batch test results indicating TOC and orthophosphate concentrations during high EBPR performance. Acetate was added at time zero. Error bars represent standard deviations from three batch tests.

Biomass glycogen and PHA contents in each reactor (anaerobic, anoxic and aerobic zones) during the steady state operating period of the EBPR are also presented in Figure 4-9. The results support the Mino model (although detailed biochemistry of the EBPR system is beyond the scope of this research), because the biomass glycogen content in the anaerobic zone was significantly lower than that of the aerobic zone. This suggests that the glycogen might be used for reducing power to support PHA synthesis in the anaerobic zone as suggested by Mino et al. (1985).
In conclusion, all the results from steady state operation showed that the lab-scale UCT process was well stocked with active PAOs and its carbon and phosphate removal efficiencies were high and stable. Also, the daily and cumulative similarity indices were indicative of stability. The information regarding steady state operation was to be compared later with experimental data obtained during periods of dynamic operation.

4.3.3 Converting PAO-dominant Community Composition to GAO-dominant Composition

It was necessary to convert the enriched PAO-dominant community composition in the lab-scale UCT system to a GAO-dominant composition to answer the question; "Is analysis of
microbial fatty acids a useful monitoring tool for detecting the transition of an EBPR process from a desirable PAO-dominant community composition to a detrimental GAO-dominant?" (Section 4.1.3)

The factors influencing PAO or GAO growth are still not well known, although they are important for EBPR optimization. In the present study, however, three known methods were employed in order to convert the PAO-dominant community composition to GAO-dominant one. These were low P/C ratio (1.5:120) feed (Liu et al., 1996 and 1997), high temperature (30°C) operation (Wang and Park, 2002) and a mixture of glucose and acetate in the raw wastewater (Cech and Hartman, 1990). Among these three methods, only the feed containing a glucose and acetate mixture was effective in driving the PAO-dominant microbial population established in this study, to a GAO-dominant microbial community composition.

4.3.3.1 Impact of Low P/C Ratio Feed

There have been several reports that a GAO-dominant microbial community and a PAO-dominant microbial community were successfully constructed by controlling phosphorus and carbon mass ratio (P/C ratio) in the feed (Liu et al., 1997; Liu et al., 2000). In those experiments, PAO-dominant and GAO-dominant microbial communities were achieved using three identical lab-scale sequencing batch reactor (SBR) systems by feeding different P/C ratios (for PAO-dominant composition; 12:100 and 20:100, for GAO-dominant composition; 2:100).
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In this study, a similar strategy was employed to convert the PAO-dominant microbial community composition to a GAO-dominant one using the lab-scale UCT process. On day 52, the feed for the well established EBPR UCT process was changed from high P/C ratio (20:120) to low P/C ratio (1.5:120) for 58 days.

However, this attempt didn’t convert the PAO-dominant composition to a GAO-dominant composition efficiently. For the first 15 days of the low P/C feed period, P release in the anaerobic zone occurred continuously (Figure 4-10). The phenomenon was attributed to the fact that the PAOs released ortho-P in the anaerobic zone by recycling ortho-P that they accumulated in the aerobic zone, even after the high exogenous ortho-P supply was reduced.

![Figure 4-10. The concentrations of ortho-P in influent, anaerobic zone and effluent for the first 15 days after P/C ratio changed.](image)
During the low P/C period, a batch test was executed once every two weeks to examine the ability of the biomass to assimilate orthophosphate. A typical result of the batch tests is illustrated in Figure 4-11.

![Figure 4-11. Typical aerobic batch test result between TOC and orthophosphate concentrations during low P/C feed period. Error bars represent standard deviations from three batch tests (n=4).](image)

From the results, it can be seen that the P-assimilating ability of the biomass did not diminish in spite of the low P/C ratio feed, because the biomass took up ortho-P under aerobic conditions similarly for 15 days, even without an exogenous carbon source. Furthermore, MFA analysis (daily similarity index and cumulative similarity index) did not show any noticeable increase in the shift rate of microbial community composition compared to those under steady state conditions, although MFA data did indicate unusual low daily similarity values for unknown reasons three times (Figure 4-12). The ortho-P
concentrations in the effluent during this period were always lower than 0.05 mg/L and carbon removal efficiencies were always high (Figure 4-12).

![Figure 4-12](image)

Figure 4-12. Daily and cumulative similarity indices, and carbon and ortho-P removal efficiencies under low P/C feed conditions from day 52 to day 110.

On day 110, after the 58-day trial period, the feed was changed back to the original high phosphorus condition. This time, however, it took only 4 days for the system to again show the full EBPR activity observed during the steady state period. This quick recovery of full EBPR activity further indicated that the microbial community had previously not changed into a GAO-dominant composition. The four-day delay in recovering EBPR activity may indicate that EBPR activity is inducible, because during those four days, no significant
changes in daily and cumulative SI were observed (Figure 4-13).

