# Community Structure and Microbial Activity of Sulfate Reducing Bacteria in Wastewater Biofilms and Mature Fine Tailings Analyzed by Microsensors and Molecular Biology Techniques

by

Hong Liu

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Environmental Engineering

Department of Civil and Environmental Engineering

University of Alberta

## Abstract

Sulfate reducing bacteria (SRB) play a significant role in complex microbial environments such as wastewater biofilms and mature fine tailings (MFT). A unique characteristic of these complex microbial environments is that stratified structure containing both oxic and anoxic zones could be formed; the anoxic zones were expected to provide microenvironment for the growth and function of SRB. SRB utilize sulfate as a terminal electron acceptor during dissimilatory sulfate reduction for the degradation of organic compounds. However, hydrogen sulfide ($H_2S$) gas is generated during the biological sulfate reduction process. The produced toxic $H_2S$ itself is one of the concerns; in addition, the generated $H_2S$ leads to an increase in oxygen ($O_2$) consumption due to the internal re-oxidation of $H_2S$. To address the SRB problem in municipal wastewater, in MFT, and in oil sands process-affected wastewater (OSPW) generated from the industrial extraction of bitumen, the functional diversity and in situ activity of SRB were investigated in $O_2$ based membrane aerated biofilm (MAB), MFT, and biofilm grown in OSPW. $H_2S$ microsensor was used for in situ measurements of SRB microbial activity. PCR-DGGE-FISH (polymerase chain reaction-denaturing gradient gel eletrophoresis-fluroscence in situ hybridization) and DGGE-qPCR were applied to investigate the functional diversity and abundance of SRB in biofilms and MFT, respectively. In the MAB sample, the $O_2$ concentration profile in MAB revealed the presence of oxic and anoxic zones. The $H_2S$ concentration

profile showed that $H_2S$ was produced in the upper region of the biofilm and penetrated 285 μm below the interface between biofilm and bulk liquid, indicating a high activity of SRB in this region. DGGE of the PCR-amplified dissimilatory sulfite reductase subunit β (dsrB) gene and FISH showed an uneven spatial distribution of SRB communities in terms of functional diversity and biomass. The maximum SRB biomass was located in the upper biofilm. In the MFT sample, a higher diversity of SRB was present and more $H_2S$ was produced in gypsum amended MFT than in unamended MFT. Based on the combined techniques, a higher sulfate reduction activity in gypsum amended MFT than in unamended MFT was indicated; in addition, more $H_2S$ was produced in the deeper regions of the MFT samples. In the OSPW biofilm sample, multispecies biofilm in OSPW was developed on engineered biocarriers and was capable of simultaneous removal of chemical oxidation demand (COD), sulfate, and nitrogen from OSPW. $H_2S$ was observed in the deeper anoxic zone from around 750 μm to 1000 μm below the interface, revealing in situ sulfate reduction in the deeper zone of the stratified biofilm.

**Acknowledgment**

It has been a great privilege to spend six years in the Department of Civil and Environmental Engineering at University of Alberta. I would like to take this opportunity to acknowledge all the persons who have made it possible for me to complete my PhD program.

My first gratitude to my supervisors, Dr. Tong Yu and Dr. Yang Liu, for their continuous support, motivation, and helpful guidance over the past six years. I would not have been able to proceed through the doctorial program and complete my dissertation without their priceless advice and encouragement. They are not only as supervisor on research assistantship, but also as mentor on my professional and personal life helping me to get through those most rough roads.

I would also like to thank Dr. Evan Davies and Dr. Zhenghe Xu for serving as my committee members, for their encouragement, insightful comments, and suggestions. Sincere appreciation is due to Dr. Baikun Li of University of Connecticut for serving as the external examiner for this dissertation. Constructive and professional comments from committee members are highly appreciated.

My thanks to my fellow labmates in Environmental Engineering Group: Dr. Kusumakar Sharma, Dr. Shuying Tan, Shujie Ren, Lei Zhu, Miao Yu, and Yijun

Chen for the stimulating discussions, for the time we were working together, and for all the fun we have had during my PhD study. In particular, I am grateful to Zhiya Sheng, Jody Yu, and Elena Dlusskaya for teaching me molecular biology techniques and for helpful suggestions throughout my experiments.

My sincerest thanks to the financial supports from Natural Sciences and Engineering Research Council (NSERC) of Canada, and China Scholarship Council (CSC).

Last but not least, a special thanks to my family. Words cannot express how grateful I am to my parents, my brother, and my husband. Your love, unconditional support, encouragement, and all of the sacrifices you have made provided my inspiration and allowed me to finish this journey.

# Table of Content

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| BLAST | Basic Local Alignment Search Tool |
| CBD | Calgary Biofilm Device |
| CLSM | Confocal Laser Scanning Microscopy |
| CT | Composite Tailing |
| COD | Chemical Oxygen Demand |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DNA | Deoxyribonucleic Acid |
| DsrB | Dissimilatory Sulfite Reductase β-Subunit |
| FISH | Fluorescence *in situ* Hybridization |
| LSM | Laser Scanning Microscopy |
| MABR | Membrane Aerated Biofilm Reactor |
| MBBR | Moving Bed Biofilm Reactor |
| MFT | Mature Fine Tailing |
| MLSB | Mildred Lake Settling Basin |
| MPN | Most Probable Number |
| NAs | Naphthenic Acids |
| NCBI | National Center for Biotechnology Information |
| OSPW | Oil Sands Process-affected Wastewater |
| PAHs | Polycyclic Aromatic Hydrocarbons |
| PCR | Polymerase Chain Reaction |
| qPCR | Quantitative Polymerase Chain Reaction |
| RBC | Rotating Biofilm Contactor |
| SRB | Sulfate Reducing Bacteria |

# Chapter 1 Introduction

## 1.1 Background

Sulfate reducing bacteria (SRB) have been defined as "bacteria and archaea that can obtain energy by oxidizing organic compounds or molecular hydrogen ($H_2$) while reducing sulfate ($SO_4^{2-}$) to hydrogen sulfide ($H_2S$)" (Schulze and Mooney 1993). SRB are widely distributed in anaerobic environments such as marine sediments (Jorgensen 1982), microbial mats (Minz *et al.,* 1999), wastewater biofilms (Okabe *et al.,* 2003), and mature fine tailings (MFT) (a nuisance product of industrial bitumen extraction) (Holowenko *et al.,* 2000). In these complex microbial environments, the SRB role in biomineralization is important in sulfur and carbon cycles. SRB utilize sulfate as a terminal electron acceptor during sulfate reduction in the degradation of organic compounds.

Wastewater biofilms contain diversity of microorganisms with a complex and heterogeneous structure (Debeer *et al.,* 1994). Within a few millimeters thickness of biofilm, substrate and oxygen are diffused and consumed into the biofilm layers. As a result, even though the bulk water is in oxic condition, sulfate reduction can be anticipated to take place in the anoxic strata in deeper biofilm. Oil sands MFT in Alberta are highly stratified because not only the solids content and chemicals but also microbial activities are present as a function of depth of

the ponds (Foght *et al.,* 1985; Li 2010). Some tailing ponds were treated with gypsum ($CaSO_4 \bullet 2H_2O$) to accelerate the densification process in order to increase water release and to reduce the volume of settled sediments (List and Lord 1997). As a result, the supplement of gypsum introducing $SO_4^{2-}$ would facilitate the growth of SRB and sulfate reduction activity.

However, during the biological sulfate reduction process in these microbial environments, $H_2S$ is generated which is biologically toxic (Barton and Fauque 2009), promotes corrosion of the facilities (Pol *et al.,* 1998), and leads to an increase in oxygen consumption due to the internal reoxidation of $H_2S$ (Okabe *et al.,* 1998). Therefore, the understanding of the presence of SRB and its activities in these complex microbial environments is necessary to provide information for future improvements in the design and operation of wastewater biofilm reactors and the long-term plan for oil sands tailings reclamation.