Figure 4-13. Daily and cumulative similarity indices, and ortho-p concentrations of influent, anaerobic zone and effluent after p/c ratio in influent changed: The dotted vertical line represents the day when the ratio changed.

Overall, it was concluded that the low P/C feed did not drive the PAO-dominant microbial community composition into a GAO-dominant composition, as shown in the literature. This different result may be attributed to different microbial populations in a system.
4.3.3.2 Impact of High Temperature

It has been reported that an increase in temperature (from 20°C to 30°C) impacted an EBPR system (Wang and Park, 2002). In their study using two identical lab-scale sequencing batch reactor systems, the one system operated at 20°C showed typical EBPR activities (biological P-release in anaerobic zone and P accumulation in aerobic zone), while the other system, operated at 30°C, showed no detectable P-release during an anaerobic period and no phosphate uptake during an aerobic period.

In the present experiment, the same approach was applied in order to convert the PAO-dominant microbial population to a GAO-dominant population. The operating temperature was increased from 20°C to 25°C on day 120 and the temperature was further increased to 30°C on day 125, for 15 days. However, a GAO-dominant community composition did not arise following the increase in temperature. The P-release in the anaerobic zone remained high, even at operating temperatures of 25°C to 30 °C (Figure 4-14). Carbon and phosphorus removal efficiencies did not decrease during the period either (Figure 4-15).
Figure 4-14. The ortho-P in influent, anaerobic zone and effluent during the period of changes in operating temperature.

Figure 4-15. The carbon and ortho-P removal efficiencies during the period of changes in operating temperature.
Both the daily and cumulative similarity indices suggested a significant increase in population shift during the high-temperature operating period (Figure 4-16). To compare the shift rate of the microbial community composition under the high-temperature operating conditions to that of steady state operation, the daily and cumulative similarity indices calculated under steady state EBPR operation, days 31 to 51, are illustrated in Figure 4-16, together with comparable data for high-temperature operation.

![Figure 4-16. Daily and cumulative similarity indices during the high-temperature operating period. Control daily and cumulative SI represents the similarity index measured during steady state operation from day 31 to day 51.](image)

The average daily similarity index value during the high-temperature operation was 94.9%, while that of steady state operation, which was observed between days 31 and 51, was 96.2%. The cumulative similarity index demonstrated that the microbial community
composition was much more dynamic during the high-temperature operation than during the steady state operation. For twenty days, the cumulative similarity indices were 71% and 82% for the high temperature operation and the steady state operation, respectively. The finding that the PAO activity was maintained even when population dynamics increased, may suggest that the increase in temperature induced a growth of other PAO species that were distinct from those that were dominant at the 20°C operating condition; different PAOs may have different MFA compositions.

Overall, it was concluded that elevation of operating temperature from 20°C to 30°C for 20 days did not drive the PAO-dominant microbial community composition to a GAO-dominant composition, as was demonstrated by Wang and Park (2002), although some increase in the shift rate of the microbial community composition was detected. This observation may be attributed to the fact that the microbial population used for the current experiment was different from the microbial population used by Wang and Park (2002). Different microbial community compositions, consisting of different microbial species, may respond differently to the same change in operating factors.

4.3.3.3 Impact of Substrate Change to Glucose and Acetate Mixture

Cech and Hartman (1990, 1993) reported a breakdown of EBPR due to domination by GAOs when an acetate and glucose solution was fed to the system. This experimental finding has been confirmed by several subsequent reports (Randall et al., 1994; Satoh et al., 1994).
On the other hand, there have been several reports indicating that excellent EBPR performance could be achieved with a feed of acetate and glucose, or with glucose as the sole carbon source (Jeon and Park, 2000). In an experiment using $^{13}$C-NMR, Jeon and Park (2000) suggested that a lactic acid-producing organism (LPO) played an essential role in a glucose-fed EBPR system. In the metabolic model proposed, the LPO stored glucose as glycogen in the cell and released lactate outside, which was converted into PHAs by PAO. The finding could explain the conflicting results, whereby a feed containing glucose and acetate caused an EBPR system to failure in some experiments, but in other experiments, EBPR activity was satisfactory. If there are no fermentative organisms that produce short-chain fatty acids (SCFA) as a by-product of glucose assimilation, such as LPOs, PAOs may not be able to survive competition with GAOs, because GAOs are able to assimilate and store glucose as glycogen directly. This would provide an important advantage for GAOs to out-compete PAOs in a mixed culture system.