## 1.2 Objectives

This research aims to expand the fundamental understanding of the structure and function of microorganisms in complex microbial environments. Three different samples, including municipal wastewater membrane aerated biofilm (MAB), mature fine tailings (MFT), and oil sands process-affected wastewater (OSPW) biofilm were used as the examples of complex microbial environments

in this study of the community structure and microbial activity of SRB. The specific objectives of the research were:

1. To obtain concentration profiles of the sulfate reduction end product $H_2S$ based on $H_2S$ microsensor measurements along the depths of MAB, MFT, and OSPW biofilm;

2. To investigate the presence, the functional diversity, and the abundance of SRB by molecular biology techniques;

3. To correlate and elucidate the relationship between the microbial characteristics of SRB and their activities in these three microenvironments.

**1.3 Significance of the Research**

This is one of the few studies that combines the techniques of microsensors and molecular biology and applies them in a variety of complex microbial environments. The knowledge obtained will enable the future development of strategies for wastewater and tailings management. This is the first detailed investigation of in situ activity and functional diversity of SRB in wastewater membrane aerated biofilm, in OSPW biofilm and in mature fine tailings microenvironments. The microsensor techniques used here provide the first evidence of in situ microbial activity of SRB in microbially active tailings. The results of this study will benefit the modeling, design, and operation of biofilm

reactors that treat wastewater, and long-term plans for tailings management can be assessed more wisely with the information provided in this study.

## 1.4 Thesis Outline

An overall framework of the research is provided in Figure 1-1. The three complex microbial environments were developed in a membrane aerated biofilm reactor (MABR), MFT settling columns, and an OSPW biofilm reactor.

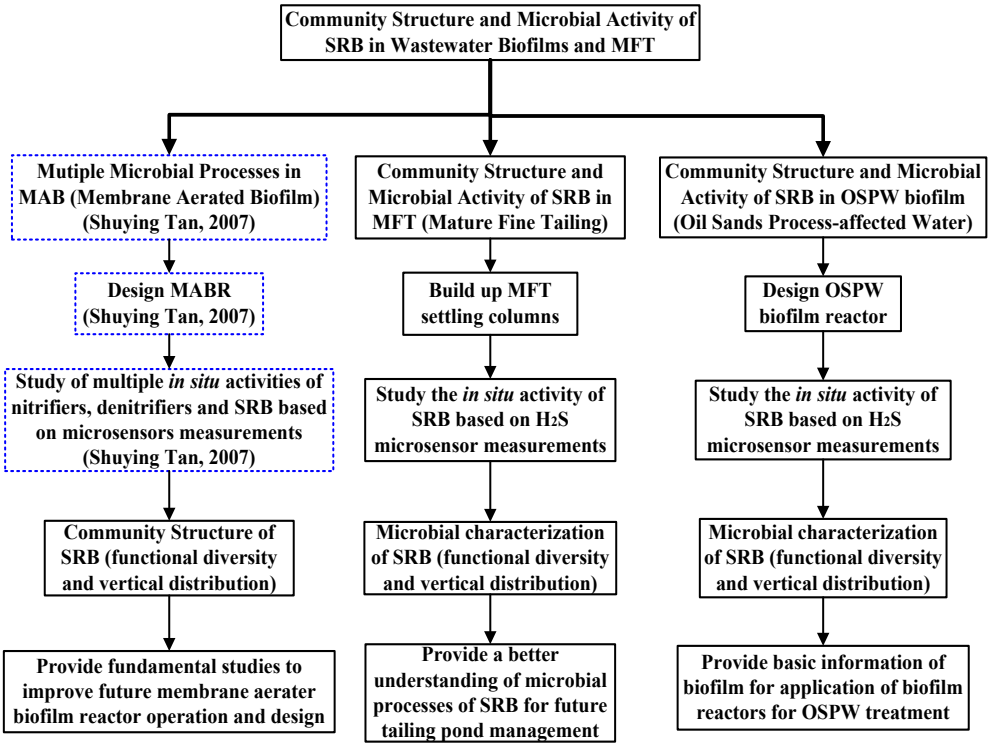



**Figure 1-1. Framework of the Study.**

In each complex microbial environment, the community structure and microbial activity of SRB were analyzed based on microsensor measurements and molecular biology analysis. The $H_2S$ microsensors were used to measure chemical gradients within the three complex microbial environments and to indicate the occurrence of sulfate reduction. The DNA of the microorganisms in these microbial environments was extracted and subjected to PCR, DGGE, FISH, or qPCR to elucidate the community structures and biomass distribution of SRB in the bioreactors and settling columns. The structure of the dissertation is presented below.

Chapter 1 introduces the background, research needs, and significance of this study. A framework of the content of the study is provided.

Chapter 2 reviews previous research in three sections: (1) complex microbial environments; (2) community structure and activity of microorganisms in different complex microbial environments, including wastewater biofilms and oil sands tailings; and (3) studies of SRB and sulfate reduction activity in wastewater biofilms and oil sands tailings.

Chapter 3 describes the research tools used to characterize microorganisms and study their microbial activity in complex microbial environments. The basic principles of the microsensor measurements and molecular biology techniques

(PCR-DGGE, FISH, real-time qPCR), and selected applications of these techniques in complex microbial environments are briefly reviewed.

Chapter 4 presents a study of community structure and microbial activity of SRB in a membrane aerated wastewater biofilm. The oxygen impact on SRB sulfate reduction activity within the biofilm is evaluated.

Chapter 5 investigates the community structure and microbial activity of SRB in stratified mature fine tailings and also examines the effect of gypsum addition on the acceleration of SRB sulfate reduction activity.

Chapter 6 shows the presence of SRB and measures the SRB activity in OSPW biofilm.

Chapter 7 provides the conclusions of the study and areas that could benefit from future studies are suggested.

## References


Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology. **68**: 41-98.

Debeer, D., Stoodley, P., Roe, F. and Lewandowski, Z. 1994. Effects of biofilm structures on oxygen distribution and mass-transport. Biotechnology and Bioengineering. **43**(11): 1131-1138.

Foght, J. M., Fedorak, P. M. and Westlake, D. W. S. 1985. Microbial content and metabolic activities in the Syncrude tailings pond, Aostra Journal of Research. **1**:139-146.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology. **46**(10): 927-937.

Jorgensen, B. B. 1982. Mineralization of organic matter in the sea bed - the role of sulfate reduction. Nature. **296**(5858): 643-645.

Li, C. 2010. Methanogenesis in oil sands tailings—An analysis of the microbial community involved and its effects on tailings densification. Master Thesis. University of Alberta. Edmonton, Canada.

List, B. R. and Lord, E. R. F. 1997. Syncrude's tailings management practices from research to implementation. CIM Bulletin. **90**(1010): 39-44.

Minz, D., Flax, J. L., Green, S. J., Muyzer, G., Cohen, Y., Wagner, M., Rittmann, B. E. and Stahl, D. A. 1999. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. Applied and Environmental Microbiology. **65**(10): 4666-4671.

Okabe, S., Ito, T. and Satoh, H. 2003. Sulfate reducing bacterial community structure and their contribution to carbon mineralization in a wastewater biofilm growing under microaerophilic conditions. Applied Microbiology and Biotechnology. **63**(3): 322-334.

Okabe, S., Matsuda, T., Satoh, H., Itoh, T. and Watanabe, Y. 1998. Sulfate reduction and sulfide oxidation in aerobic mixed population biofilms. Water Science and Technology. **37**(4-5): 131-138.

Pol, L. W. H., Lens, P. N. L., Stams, A. J. M. and Lettinga, G. 1998. Anaerobic treatment of sulfate-rich wastewaters. Biodegradation. **9**(3-4): 213-224.

Schulze, E.-D. and Mooney, H. A. 1993. Biodiversity and ecosystem function, Springer-Verlag.

# Chapter 2 Literature Review

## 2.1 Complex Microbial Environments

Microorganisms are found naturally on the planet Earth. Microbes continually evolve to adapt to unique habitats and their metabolic processes also influence their habitats; the result is diversity and complexity of the microbial species within an ecosystem.