In the present experiment, the carbon source was changed partially from acetate to glucose through a step change on day 175. Ultimately, the glucose concentration was increased to 80% (w/w) of the total carbon in the feed. Figure 4-17 shows the carbon and orthophosphate removal efficiencies and the daily similarity index during this period of experimentation.
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Figure 4-17. Carbon and orthophosphate removal efficiencies and daily similarity index with the changes in glucose and acetate ratio in the feed.

Figure 4-18. The ortho-P in influent, anaerobic zone and effluent during glucose and acetate mixture feed.
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The input of 20% glucose for 4 days did not result in noticeable effects on P-removal and the microbial community composition. However, a feed containing 40% glucose carbon affected both the daily similarity index and the measured P-removal. One day after the 40% glucose feed was started, the similarity index began to decrease (< 90%). Two days later, the P-release in the anaerobic reactor stopped, and the P-removal efficiency decreased to 60% (Figure 4-17, 4-18). After the glucose content was increased to 60%, no P-release in the anaerobic reactor was observed, and P-removal efficiencies were less than 20%. The daily similarity index decreased to 86%. However, the sudden large decrease in daily similarity index that was observed in Chapter 2 when the biomass experienced new substrate, was not observed here, although P-removal efficiency decreased rapidly. These results may indicate that a glucose feed first suppressed EBPR activities by limiting the availability of short-chain fatty acids, resulting in reduced P-releasing and P-accumulating activities of PAOs. Consequently, PAOs lost their competitive advantage against GAOs. On the other hand, GAOs could proliferate under the high glucose condition because they were able to assimilate and store glucose as glycogen, which would be consistent with several reported experimental results (Cech and Hartman, 1993; Kong et al., 2002).

On days 190, 195 and 198, biomass PHA and biomass glycogen contents (w/w) from the lab-scale UCT bioreactor were analyzed, and the results are presented in Figure 4-19.
The results showed that more glycogen was stored inside the biomass in the aerobic reactor than in the EBPR biomass during the steady state period. The average glycogen content for GAO-dominant population was 18.8%, whereas that of the PAO-dominant population observed during the steady state period was 13.2% (refer to Figure 4-9 in Section 4.3.3.2). This stored glycogen was consumed for nutrient uptake and PHA accumulation in the anaerobic reactor, according to the Mino model (Mino et al., 1998). However, the biomass PHA contents in each reactor were considerably lower than those during the high EBPR steady state period (refer to Figure 4-9). The average biomass PHA concentrations of anaerobic, anoxic and aerobic zone during the steady state period were 15.4%, 9.3% and 4.1%, respectively. The increase in biomass glycogen and decrease in biomass PHA
concentrations in the GAO-dominant microbial community composition compared to the PAO-dominant community composition, were also reported in the experiment by Wang and Park (1998). This fact indicated that the glucose-carbon was not efficiently assimilated into PHA; rather, it was stored as glycogen in the GAO-dominant microbial community composition.

The MFA analysis expressed by the daily and cumulative similarity indices clearly detected an abnormal increase in the shift rate of microbial population during the experimental period. Figure 4-20 illustrates that the community composition changed during the shift from efficient to inefficient P-removal. When the system was fed with pure acetate as a carbon source during the steady state period between days 21 and 51, the microbial community composition was quite stable. However, when the glucose-carbon content of the feed was increased to more than 40%, the composition shifted quickly from PAO-dominant to GAO-dominant community compositions, as observed through carbon removal and ortho-P removal efficiencies.
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Figure 4-20. Cumulative similarity index change the changes in glucose and acetate ratio in the feed between day 174 and day 194. Control cumulative SI represents the similarity index measured during steady state operation for 20 days from day 31 to day 51.

Principal component analysis (PCA), which has been used for analyzing microbial community composition data in most literature reports, also showed a similar result (Figure 4-21). According to the PCA, the microbial community composition changed slowly with an acetate only feed and with the 20% glucose feed for six days (group A: from day 173 to day 179). However, after the glucose-carbon content of the feed was increased to 40%, the microbial population quickly demonstrated very different fatty acid profiles. The shift decreased again at 80% glucose content. These results were used to calculate “shift rate of microbial community composition”, expressed as “the distance between two points that represent two consecutive days of microbial community compositions on the PCA graph (Figure 4-22).
Figure 4-21. PCA results of the microbial fatty acid analysis with the change in glucose and acetate ratio in the feed. Group A represents PCA results from day 173 to day 179.