Biofilm is a complex microbial environment in which bacteria adhere to a surface (metal, plastic, etc.) in a moist environment by excreting extracellular polymeric substances (EPS) that hold the cell aggregates together. The vast majority of microbes in aqueous environments live as biofilms rather than planktonic forms (Hall-Stoodley *et al.,* 2004). Biofilm can be found wherever there is availability of moisture, nutrients, and a surface. Once the bacterial cells develop the biofilm matrix, they will interact with each other and express phenotypic traits that are often different from those expressed by planktonic cells; these traits depend on the type of bacteria species, the available nutrients, the surface to which the cells are attached, temperature, and light. Thus biofilms vary in temporal and spatial characteristics according to their community composition, internal structure, and metabolism activity.

Other forms of complex microbial environments include sediments, oil sands tailings, and hydrothermal vents. The physiochemical conditions of these microbial environments are also inherently heterogeneous and have continually changing conditions temporally and spatially; as a result, the microorganisms present in these microbial environments are various in terms of their abundance, distribution, and metabolic activity.

## 2.2 Community Structure and Microbial Activity of Microorganisms in Complex Microenvironments

### 2.2.1 Structure and Activity of Microorganisms in Wastewater Biofilms

A biofilm is a dynamic, living microbial system composed mainly of multispecies microorganisms, EPS, and water. The competition for substrate of a large number of different kinds of microorganisms living together is determined by mass transport and substrate utilization rate. As a result, different types of microorganisms will find their ecological niche at different locations within the biofilm. In turn, the spatial distribution and activity of the microorganisms within the biofilm matrix may influence biofilm functions and can affect the performance of the biofilm in a bioreactor that treats wastewater.

Biofilms have been used for wastewater remediation since the mid-1800s because biofilms play a role in the biodegradation of carbon compounds that pollute the water. Researchers have developed a variety of biofilm systems to aid

in wastewater treatment (Ibrahim *et al.,* 2012). For example, in biofilm systems such as the granular media biofilter, rotating biological contactors (RBC) and trickling filters (TF), microbial biomass in a film that grows on the surface of an inert, solid medium. Purification is achieved when the wastewater is brought into contact with this microbial film. In other wastewater treatment systems, including a biofilm upflow sludge blanket (USB), a fluidized bed (FB), an expanded granular sludge blanket (EGSB), and a biofilm airlift suspension (BAS), particles are kept fluidized by upflowing influent. The moving bed biofilm reactor (MBBR) is a continuous flow-through process that combines the benefits of fixed film and suspended microorganism growth. Microbial fuel cells have shown great potential in selective, low-cost catalysis and energy conversion in biofuel production and microbial driven batteries. Biofilm systems are developed and optimized based on what is understood about the dynamic nature and characteristics of microorganisms within the biofilm and the effects of reactor operation conditions on the microorganisms.

In the last 20 years, researchers have revealed the remarkable heterogeneity of biofilms in terms of their composition, structure, and in situ activity (Debeer *et al*. 1994). The microscale heterogeneity in solute chemistry that is present within a biofilm has been recognized, and microsensors have been developed to measure the chemical gradient within a biofilm. Revsbech and Jorgensen (1986) introduced the application of microsensors in microbial environments, mostly in

sediments and microbial mats. They also addressed the potential relevance of microsensor techniques to microbial ecology. Later, microsensor measurements were used to report concentration gradients of metabolic substrates and products in many studies of wastewater biofilms.

de la Rosa and Yu (2006) mapped the three-dimensional dissolved oxygen (DO) distribution in a wastewater biofilm grown on a rotating biological contactors (RBC) disk in a municipal wastewater treatment plant using an oxygen microsensor combined with an automation system. It was demonstrated that the DO was unevenly distributed along the depth of the wastewater biofilm. The oxygen concentration decreased with the depth into the biofilm. To obtain the oxygen distribution and to understand mass transport in the complex heterogeneous biofilm microenvironment, the oxygen concentration contour and local gradients were plotted within an aerated biofilm using an oxygen microsensor (deBeer *et al*., 1994). Oxygen profile measurements in these biofilms typically revealed a steadily declining oxygen concentration as the microelectrode progressed from the fluid above the biofilm into the biofilm depths. Oxygen fails to penetrate because it is actively respired by cells in the upper layers of the biofilm; oxic zones that have dimensions of tens to a few hundred microns are commonly reported. deBeer *et al*. (1997) studied nitrification in an environmental biofilm with nitrite and nitrate microsensors. The profiles were used to discuss the conversion of ammonium to nitrite and nitrite to nitrate. To better understand the

nitrification process in biofilms, Zhang and Bishop (1996) studied the effect of pH on nitrification in a biofilm sample using microsensors to obtain the gradient profiles of pH, ammonium, and nitrate within the biofilm. Yu and Bishop (2001) investigated the stratification of microbial processes indicated by the oxidation-reduction potential (ORP) within an aerobic sulfate reducing biofilm. It was found that aerobic oxidation occurred in a surface layer of the biofilm and sulfate reduction took place in the deeper anoxic zone. Tan and Yu (2007) studied microbial sulfate reduction activity in a membrane aerated biofilm based on $H_2S$ microsensor measurements. The production of $H_2S$ in the anoxic zone of the biofilm indicated that active sulfate reduction activity was occurring there. Yu (2000) applied oxygen, sulfide, pH, redox potential, nitrate, and ammonium microsensors to investigate simultaneous microbial processes including nitrification, denitrification, sulfate reduction, and the stratification of these processes in wastewater biofilms. Tan (2012) also investigated the simultaneous occurrence of multiple microbial processes including nitrification, denitrification, sulfate reduction, and their stratification inside one piece of membrane aerated biofilm using oxygen, ORP, nitrate, pH, ammonium, and $H_2S$ microsensors.

The development of molecular biology methods in microbial ecology made it possible to identify bacteria and characterize microbial consortia structure without using the traditional microbiology techniques of isolation and cultivation. During the last two decades, researchers have used molecular biology methods with

microsensor measurement techniques to reveal the distribution and heterogeneity of microorganisms present in biofilms, and to determine the relationship between biofilm structure and microorganism activity. These studies have made it possible to better control the performance of wastewater treatment systems.

Santegoeds *et al*. (1999) found a stratified structure of methanogenesis and sulfate reduction in an anaerobic aggregate from an upflow anaerobic sludge bed (UASB) reactor with SRB in the outer 50 to 100 μm and methanogens in the inner zone of the aggregate; correspondingly, the production of sulfide resulting from microbial sulfate reduction was located in the outer 50 to 100 μm, while methane production resulting from methanogenesis was exclusively detected below a depth of 100 μm. Schramm *et al*. (1999) investigated the in situ activity, abundance, and distribution of nitrifying bacteria in a nitrifying fluidized bed reactor biofilm with a combination of microsensor measurements and fluorescence in situ hybridization. Profiles of $O_2$, $NH_4^+$, $NO_2^-$, and $NO_3^-$ indicated that oxygen consumption and ammonium and nitrite depletion were restricted to a surface zone of 100 to 150 μm of the biofilm aggregate; accordingly, the ammonia oxidizing bacteria *Nitrosospirasp* and the nitrite oxidizing bacteria *Nitrosospira* were identified within this narrow surface zone of the aggregates. Schramm *et al*. (2000) studied the distribution of nitrifying bacteria in a membrane-bound biofilm, it was found that the oxic part of the biofilm which was subjected to high nitrite and nitrate concentrations was dominated by the nitrifying bacteria genera

*Nitrosomonas*, *Nitrosospira sp.*, and *Nitrobacter*. In Okabe and Watanabe (2000)' study, the in situ spatial organization of ammonia oxidizing and nitrite oxidizing bacteria in domestic wastewater biofilms was studied using microsensors and 16S rRNA-targeted oligonucleotide probes. Based on microsensor measurements, the active ammonia oxidizing zone in this stratified biofilm was located in the outer part of the biofilm, whereas the active nitrite oxidizing zone was located just below the ammonia oxidizing zone. In situ hybridization revealed that ammonia oxidizing bacteria were dominated by the genus *Nitrosomonas*, most of the ammonia oxidizing bacteria were present throughout the biofilm, and *Nitrospira*-like bacteria were the main nitrite-oxidizing bacteria. No *Nitrobacter* were detected in this biofilm, and nitrite oxidizing bacteria were restricted to active nitrite oxidizing zones.