Figure 4-22. The state speed of the microbial community composition from the PCA results during acetate and glucose feed.
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The distance between two points that represent two consecutive days on the PCA plot was calculated using the X, Y values (x, y) on the PCA graph. Since the X-axis represents 53.5% and Y-axis represents 15.6% of the whole variability of the data, the distance on each X-axis and Y-axis was weighted according to its proportionality. For example, the state speed for day 179 was calculated with the distance of two coordinates between day 178 (-4.25, -0.58) and day 179 (-4.19, -0.18).

\[
\sqrt{(-4.19 + 3.93)^2 \times \frac{53.5}{69.1} + (-0.18 + 0.87)^2 \times \frac{15.6}{69.1}} = 0.48
\]

The results of the conventional PCA method and its derivative "state speed" calculation demonstrated that simple MFA analysis may be a good monitoring tool for detecting a change in microbial populations from PAO-dominant to GAO-dominant cultures, which would be useful in predicting possible deterioration of an EBPR process.

Overall, it was concluded that the feed containing a high ratio of glucose to acetate drove a functioning EBPR system into a deteriorated GAO-dominated state. As explained earlier, this result may be associated with an absence of fermentative micro-organisms, which could assimilate the glucose and produce SCFAs in the system (Jeon and Park, 2000). The similarity indices (daily and cumulative), based on simple MFA analysis, showed that the shift rate of the microbial community composition with a high glucose-carbon feed significantly increased, compared to that with an acetate feed under steady state operating conditions. This indicates that the new monitoring method using MFA analysis could be
applied for detecting EBPR deterioration resulting from GAO domination.

The observation of prolonged effects of 20% glucose feed would be recommended, because 20% glucose feed may affect P-removal activity in the long run. If MFA technique would detect the changes in microbial population before the perturbation in the system performance associated with 20% glucose feed, which would prove more clearly that MFA technique is useful in predicting future system upsets.
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4.4 Conclusions

An EBPR system, using a simplified lab-scale UCT process, was successfully established with acetate-based synthetic feed after one month of operation. In order to convert the PAO-dominant EBPR system to a GAO-dominant, non-EBPR system, three perturbation approaches were tried: (1) low phosphate-to-carbon ratio feed (P/C = 1.5:120); (2) high temperature operation (25 °C and 30 °C); and, (3) a high glucose to acetate ratio feed. Among these, only the last approach successfully changed the PAO-dominant microbial community composition to a GAO-dominant one. The reason that the first two trials were not effective may be the fact that similar or identical treatment systems can have very different microbial community compositions; therefore, their responses to changes in operating conditions could be different.

During the period of low P/C feed, it is supposed that EBPR activity was induced by the available orthophosphate retained in the system, because the EBPR activity disappeared and re-appeared without noticeable changes in the microbial population composition. This possibility needs more study.

Daily and cumulative similarity indices, based on the MFA analysis technique (which was applied effectively for monitoring of microbial community composition in previous chapters), successfully detected the sudden increase in the shift rate of the PAO-dominant microbial community composition in parallel with EBPR deterioration. Further the results were confirmed by PCA and its derivative “state speed” analysis.
These experimental results demonstrate that the MFA technique is a useful monitoring tool thatdetects change in a PAO-dominant microbial community composition (EBPR) to a GAO-dominant (non-EBPR) composition, with a correlation to EBPR performance. From the operation perspective, it would be easier to run an EBPR system if the change in microbial population into a GAO-dominant microbial community composition can be detected. As shown through the experiments in Chapters 2 to 5, the sudden increases in shift rates of microbial community compositions were related to changes in operating factors, which may result in low system performance. In other words, sudden changes in microbial community compositions may reflect changes in operating factors that may be harmful to normal system operation.
4.5 References


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The hypothesis of this dissertation was that if microorganisms in a wastewater treatment system are maintained in a very stable operating condition, the efficiency of the system may be satisfactory and the population may remain stable. In other words, the fluctuations in an operating system such as pH, DO and other operating factors may affect stable system performance through increased microbial population changes in a system. Therefore, a monitoring technique that is able to detect changes in a microbial population could be a very useful tool to diagnose system stability and predict possible system problems in the future.

A new technique for monitoring microbial population changes using MFA analysis was developed in this study. To convert MFA analysis results to an expression of microbial stability, simple similarity indices (daily and cumulative) were employed. The main experimental results were reported in Chapters 2, 3 and 4.

In Chapter 2, it was demonstrated using two identical conventional activated systems (CAS), fed with acetate-based synthetic wastewater, that the MFA monitoring technique was a useful monitoring tool to detect system problems associated with undesirable or perturbed microbial populations. The technique could quantify changes in microbial community composition by relating changes in microbial fatty acid profiles of microbial
communities with variations in operating parameters such as pH, substrate loading, substrate composition and a toxic material input. More traditional methods such as principal component analysis (PCA) and ribosomal intergenic spacer analysis (RISA) supported the experimental results acquired from MFA monitoring technique.

From the results, it was shown that microbial community compositions were constantly changing and that their shift rates increased under dynamic operating conditions. Even under steady state operating conditions, microbial community compositions still changed, which was demonstrated by daily and cumulative similarity indices. The reason why they were changing should be further studied. After biomass transfer from the pilot plant to seed the lab-scale bioreactors, large changes in microbial community composition occurred due to the mixed effects of different substrate characteristics and different system configurations between the pilot plant and the lab-scale bioreactors. Subsequent experiments revealed that the main cause of the observed MFA compositional change was due to the change in substrate composition rather than to the changes in system configuration. These results showed that lab-scale experiments using synthetic wastewater might have limited value due to different substrate compositions resulting in different microbial community composition. A full-size treatment plant would have a microbial community composition that is dissimilar to that in lab-scale bioreactors, unless real wastewater is used for designing and modeling of the real wastewater treatment.

Impacts of chlorine as a toxic material and pH shocks increased the shift rates of microbial
populations in the systems noticeably, which was detected by the MFA monitoring technique efficiently. The changing patterns, although it was studied in terms of increased or decreased fatty acids only in current study, should be further examined through long term and repeated experiments in terms of marker fatty acids. For example, C16ω7c and C18ω7c could be marker fatty acids for well established acetate-fed lab-scale reactor in the present study (Figure 2-13). If the mole percents of these two fatty acids decrease, that may indicate that an abnormal operating condition is not suitable to the reactor. The research would be useful to predict possible system problems with regard to the marker fatty acid.

If microbial fatty acid analyses are measured on a routine basis as a monitoring tool, this technique could also be used as a good estimator for the mixed liquor volatile suspended solid (MLVSS) concentration in a wastewater treatment system, minimizing the time and effort required for the conventional measurement of MLVSS. However, the lab-scale bioreactors fed synthetic wastewater (acetate-based) did not demonstrate any deterioration in system performance associated with changes in operating conditions. It was assumed that the organic material used for the experiment was so simple that its removal rate was not affected by the operating changes imposed. This assumption partly motivated the experimental plan of Chapter 3, whereby two lab-scale conventional activated sludge (CAS) bioreactors were fed with real sewage wastewater.

In Chapter 3, the main objective of the experiments was the determination of the applicability of the MFA monitoring technique as a monitoring tool for a lab-scale reactor
that was subjected to dynamic operating conditions in terms of raw wastewater characteristic provided.

Two CAS systems were fed with real municipal wastewater of varying composition and strength. The MFA technique could successfully quantify changes in microbial community compositions associated with variations in operating parameters such as pH, DO, substrate composition and chlorine additions, through a simple MFA analysis.

The average values of the daily similarity index and carbon removal efficiency during steady state operation were lower than those observed during a previous experiment using bioreactors fed with a simple synthetic wastewater in Chapter 2. The difference was attributed to the complex organic materials in the real municipal wastewater used in the second phase of experiments. However, the strength of the municipal wastewater used also varied widely, although the similarity indices remained relatively stable. This indicated that the microbial community was more sensitive to changes in the composition of the substrate, than to changes in the strength of the influent.

The daily similarities measured in Chapter 3 following biomass transfer from the pilot plant to the laboratory, were much higher than those measured in section 2.3.4.1. That was attributed to the fact that the raw wastewater used for the lab-scale reactor in Chapter 3 was the same municipal wastewater treated in the pilot-scale system from which the biomass was transformed.
In order to investigate the influence of a sudden pH increase on the microbial community populations in CAS-1, the pH was increased from 6.5 to 8.5 for 7 days. This increase in pH gave rise to a significant change in the microbial community population of CAS-1. The cumulative SI, as well as the daily SI for CAS-1, showed that the microbial community population in CAS-1 became very dynamic (unstable) after the change in pH. According to the results of the cumulative SI graph for CAS-1, the second change of pH from 8.5 to 6.5 drove the microbial population back towards the original microbial community composition that had been established at pH 6.5, before the increase in pH was implemented. This indicates that pH may be an important factor in determining a microbial population.

Carbon removal efficiency was strongly affected by the change in pH, which did not occur in the experiments using a synthetic wastewater feed in Chapter 2. These results observed in CAS-1 were likely due to the complex organic materials in the municipal wastewater. Some materials change their metabolic characteristics according to the pH (e.g. ionized or tautomerized forms), which also affects their degradability by micro-organisms. Therefore, it was assumed that some bacterial groups used to treat the wastewater had been affected by the increase in pH either physically and/or metabolically.