### 2.2.2 Structure and Activity of Microorganisms in Oil Sands Tailings

Alberta has the largest oil sands deposit in the world. Three major distribution areas in Alberta—Athabasca, Cold Lake, and Peace River—contain 270 billion cubic meters of crude bitumen (Chalaturnyk *et al.,* 2002). Bitumen is extracted from oil sands with hot caustic water and chemical additives. Four liters of hot water is needed to obtain one liter of crude oil (Allen, 2008). The hot water extraction process results in a slurry that contains coarse sand, fine particles, water, and residual bitumen. The produced water—identified as "oil sands process-affected water (OSPW)"—has to be discharged into a designated pond because

the policy of the Alberta government forbids the discharge of polluted water to the environment. The coarse sand and fine particles (tailings) contained in the OSPW settle with time and the released water is recycled for further usage, or treated to remove chemical pollutants.

The oil sands tailing ponds provide unique microbial environments for microorganisms due to the availability of hydrocarbons and water for bacterial growth. Foght *et al.* (1985) detected both aerobic and anaerobic microbes in the Mildred Lake Settling Basin (MLSB) owned by Syncrude Canada Ltd. (Foght *et al*., 1985; Li, 2010). Researchers have identified numerous bacterial species inhabiting oil sands tailings ponds, including methanogens, SRB, iron reducing bacteria, and nitrite reducing bacteria (Penner and Foght, 2010).

The microbial communities in tailings ponds have high bacterial diversity and are microbiologically active and highly stratified due to the stratification of hydrocarbons, metals, and electron donors presenting as a function of depth of the ponds. Several studies revealed significant stratification in terms of structure and activity of microorganisms in tailings ponds. Holowenko *et al.* (2000) profiled methanogens and SRB populations at 1, 5, 10, 15, and 20 meters below the surface of the Syncrude MLSB tailings pond based on a standard most probable number (MPN) method. Ramos-Padron *et al*. (2011) investigated microbial communities and their activities as a function of tailing pond depth. Based on pyrosequencing of 16S rDNA analysis, differences in microorganism community

composition were observed for samples at 1.5 meter intervals of tailing pond depth.

Indigenous microorganisms in the tailing ponds play significant roles in OSPW remediation. Some studies have demonstrated that bacteria in the tailings ponds are capable of catalyzing hydrocarbon biodegradation (Herman *et al.,* 1994; Holowenko *et al.,* 2002; Quagraine *et al.,* 2005; Siddique *et al.,* 2010).

Researchers have tried to link bacteria groups with functions in oil sands tailings management—i.e., the settling of tailings in the ponds. For example, methanogens are evidently responsible for accelerated densification of mature fine tailings (MFT) by producing methane and generating channels in the tailings where gas bubbles rise, allowing the water to be released to the surface. Bordenave *et al*. (2010) studied the relationship between anaerobic microbial populations in an oil sands tailings pond and the sedimentation of the tailings. It was found that the addition of the nitrate reducing bacteria *Thauera sp.*, the SRB *D. vulgaris*, or the acetotrophic methanogen *M. barkeri* promoted clay aggregation, and thus increased the sedimentation rate of the tailings compared to that of sterilized tailings samples. In addition, it was postulated that bacteria capable of producing bioflocculant would adsorb clays on their surface and within their EPS, and thus enhance the aggregation and sedimentation of the fine solids.

## 2.3 SRB and Sulfate Reduction in Complex Microbial Environments

### 2.3.1 SRB and Sulfate Reduction in Wastewater Biofilms

Biofilms are widely utilized in engineered environmental systems for biodegradation of contaminated water. Biofilm reactors such as the conventional RBC and TF have been used in wastewater treatment for decades. Innovative technologies are continually sought to obtain better treatment efficiency and reduce operating costs. However, the structure and function of the biofilms must be elucidated to improve existing technologies.

#### 2.3.1.1 SRB and Sulfate Reduction in Conventional Biofilms

Previous studies have recognized the complex structure and heterogeneity of wastewater biofilms (Debeer *et al*., 1994). Yu (2000) demonstrated the stratification of conventional biofilms, which could be caused by combined microbial processes, and could include aerobic oxidation, nitrification, denitrification, and sulfate reduction.

In the treatment of sulfate enriched wastewater, SRB play important roles in the mineralization of organic matter, and compete with methanogens or denitrifying bacteria to utilize organic compounds as electron donors. Studies on the microbial characteristics and activity of SRB in conventional wastewater biofilms are summarized in the literature cited below.

Early in the 1980s, the presence of SRB in RBC was investigated by Alleman *et al.* (1982). Based on scanning electron microscopy (SEM), it was found that *Desulfovibrio* were present in an anaerobic layer in a deep zone of the biofilm. With the development of biofilm analysis techniques, SRB in wastewater biofilms could be further investigated. Kuhl and Jorgensen (1992) studied sulfate reduction and its interaction with other respiratory processes in TF biofilms using $S^{2-}$ and $O_2$ microsensors. The study demonstrated the occurrence of sulfate reduction in deep anoxic zones of the biofilm where there was low oxygen respiration. Yu and Bishop (2001) studied the stratification and ORP change in an RBC biofilm using ORP, $O_2$, and $S^{2-}$ microsensors; it was found that sulfate reduction took place in deep anoxic zones of the biofim. Ramsing *et al.* (1993) were the first to use oligonucleotide probes and microsensors to study SRB in a TF wastewater biofilm. It was demonstrated that the presence of SRB cells was negatively related to the oxygen concentration in the biofilm. Okabe *et al*. (1999) studied the spatial distribution and potential activity of SRB in RBC biofilm with microsensors and 16S rRNA-targeted oligonucleotide probes. Results indicated that both SRB and SRB activity were restricted to a narrow zone (150~300 μm) in the middle part of the biofilm, below the biofilm surface, then decreased in the bottom of biofilm due to the transportation limitation of electron donors. Ito *et al*. (2002) also demonstrated that active sulfide production was correlated with an increase in SRB populations in a RBC biofilm.

#### 2.3.1.2 SRB and sulfate reduction in membrane aerated biofilms

A membrane aerated biofilm reactor (MABR) is a promising alternative in wastewater treatment because of its high gas transfer efficiency and low cost (Cote *et al*., 1988; Brindle and Stephenson, 1996). An MABR has a special microbial configuration because the oxygen and substrates are supplied from opposite sides of the biofilm (Syron and Casey, 2008); therefore, simultaneous aerobic and anaerobic processes can be realized in a single biofilm cultured in one vessel. Within the membrane aerated biofilm (MAB), a very different stratification in terms of substrates concentration and bacterial activities can be established compared to biofilm attached to solid surfaces (Essila *et al.,* 2000).

It was first reported in 1988 that nitrification, aerobic oxidation, and denitrification simultaneously occurred in a single MAB (Timberlake *et al.,* 1988). Since then, studies and applications of MABRs in wastewater treatment have focused on organic carbon removal (Brindle *et al.,* 1999) and simultaneous COD and nitrogen removal (Yamagiwa *et al*., 1994; Brindle and Stephenson, 1996; Semmens *et al*., 2003). These studies demonstrated that high rates of carbon and nitrogen removal could be achieved in a single MAB. Microbial distributions and activities of nitrifiers and denitrifiers within an MAB were also characterized (Cole *et al.,* 2002; Terada *et al.,* 2003; Cole *et al.,* 2004; Satoh *et al.,* 2004). It was shown in these studies that ammonia oxidizing bacteria (AOB) existed near the membrane side while denitrifiers preferred the anaerobic environment near the

surface of the biofilm. Nevertheless, there is a lack of information on the community structure and corresponding microbial activity of SRB in MABs.