Carbon removal efficiency gradually recovered at pH 8.5. The seventh day after the change, the removal efficiency was 87%. Although this was still below the average of the previous period of steady state operation, it might indicate that pH 8.5 was not very inhibitory to
biological treatment systems.

After the pH set-point was returned to 6.5 in CAS-1, the system performance was again negatively affected. Carbon removal efficiency one day after the change, dropped from 87% to 79%, and it took seven days to return to normal removal efficiency. This finding confirms that pH is an important factor affecting microbial community populations and stable system performance.

Microbial community composition was more sensitive than system performance to variations in operating conditions through the stepped increase of chlorine additions. The daily similarity index and cumulative similarity index showed that the microbial community population was more affected than was the system performance, following a weak chlorine addition (2 mg/L). According to the carbon removal efficiency graph during the 2 mg/L chlorine spiking period, the system performance based on carbon removal efficiency did not seem to be affected. The results demonstrate a possibility that the monitoring technique based on microbial fatty acid analysis can be used to predict future system problems, with advanced detection of symptoms before the problems actually happen. Although a treatment system may not show any changes in its performance within a short period, more changes in microbial community composition may have adverse effects on system performance in the long run.

After the chlorine addition was stopped, the microbial community became stable relatively quickly. However, the cumulative similarity index showed that the community composition
affected by the chlorine spiking did not trend back to the original microbial community composition once the chlorine addition was stopped. This is a different result than that observed during the pH shock experiments. After chlorine addition was stopped, almost 10 days was required before carbon removal returned to the same steady state level as the control reactor. The response characteristics for microbial community population and system performance were very different from the experimental results noted for the bioreactor fed with synthetic wastewater in Chapter 2. The different results with real wastewater may be attributed to different exposure times of chlorine to biomass and to the different characteristics of the influent substrate. Apparently, real municipal wastewater is harder for biomass to treat than acetate-based synthetic wastewater. Although there was a big population change associated with chlorine spiking, in the case of synthetic wastewater, most bacteria could assimilate acetate easily, resulting in little difference in system performance. However, in the case of real municipal wastewater, if some of the bacterial groups that were mainly responsible for removing carbon sources in the wastewater were damaged by the chlorine spiking, system performance could be affected.

The exposure time of the biomass to chlorine may be one reason that the microbial community population did not return to the original population after chlorine spiking was stopped. In the present experiment, the chlorine spiking was executed over nine days in a row with different chlorine concentrations, instead of over four days as described in Chapter 2. It could be assumed that if some microbial populations were damaged seriously by the longer exposure to chlorine, it would be more difficult for the populations to return
to the norm that was established under steady state conditions. However, if the exposure time was not long enough to damage the population, the populations may have returned to normal. These results may be confirmed with the combination of some molecular technologies such as 16S rDNA/RNA sequencing with DGGE or RISA.

In order to determine if the MFA monitoring technique is able to detect the influence that a decrease in DO concentration may have on the fatty acid profiles of aerobic microbial community population, a very low level of DO (≤ 0.1 mg/L from 2.0 mg/L of normal DO concentration) was applied to CAS-1, by manual control, for 3 days.

The impact was significant, on both microbial community composition and system performance. After the change in DO set-point, the similarity index suddenly dropped from 93% to only 75% in just one day. System performance, based on TOC measurements, showed a very low removal efficiency of only 69%. The sudden drop in removal efficiency was apparently due to the change in DO, since the control bioreactor (CAS-2) showed a stable TOC removal efficiency and similarity index during the same period.

The results of the low DO operation on microbial community composition were unexpectedly significant. The biomass studied had been originally drawn from the UCT pilot plant at UBC, in which most of micro-organisms were thought to be facultative. RISA analysis and MFA analysis of the original biomass (day 0) and the biomass samples before (day 63) and after (day 67) the low DO experiment showed very different results. The
similarities based on MFA analysis were 51.4% between day 0 and day 63, and 64.5% between day 63 and day 67. However, the RISA similarities between day 0 and day 63, and between day 63 and day 67 were 62.3% and 92%, respectively.

The importance of the results is two-fold. First, the microbial community population changed significantly during the first two months (although it was difficult to tell whether the community population was a facultative-dominant or aerobe-dominant population). Second, the results suggest an interesting possibility that the changes in MFA profile associated with the sudden decrease in DO concentration were not wholly due to changes in microbial community composition, but perhaps substantially due to membrane adaptation. MFA analysis showed large changes in MFA profiles before and after the decrease in DO, while RISA did not. The analysis of carbon removal efficiency showed that the negative impact on system efficiency, which occurred on the first day of the low DO operation, quickly disappeared within a few days of the DO level being returned to normal. This characteristic may confirm that the microbial population did not actually change due to the sudden decrease in DO level. However, this interpretation should be confirmed by repeated experiments, since a relationship between membrane adaptation and dissolved oxygen level has not been shown clearly in the literature.

The important finding derived from the experiment was that MFA analysis could detect a system perturbation that was not detectable by RISA, suggesting an advantage of MFA analysis as a monitoring technique over other molecular techniques, in addition to such
advantages as simplicity, economy and rapid turnaround of results. However, the relationship between MFA analysis and RISA should be studied more thoroughly using model microbial systems, as was done in Chapter 2.

These experimental findings support the MFA application of the monitoring method, developed in the previous group of experiments, to a system that was subject to very dynamic operating conditions. An EBPR system, using a simplified lab-scale UCT process, was successfully established with acetate-based synthetic feed after one month of operation in Chapter 4. After start-up period, the system reached a steady state condition. The system showed typical EBPR activities. Daily SI changes and carbon and phosphorus removal efficiencies were very stable during the steady state period. On day 47, biomass from R3 (aerobic zone) stained with Neisser staining solution demonstrated that most of the biomass during the steady state condition was composed of PAOs.

During the steady state period, weekly batch tests were completed in order to estimate the amount of P released per unit mass of acetate consumed. The mass of ortho-P released per unit mass of acetate consumed was determined from the graph, and the ratio was calculated to be 0.72 g P released/g acetate consumed. This present result confirmed that the lab-scale simplified UCT system used was enriched with PAOs.

In order to convert the PAO-dominant EBPR system to a GAO-dominant, non-EBPR system, three perturbation approaches were tried: (1) low phosphate-to-carbon ratio feed
(P/C = 1.5:120); (2) high temperature operation (25 °C and 30 °C); and, (3) a high glucose to acetate ratio feed. Among these, only the last approach successfully changed the PAO-dominant microbial community to a GAO-dominant one. The reason that first two trials were not effective may be the fact that similar or identical treatment systems can have very different microbial community compositions as tested described in the literature; therefore, their responses to changes in operating conditions could be different.

In the present experiment, the carbon source was changed partially from acetate to glucose through a step change on day 175. Ultimately, the glucose concentration was increased to 80% (w/w) of the total carbon in the feed.

The input of 20% glucose for 4 days did not result in noticeable effects on P-removal and the microbial community structure. However, a feed containing 40% glucose carbon affected both the daily similarity index and the measured P-removal. One day after the 40% glucose feed was started, the similarity index began to decrease (< 90%). Two days later, the P-release in the anaerobic reactor stopped, and the P-removal efficiency decreased to 60%. After the glucose content was increased to 60%, no P-release in the anaerobic reactor was observed, and P-removal efficiencies were less than 20%. The daily similarity index decreased to 86%. However, the sudden large decrease in daily similarity index that was observed in Chapter 2 when the biomass experienced new substrate, was not observed here, although P-removal efficiency decreased rapidly. These results may indicate that a glucose feed first suppressed EBPR activities by limiting the availability of short-chain fatty acids,
resulting in reduced P-releasing and P-accumulating activities of PAOs. Consequently, PAOs lost their competitive advantage against GAOs. On the other hand, GAOs could proliferate under the high glucose condition because they were able to assimilate and store glucose as glycogen, which would be consistent with several reported experimental results.

The results of the conventional PCA method and its derivative "state speed" calculation demonstrated that simple MFA analysis may be a good monitoring tool for detecting a change in microbial populations from PAO-dominant to GAO-dominant cultures, which would be useful in predicting possible deterioration of an EBPR process.

It was concluded that the feed containing a high ratio of glucose to acetate drove a functioning EBPR system into a deteriorated GAO-dominated state. As explained earlier, this result may be associated with an absence of fermentative micro-organisms, which could assimilate the glucose and produce SCFAs in the system (Jeon and Park, 2000). The similarity indices (daily and cumulative), based on simple MFA analysis, showed that the shift rate of the microbial community structure with a high glucose-carbon feed significantly increased, compared to that with an acetate feed under steady state operating conditions. This indicates that the new monitoring method using MFA analysis could be applied for detecting EBPR deterioration resulting from GAO domination.

Another interesting finding was that it was supposed that EBPR activity was induced by the available orthophosphate retained in the system, because the EBPR activity disappeared and re-appeared without noticeable changes in the microbial population structure. This
possibility needs more study.
5.1 Overall Conclusions

Development of monitoring method for biological wastewater systems is important because the systems are not always stable. If we could diagnosis the systems, that would be a one more step for stable operation of biological treatment system. In present study, MFA technique was developed and evaluated for the monitoring technique which can diagnosis the system stability.