#### 2.3.1.3 SRB and Sulfate Reduction in Oil Sands Process-Affected Water Biofilms

Treatment of OSPW is a critical issue in the oil sands industry. The zero discharge policy of the Alberta government mandates that OSPW must be delivered to onsite tailings ponds. Continuous recycling of tailings pond water (~ 80%) leads to a decline in water quality as the pollutants become concentrated. Failure to purify OSPW has negative consequences for bitumen recovery and pure water consumption. An evaluation of the processed water quality (organic and inorganic chemistry) and toxicity data from two long-term oil sands mining projects have been compiled to identify target pollutants and establish water treatment priorities (Allen, 2008). The presence in OSPW of (1) NAs which are toxic and not easily biodegraded, (2) heavy metals which are also toxic and difficult to remove, and (3) high salinity which is environmentally undesirable suggested that water treatment technologies need to evolve to meet the water management needs of the oil industry.

To minimize the environmental impact and to increase the efficiency of water reuse, early tests of OSPW treatment included adsorption, coagulation and flocculation, membrane filtration, freeze-thawing, and advanced oxidation (Allen 2008). Biofilm reactors can now be added to physical and chemical technologies

or can be combined with physicochemical treatments for OSPW remediation. Application of biological treatment is to some extent hindered by the sensitivity of microorganisms to the toxic substances contained in the OSPW. However, the existing microorganisms in tailings ponds are believed to tolerate the toxicity and to some extent are capable of degrading the pollutants. Studies have investigated the degradation of hydrocarbons in tailings by endogenous microorganisms (Herman *et al*., 1994). Golby *et al*. (2011) demonstrated that oil sands' indigenous microorganisms could form a biofilm in the Calgary Biofilm Device (CBD). They revealed that the biofilm with 1-3 cells layers thickness contains dominant species belonging to *Pseudomonas*, *Thauera*, *Hydrogenophaga*, *Rhodoferax*, and *Acidovorax*. A variety of biofilm reactors have been proved useful for treating OSPW. Islam *et al*. (2014) evaluated the treatment of OSPW using a fluidized bed biofilm reactor (FBBR) with granular activated carbon (GAC) as support media. The biofilm with 30-40 μm thickness after 120 day's treatment was found has a diverse community including *Polaromonas jejuensis*, *Algoriphagus sp*., *Chelatococcus sp*. and *Methylobacterium fujisawaense*. Choi *et al*. (2014) incubated 20-30 μm thickness biofilms for treating OSPW. It was revealed that the reactor biofilms contain *Flavobacterium, Rhizosphere soil bacteria*, *Rhizobium, Azoarcus, Stigmatella aurantiaca, Actinobacterium,* and *Sulfuritaleahydrogenivorans.* Despite the successful development and characterization of biofilms applied in OSPW treatment, the community structure and the activities of SRB in OSPW biofilms are far from clear and need to be

explored.

### 2.3.2 SRB and Sulfate Reduction in Mature Fine Tailings

Oil sands tailings ponds in Northern Alberta that receive and store wastes from the bitumen extraction process contain large amounts of recalcitrant and toxic organics. The current total volume of the fine tailings has exceeded 700 $m^3$, and this volume keeps increasing as mining operations proceed because each cubic meter of mined oil sands uses 3 $m^3$ of water. One of the problems is the very slow settlement of fine clay particles in oil sands tailings ponds. The fine clay particles that stay in suspension in tailings ponds are called mature fine tailings (MFT). It has been estimated that a serviceable densification of the fine clay would need over a hundred years (Eckert *et al.,* 1996). Technologies being applied to solve the tailings densification problem include physical mechanical processes, chemical amendments, natural processes such as freeze-thaw, and in situ biological treatments (Powter, 2010). Management of MFT has lately turned toward biological treatment technology. Researchers are interested to find whether microorganisms endogenous in MFT can be utilized to aid tailings densification.

Foght *et al*. (1985) detected both aerobic and anaerobic microbes present in Syncrude Canada's MLSB. Later investigations discovered the presence of SRB, methanogens, and iron reducing bacteria in MFT, and noted that the endogenous microorganisms were able to adapt to the toxic and harsh environments in the

tailings ponds. However, the degradation activities of the existing microorganisms was very slow and had not been seriously noticed until the early 1990s when gas bubbles were observed at the surface of MLSB tailings ponds. Holowenko *et al*. (2000) reported that 60–80% of the observed gas flux to the atmosphere across the surface area of the MLSB was methane. It has been estimated that a daily flux of 12 g $CH_4$ $m^{-2}$ was produced in a single pond, evidence of a very active methanogenesis in the pond.

Several studies investigated the relationship between methanogenesis and MFT densification. Fedorak *et al*. (2003) demonstrated that the MFT densification rate could be significantly accelerated due to the $CH_4$ gas produced by methane-producing archea (methanogens). Guo (2009) studied the influence of microbial activity on rapid MFT densification and found that water drainage from MFT increased with an increase in microbial activity and $CH_4$ gas generation. There was a clear relationship between the intense microbial activity zone and the rapid tailings densification zone.

In some experiments tailings were mixed with gypsum ($CaSO_4 \cdot 2H_2O$) to yield "consolidated" or "composite" tailings (CT) that would accelerate the densification rate of the solids. The addition of gypsum resulted in a high concentration of sulfate (1,300–8,000 mg $L^{-1}$) in the released water. Such a high concentration of sulfate can promote SRB growth and inhibit methanogenesis in MFT. Several studies have been conducted to demonstrate the inhibition of

methanogenesis by sulfate.

Holowenko *et al*. (2000) profiled the MPN of SRB and methanogens as a function of depth in a tailings pond. They found that MPN values of SRB were higher than those of methanogens when sulfate consumption was occurring, and that MPN values of SRB were lower than those of methanogens when sulfate was depleted. Salloum *et al*. (2002) also found that the addition of sulfate inhibited methanogenesis. In $SO_4^{2-}$ amended MFT, there was a decrease in the sulfate concentration of the pore water, and no $CH_4$ was detected in the headspace. Ramos-Padron *et al*. (2011) also showed that sulfate inhibited methanogenesis, and updated the assessment of microbial communities in relation to their activities as a function of depth in a tailings pond. The results indicated that at depths where sulfate reducers were high, there were fewer methanogens detected. Moreover, a high sulfate reduction rate was accompanied by a low methanogenesis rate.

Up to now, most studies have focused on the sulfate inhibition of methanogenesis, but researchers need to investigate the effects of sulfate on SRB community structure and in situ activities in fine tailings.

## References

Alleman, J. E., Veil, J. A. and Canaday, J. T. 1982. Scanning electron-microscope evaluation of rotating biological contactor biofilm. Water Research. **16**(5): 543-550.

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science. **7**(2): 123-138.

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: II. A review of emerging technologies. Journal of Environmental Engineering and Science. **7**(5): 499-524.

Bordenave, S., Kostenko, V., Dutkoski, M., Grigoryan, A., Martinuzzi, R. J. and Voordouw, G. 2010. Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings. Chemosphere. **81**(5): 663-668.

Brindle, K. and Stephenson, T. 1996. The application of membrane biological reactors for the treatment of wastewaters. Biotechnology and Bioengineering. **49**(6): 601-610.

Brindle, K., Stephenson, T. and Semmens, M. J. 1999. Pilot-plant treatment of a high-strength brewery wastewater using a membrane-aeration bioreactor. Water Environment Research. **71**(6): 1197-1204.

Chalaturnyk, R. J., Scott, J. D. and Ozum, B. 2002. Management of oil sands tailings. Petroleum Science and Technology. **20**(9-10): 1025-1046.

Choi, J., Hwang, G., Gamal El-Din, M. and Liu, Y. 2014. Effect of reactor configuration and microbial characteristics on biofilm reactors for oil sands process-affected water treatment. International Biodeterioration and Biodegradation. **89**:74-81.

Cole, A. C., Semmens, M. J. and LaPara, T. M. 2004. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. Applied and Environmental Microbiology. **70**(4): 1982-1989.

Cole, A. C., Shanahan, J. W., Semmens, M. J. and LaPara, T. M. 2002. Preliminary studies on the microbial community structure of membrane-aerated biofilms treating municipal wastewater. Desalination. **146**(1-3): 421-426.