In Chapter 2, it was concluded from the experiments using two identical conventional activated systems (CAS), fed with acetate-based synthetic wastewater, that the MFA monitoring technique could quantify changes in microbial community composition by relating the changes in microbial fatty acid profiles to variations in operating parameters such as pH, substrate loading, substrate composition and a toxic material input. Also, the MFA data were easily interpreted by a simple calculation and graphs, using a similarity index. It was also shown that microbial community compositions were constantly changing and that their shift rates increased under dynamic environmental conditions. If microbial fatty acid analyses are measured on a routine basis as a monitoring tool, this technique could also be used as a good estimator for the mixed liquor volatile suspended solid (MLVSS) concentration in a wastewater treatment system, minimizing the time and effort
required for the conventional measurement of MLVSS.

In Chapter 3, the two CAS systems were fed with real municipal wastewater to determine if the MFA monitoring technique was applicable to systems treating wastewater with varying composition and strength. The MFA technique could successfully quantify changes in microbial community structures associated with variations in operating parameters such as pH, DO, substrate composition and chlorine additions, through the same simple MFA analysis. The average values of the daily similarity index and carbon removal efficiency during steady state operation were somewhat lower than those observed during the previous experiment using bioreactors fed with a simple synthetic wastewater. With the municipal wastewater feed, system performance was strongly influenced by changes in operating factors. The performance changes were effectively mirrored by the results of the new monitoring tool, based on the analysis of microbial fatty acids of biomass. These experimental findings support the MFA monitoring technique, developed in the current study, as also being applicable to a system that was subjected to more dynamic operating conditions. This indicated the potential that this technique may be used for pilot-scale or full-scale wastewater treatments.

In Chapter 4, the application of this technique was extended to an enhanced biological phosphorus removal (EBPR) process. During the experiment, three perturbation approaches were tried: (1) low phosphate-to-carbon ratio feed (P/C = 1.5:120); (2) high temperature operation (25°C and 30°C); and, (3) a high glucose-to-acetate ratio feed. Among these
methods, only the last approach successfully changed the PAO-dominant microbial community structure to a GAO-dominant one. The MFA monitoring technique, which was effectively applied to the monitoring of microbial community composition in previous chapters, successfully detected the sudden increase in the shift rate of the PAO-dominant microbial community structure, in parallel with EBPR deterioration.

Throughout the whole experimental process of this study, it was successfully demonstrated that the MFA monitoring technique is a potential monitoring tool that detects microbial community response to a change in operating conditions, which may affect system performance immediately, or in the future.

From a treatment plant operation perspective, the technique is advantageous because it allows easier management of biological wastewater systems. For example, if a change in microbial population into a GAO-dominant microbial community can be detected early, an operator may cope with the problem by analyzing operating conditions such as HRT, temperature, sludge age, etc. An early indication of impending system malfunction could be gained in two ways. First, an abnormal increase in the microbial population shift rate could be a warning of possible future problems with an operating treatment system. An increase in population shift rate is most likely to occur in association with changes in operating conditions, which may result in system perturbations. In the present experiments, many abnormal increases in population shift rates were detected concomitantly with changes in operating conditions. However, when the changes in operating conditions were imposed
through step increases, the shift rates responded more quickly than system performance (for example, the step increase in chlorine addition in Chapter 3 and step increase in glucose feed in Chapter 4). For those experiments, a 2 ppm chlorine addition and a 20% glucose feed increased the shift rates of microbial populations, even though the observed carbon removal efficiencies did not change. For the detection of abnormal shift rates of microbial populations, it is important to know the normal or background shift rates for biological systems because the “normal shift rate” of a biological system may differ with operating conditions.

Secondly, specific fatty acid peaks may be an important indicator of system stability. For example, C16:ω7c could be an indicator of well-established PAO-dominant microbial population in this study. It is important, therefore, to obtain information on the dominant fatty acid peaks in each microbial population.

Furthermore, efforts correlating community changes to operating conditions may lead to the best operational adjustment, which may be specific to each system.

It is hoped that the current study will be a stepping stone to bridge the gaps between scientific information regarding microbial community structure and biological wastewater treatment system.
Chapter 6 – Conclusions and Recommendations

5.2 Research Recommendations

It is recommended that further efforts are necessary to extend the present knowledge regarding the MFA monitoring technique and to enrich the scope of application.

1. Full-scale study of the MFA monitoring technique to extend its applicability.

2. Molecular level study to determine whether EBPR activity is inducible.

3. Automation of MFA analysis.


5. Parallel study of the MFA technique with several molecular techniques to distinguish between population change and membrane adaptation.

6. Prolonged observation of 2 ppm addition of chlorine and 20% glucose feed in terms of system performance versus microbial population stability.