Cote, P., Bersillon, J. L., Huyard, A. and Faup, G. 1988. Bubble-free aeration using membranes - process analysis. Journal Water Pollution Control Federation. **60**(11): 1986-1992.

de la Rosa, C. and Yu, T. 2006. Development of an automation system to evaluate the three-dimensional oxygen distribution in wastewater biofilms using microsensors. Sensors and Actuators B-Chemical. **113**(1): 47-54.

deBeer, D., Schramm, A., Santegoeds, C. M. and Kuhl, M. 1997. A nitrite microsensor for profiling environmental biofilms. Applied and Environmental Microbiology. **63**(3): 973-977.

Debeer, D., Stoodley, P., Roe, F. and Lewandowski, Z. 1994. Effects of Biofilm Structures on Oxygen Distribution and Mass-Transport. Biotechnology and Bioengineering. **43**(11): 1131-1138.

Eckert, W. F., Masliyah, J. H., Gray, M. R. and Fedorak, P. M. 1996. Prediction of sedimentation and consolidation of fine tails. AIChE Journal. **42**(4): 960-972.

Essila, N. J., Semmens, M. J. and Voller, V. R. 2000. Modeling biofilms on gas-permeable supports: Concentration and activity profiles. Journal of Environmental Engineering-ASCE. **126**(3): 250-257.

Fedorak, P. M., Coy, D. L., Dudas, M. J., Simpson, M. J., Renneberg, A. J. and MacKinnon, M. D. 2003. Microbially-mediated fugitive gas production from oil sands tailings and increased tailings densification rates. Journal of Environmental Engineering and Science. **2**(3): 199-211.

Foght, J. M., Fedorak, P. M. and Westlake, D. W. S. 1985. Microbial content and metabolic activities in the Syncrude tailings pond, Aostra Journal of Research. **1**:139-146.

Golby, S., Ceri, H., Gieg, L. M., Chatterjee, I., Marques, L. L. R. and Turner, R. J. 2011. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. FEMS Microbiology Ecology. **79**(1): 240-250.

Guo, C. 2009. Rapid densification of the oil sands mature fine tailings (MFT) by microbial activity. PhD Thesis. University of Alberta. Edmonton, Canada.

Hall-Stoodley, L., Costerton, J. W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology. **2**(2): 95-108.

Herman, D. C., Fedorak, P. M., Mackinnon, M. D. and Costerton, J. W. 1994. Biodegradation of naphthenic acids by microbial-populations indigenous to oil sands tailings. Canadian Journal of Microbiology. **40**(6): 467-477.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology. **46**(10): 927-937.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research. **36**(11): 2843-2855.

Ibrahim, H. T., He, Q., Al-Rekabi, W. S. and Yang, Q. 2012. Improvements in biofilm processes for wastewater treatment. Pakistan Journal of Nutrition. **11**(8): 610-636.

Islam, S., Dong, T., Sheng, Z., Zhang, Y., Liu, Y. and Gamal El-Din, M. 2014. Microbial community structure and operational performance of a fluidized bed biofilm reactor treating oil sands process-affected water. International Biodeterioration & Biodegradation. **91**: 111–118.

Ito, T., Okabe, S., Satoh, H. and Watanabe, Y. 2002. Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. Applied and Environmental Microbiology. **68**(3): 1392.

Kuhl, M. and Jorgensen, B. B. 1992. Microsensor measurement of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. Applied and Environmental Microbiology. **58**(4): 1164-1174.

Li, C. 2010. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification. Master Thesis. University of Alberta. Edmonton. Canada.

Okabe, S., Itoh, T., Satoh, H. and Watanabe, Y. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Applied and Environmental Microbiology. **65**(11): 5107-5116.

Okabe, S. and Watanabe, Y. 2000. Structure and function of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **42**(12): 21-32.

Penner, T. J. and Foght, J. M. 2010. Mature fine tailings from oil sands processing harbour diverse methanogenic communities. Canadian Journal of Microbiology. **56**(6): 459-470.

Powter, C. B., Biggar, K.W., Silva, M.J., McKenna, G.T and Scordo, E.B. 2010. Review of oil sands tailings technology options. The 14th International Conference on Tailings and Mine Waste: 381-391.

Quagraine, E. K., Peterson, H. G. and Headley, J. V. 2005. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances and Environmental Engineering. **40**(3): 685-722.

Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I. M., Dong, X., Sensen, C. W., Fournier, J., Voordouw, G. and Gieg, L. M. 2011. Carbon and sulfur

cycling by microbial communities in a gypsum-treated oil sands tailings pond. Environmental Science & Technology. **45**(2): 439-446.

Ramsing, N. B., Kuhl, M. and Jorgensen, B. B. 1993. Distribution of sulfate-reducing bacteria, $O_2$, and $H_2S$ in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Applied and Environmental Microbiology. **59**(11): 3840-3849.

Revsbech, N. P. and Jorgensen, B. B. 1986. Microelectrodes - their use in microbial ecology. Advances in Microbial Ecology. **9**: 293-352.

Salloum, M. J., Dudas, M. J. and Fedorak, P. M. 2002. Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Management and Research. **20**(2): 162-171.

Santegoeds, C. M., Damgaard, L. R., Hesselink, C., Zopfi, J., Lens, P., Muyzer, G. and de Beer, D. 1999. Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. Applied and Environmental Microbiology. **65**(10): 4618-4629.

Satoh, H., Ono, H., Rulin, B., Kamo, J., Okabe, S. and Fukushi, K. I. 2004. Macroscale and microscale analyses of nitrification and denitrification in biofilms attached on membrane aerated biofilm reactors. Water Research. **38**(6): 1633-1641.

Schramm, A., De Beer, D., Gieseke, A. and Amann, R. 2000. Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. Environmental Microbiology. **2**(6): 680-686.

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S. and Amann, R. 1999. Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by *in situ* hybridization and the use of microsensors. Applied and Environmental Microbiology. **65**(8): 3690-3696.

Semmens, M. J., Dahm, K., Shanahan, J. and Christianson, A. 2003. COD and nitrogen removal by biofilms growing on gas permeable membranes. Water Research. **37**(18): 4343-4350.

Siddique, T., Penner, T., Semple, K. and Foght, J. M. 2010. Anaerobic biodegradation of longer-chain n-alkanes coupled to methane production in oil sands tailings. Environmental Science & Technology. **45**(13): 5892-5899.

Syron, E. and Casey, E. 2008. Membrane-aerated biofilms for high rate biotreatment: Performance appraisal, engineering principles, scale-up, and development requirements. Environmental Science & Technology. **42**(6): 1833-1844.

Tan, S. Y. 2012. Multiple microbial processes in membrane aerated biofilms studied using microsensors. PhD Thesis. University of Alberta. Edmonton. Canada.

Tan, S. Y. and Yu, T. 2007. Fabrication of an amperometric $H_2S$ microsensor and its application in wastewater biofilms. Proceedings of International Workshop on Monitoring and Sensors for Water Pollution Control, Beijing, China.

Terada, A., Hibiya, K., Nagai, J., Tsuneda, S. and Hirata, A. 2003. Nitrogen removal characteristics and biofilm analysis of a membrane-aerated biofilm reactor applicable to high-strength nitrogenous wastewater treatment. Journal of Bioscience and Bioengineering. **95**(2): 170-178.

Timberlake, D. L., Strand, S. E. and Williamson, K. J. 1988. Combined aerobic heterotrophic oxidation, nitrification and denitrification in a permeable support biofilm. Water Research. **22**(12): 1513-1517.

Yamagiwa, K., Ohkawa, A. and Hirasa, O. 1994. Simultaneous organic-carbon removal and nitrification by biofilm formed on oxygen enrichment memrane. . Journal of Chemical Engineering of Japan. **27**(5): 638-643.

Yu, T. 2000. Stratification of microbial process and redox potential changes in biofilm. PhD Thesis. University of Cincinnati. Cincinnati, USA.

Yu, T. and Bishop, P. L. 2001. Stratification and oxidation-reduction potential change in an aerobic and sulfate-reducing biofilm studied using microelectrodes. Water Environment Research. **73**(3): 368-373.

Zhang, T. C. and Bishop, P. L. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. Water Environment Research. **68**(7): 1107-1115.

# Chapter 3 Research Tools

Qualitative and quantitative analyses of bacteria are essential to the study of microbial communities in complex environmental samples. Different techniques have been used to study the presence, activity, and diversity of microorganisms. The presence and morphology of microbes can be determined by traditional cultivation methods. PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) can provide information about the diversity of microorganisms and allows for the possibility of a more detailed phylogenetic analysis by sequencing of the gel bands. FISH (fluorescence in situ hybridization) makes it possible to identify the abundance of particular species or taxonomic groups using specific probes, and microbial structures can be visualized when FISH is coupled with confocal laser scanning microscopy (CLSM). Quantitative real-time PCR is a highly sensitive technique to amplify and simultaneously quantify targeted DNA. Microsensors can measure the chemical gradients used to indicate microbial activities. These methods and their applications in complex microbial environments are reviewed in this chapter. Studies that illuminate the composition, distribution, and population dynamics of microbial communities and their response to environmental influences are included in this review.

### 3.1 Molecular Biology Techniques

Standard culture techniques to identify microorganisms involve isolation of individual pure cultures using growth media such as nutrient agar (Kirk *et al.,* 2004). Traditional culture-based techniques are widely used to provide information about the diversity of microbial communities. A major limitation of standard culture techniques is that over than 99% of microorganisms can not been isolated or are not cultivable because the artificial growth media is not sufficiently representative of the natural growth environment of microbes (Hugenholtz, 2002). Moreover, the complex syntrophic and symbiotic relationships among microorganisms in nature cannot be reflected by individual culture methods (Wagner *et al*., 1993). The drawbacks of the traditional culture techniques make it difficult to get a full picture of the composition and diversity of a microbial community in a complex microbial environment.

Molecular biology, a field developed during the 1990s, deals with nucleic acids—deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Molecular biology methods can overcome the drawbacks of traditional culture based techniques. Molecular biology rapidly advanced in the 2000s, initiating a new era in the study of microbial ecology.

### 3.1.1 PCR-DGGE

*Principles of PCR.* PCR is a method of amplifying DNA extracted from environmental microbe samples. The extracted DNA is used as a template and is denatured, annealed, and extended (Waters and Shapter, 2013). In the denaturing step, the double-stranded DNA template is heated at 94–98 °C which breaks the hydrogen bonds between complementary bases and yields single strands of DNA. In the annealing step, the temperature of reaction is lowered to 50–65 °C allowing primers (short DNA sequences) to bind to the single-stranded DNA. In the extension step, new double stranded DNA is formed by the complementary addition of deoxyribonucleotide triphosphate (dNTPs) to the template, a reaction which is catalyzed by the enzyme taq polymerase. The denaturing, annealing, and extending steps are repeated in a number of cycles to increase the yield of the DNA fragments. PCR amplification will produce DNA fragments of the same length but different sequences.

*Principles of DGGE.* DGGE methods are employed to separate amplified DNA (or RNA) fragments that are identical in length but differ in nucleotide sequence (Muyzer *et al*., 1993). Separation of DNA fragments is accomplished by applying an electric current to move the DNA fragments across an acrylamide gel containing a linearly increasing gradient of DNA denaturing agents (usually urea and formamide), a process called electrophoresis. In general, the melting of double stranded DNA depends on the GC content (guanine plus cytosine) in the

DNA fragment; the higher the GC content in the DNA fragment, the more stable it is to the denaturants contained in the gel; that is, DNA fragments with high GC content will not melt until they reach the higher denaturant concentrations in the gel. As a result of this melting behaviour, DNA fragments with the same length but variable sequence will stop migrating at different denaturing gradients; in this way, fragments of variable sequence can be separated from each other. Ideally, each band on the gel corresponds to one specific species. After subsequent amplification and sequencing, bands generated by electrophoresis can provide an overview of the diversity of a microbial community. Moreover, the relative abundance of microorganisms can be estimated based on the relative band intensity compared to the intensity of all bands in the same lane.

*Applications.* Although there is limited information about the activity of the detected species, and the detection sensitivity is sometimes limited due to PCR artifacts, PCR-DGGE is the method of choice to investigate the community structure in complex microbial environments. Muyzer *et al*. (1993) demonstrated the presence of different bacterial species constituting the populations in complex microbial environments, including microbial mats and wastewater biofilms, based on the PCR-DGGE technique. Nakatsu (2007) reviewed the application of PCR-DGGE in analyzing complex soil microbial communities. PCR-DGGE has also been applied in oilfield systems where the vast range of substrates and metabolites harbour a large number of complex microbial communities (Li, 2010).

16S rRNA gene based DGGE can also be applied to profile the genes that encode enzymes that catalyze specific metabolic activities in a microbial community. Such functional gene based DGGE can be applied to investigate the diversity of specific microorganisms in a microbial environment. For example, Nicolaisen and Ramsing (2002) investigated the diversity of ammonia-oxidizing bacteria (AOB) with DGGE of PCR amplicons of the ammonia monooxygenase gene (amoA). Geets *et al*. (2006) assessed the diversity of dsrB (dissimilatory sulfite reductase beta-subunit) genes to study the diversity of sulfate-reducing communities. Throback *et al*. (2004) surveyed the diversity of denitrifying community composition in environmental samples with PCR primers that targeted the dissimilatory nitrite reductase genes nirS and nirK, and the nitrous oxide reductase gene nosZ.

The PCR-DGGE technique is also suitable for monitoring microbial community changes over time or under different environmental conditions. Ferris *et al*. (1997) monitored the changes in microbial populations within a hot-springs microbial mat under different hydraulic conditions with PCR-DGGE. Ito *et al*. (2002) studied the time-dependent development of sulfate reducing bacteria (SRB) in wastewater biofilms at different stages within 8 weeks. The microbial community changes were observed based on the DGGE bands. Park *et al.* (2008) investigated nitrifying bacterial communities in an aerobic biofilm reactor under different temperature conditions with PCR-DGGE. The results demonstrated that

biomass and activity of nitrifying bacteria decreased at low temperature. Park *et al.* (2008) assessed the relationship between dissolved oxygen (DO) concentration and the characteristics of nitrifying bacterial communities in an aerobic biofilm reactor using the PCR-DGGE technique. Results indicated that there were slight changes in the AOB community and in the abundance of *Nitrosomonas sp*. in the reactor.

### 3.1.2 FISH

*Principles of FISH.* Short sequences of DNA (usually between 15 and 30 nucleotides) labeled with fluorescent dye target and hybridize with 16S rRNA sequences in fixed cells (Amann *et al.,* 2001). Four steps are involved in FISH protocol: fixation, hybridization, washing, and microscopic detection of the labeled cells (Moter and Gobel, 2000). In the fixation step, bacteria are fixed in a 4% (v/v) formaldehyde solution and permeabilized for penetration of the fluorescent probes into the cells. After fixation, hybridization is carried out at temperatures between 37 °C and 50 °C to anneal the probes to target sequences. Unbound probe is washed away with distilled water. Labeled cells are detected with CLSM, a technique that obtains high-resolution optical images with depth selectivity, allowing three-dimensional reconstruction of optical sectioned in-focus images from selected depths.

*Applications.* A large number of sequences of different microorganisms are stored in databases; as a result, specific bacteria can be identified and the spatial organization of a complex microbial community can be analyzed. The FISH technique has been widely applied to identify the abundance of specific microorganisms in complex microbial environments. The in situ identification of microorganisms in biofilm communities has been reviewed by Aoi (2002), and in Daims *et al*. (2001)'s study, 16S rRNA oligonucleotide probes for the phylum and genus *Nitrospira* were developed to investigate its architecture in biofilm. Results indicated that *Nitrospira* aggregates in the biofilm samples had a complex morphology with internal channels. Egli *et al*. (2001) used 16S rDNA sequencing and FISH to characterize enriched anammox bacteria in a rotating biological contactor (RBC) treating wastewater. In addition to the characterization of specific microorganisms in other complex microbial environments such as an oil field reservoir (Voordouw *et al.,* 1996) and a marine sediment (Llobet-Brossa *et al.,* 1998), the FISH technique was applied to study the spatial distribution and quantification of specific microorganisms within a biofilm environment. Aoi *et al*. (2000) monitored the spatial distribution of AOB and heterotrophic bacteria in wastewater biofilm under different conditions of substrate using FISH. Results indicated that when the biofilm was exposed to a feed substrate containing a high ratio of organic carbon to ammonium, heterotrophic bacteria occupied the outer part while AOB were distributed in the inner part of the biofilm. As the organic

carbon to ammonium ratio decreased, AOB were observed to colonize the outer part of the biofilm.

### 3.1.3 qPCR

*Principles of qPCR.* Real-time quantitative PCR is based on the PCR with a fluorescent signal continuously measured after each amplification cycle; the level of fluorescence in the signal corresponds to the cumulative amount of target amplicons, and enables real-time monitoring of an increase in amplicon concentration (Heid *et al*., 1996). To quantify the unknown amount of amplicons in samples, a standard curve is created using a 10-fold serial dilution series of a template with a known concentration of genomic DNA from pure-culture strains or PCR fragments of the target gene.

*Applications.* Pellicer-Nacher *et al*. (2010) used real-time qPCR to demonstrate the abundance of aerobic and anaerobic ammonium oxidizing bacteria (AOB and anAOB, respectively) performing high rate autotrophic nitrogen removal in the sequential aeration of membrane-aerated biofilm reactors. Tsushima *et al*. (2007) developed real-time qPCR to quantitate an enriched culture of anammox bacteria from an RBC biofilm and plotted the copy numbers of 16S rRNA genes of anammox bacteria against nitrogen removal rates. A linear relationship between the 16S rRNA gene abundance of anammox bacteria and the nitrogen removal rate was observed.

### 3.2 Microsensors

Microsensors can determine in situ bacterial activity by obtaining chemical profiles with high spatial resolution (Santegoeds *et al.,* 1998). The tips of microsensors are usually small enough to allow for nondestructive measurements.

Electrochemical microsensors have been developed to measure different kinds of chemical species. For example, amperometric microsensor electrodes have been fabricated to measure oxygen (Revsbech, 1989), hydrogen sulfide (Jeroschewski *et al.,* 1996), hydrogen (Ebert and Brune, 1997), and nitrous oxide (Revsbech *et al.,* 1988). Potentiometric microsensors have been developed to measure pH (Kohls *et al.,* 1997), ammonium (Debeer and Vandenheuvel, 1988), nitrate (Debeer and Sweerts, 1989), nitrite (deBeer *et al*., 1997), and sulfide (Revsbech *et al.,* 1983).

Theory, fabrication procedure, and performance all differ among different types of microsensor. For instance, a potentiometric microsensor measures membrane potential as a function of ion concentration (Lewandowski and Beyenal, 2007). The tip of a potentiometric microsensor can be as small as several micrometers, and the lifetime of this type of microsensor is usually short (1–5 days) (Revsbech, 2005). In an amperometric microsensor the limiting current is measured which is linearly proportional to the partial pressure when oxygen or $H_2S$ crosses the membrane (Jeroschewski *et al*., 1996; Lu and Yu, 2002). The tip

of an amperometric microsensor can be 10–100 μm depending on many factors including experimental requirements and fabrication skills. Oxygen and $H_2S$ microsensors have lifetimes of up to 6 months. Tan (2012) and Yu (2000) provide detailed fabrication procedures for microsensors.

The application of microsensors to the microbial ecology field was introduced in 1983 (Revsbech and Jørgensen, 1983); since then, microsensors have gained attention because of their high spatial resolution and their ability to simultaneously measure several microbial processes. Microsensor techniques have been applied in sediments, microbial mats, and biofilms to study the microbial activities in these complex environments, as reviewed in Chapter 2.

### 3.3 Combination of Microsensors and Molecular Biology Techniques

A combination of microsensor and molecular biology techniques can provide a comprehensive picture of the diversity and activity of microorganisms in environmental samples. For example, microsensors and FISH were combined to study the microbial activity and spatial distribution of nitrifiers (Schramm *et al*., 1996; Schramm *et al*., 1998; Okabe *et al*., 1999; Schramm *et al*., 1999; Schramm *et al*., 2000; Okabe *et al*., 2002; Kindaichi *et al*., 2004), and SRB (Ramsing *et al.,* 1993; Okabe *et al.,* 1999) in wastewater biofilms. Theoretically, all the techniques described here could be combined to obtain a comprehensive understanding of the in situ activity and ecophysiology of microbial communities, depending on the

samples to be investigated and results expected. For example, Kindaichi *et al*. (2006) analyzed the in situ activity and population dynamics of AOB and nitrite oxidizing bacteria (NOB) dominant in nitrifying biofilm from a submerged RBC using real-time qPCR. Time-dependent development of the autotrophic nitrifying biofilm was visualized by FISH. In addition, the consumption rate of $NH_4^+$ and $NO_2^-$, based on the concentration profiles of $O_2$, $NH_4^+$, and $NO_2^-$ determined by microsensors, explained and confirmed the stratified spatial distribution of AOB and *Nitrospira-like* NOB in the biofilm. Moreover, the real-time qPCR technique was applied to quantify the cell numbers of AOB and NOB. Results based on the culture-independent molecular techniques and results based on microsensor measurements corresponded well.

In this study, combined technologies of microsensor measurements and molecular biology analyses were applied to investigate the community structure and microbial activity of SRB in three complex microbial environments (membrane aerated biofilm reactor (MABR), an OSPW biofilm reactor, and MFT settling columns). $H_2S$, $O_2$, ORP, and pH microsensors were applied in the research. The measured $H_2S$ gradient along the depth of biofilms and tailings was used to indicate the in situ metabolic products of sulfate reduction, while $O_2$, ORP, and pH gradients implied the living environment of SRB. The dsrB gene based DGGE fingerprint was used to show the functional diversity of SRB in both biofilm and tailings environments. Quantification techniques of FISH and qPCR

were applied to investigate the vertical distribution of SRB along the depth of biofilms and tailings, respectively.

## References

Amann, R., Fuchs, B. M. and Behrens, S. 2001. The identification of microorganisms by fluorescence in situ hybridisation. Current Opinion in Biotechnology. **12**(3): 231-236.

Aoi, Y. 2002. In situ identification of microorganisms in biofilm communities. Journal of Bioscience and Bioengineering. **94**(6): 552-556.

Aoi, Y., Miyoshi, T., Okamoto, T., Tsuneda, S., Hirata, A., Kitayama, A. and Nagamune, T. 2000. Microbial ecology of nitrifying bacteria in wastewater treatment process examined by fluorescence in situ hybridization. Journal of Bioscience and Bioengineering. **90**(3): 234-240.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. and Wagner, M. 2001. In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. Applied and Environmental Microbiology. **67**(11): 5273-5284.

deBeer, D., Schramm, A., Santegoeds, C. M. and Kuhl, M. 1997. A nitrite microsensor for profiling environmental biofilms. Applied and Environmental Microbiology. **63**(3): 973-977.

Debeer, D. and Sweerts, J. P. R. 1989. Measurement of nitrate gradients with an ion-selective microelectrode. Analytica Chimica Acta. **219**(2): 351-356.

Debeer, D. and Vandenheuvel, J. C. 1988. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin. Talanta. **35**(9): 728-730.

Ebert, A. and Brune, A. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite Reticulitermes flavipes (Kollar). Applied and Environmental Microbiology. **63**(10): 4039-4046.

Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., van der Meer, J. R. and Zehnder, A. J. B. 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Archives of Microbiology. **175**(3): 198-207.

Ferris, M. J., Nold, S. C., Revsbech, N. P. and Ward, D. M. 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. Applied and Environmental Microbiology. **63**(4): 1367-1374.

Geets, J., Borrernans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D. and Vanbroekhoven, K. 2006. DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. Journal of Microbiological Methods. **66**(2): 194-205.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. 1996. Real time quantitative PCR. Genome Research. **6**(10): 986-994.

Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. Genome Biology. **3**(2).

Ito, T., Okabe, S., Satoh, H. and Watanabe, Y. 2002. Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. Applied and Environmental Microbiology. **68**(3): 1392-1402.

Jeroschewski, P., Steuckart, C. and Kuhl, M. 1996. An amperometric microsensor for the determination of $H_2S$ in aquatic environments. Analytical Chemistry. **68**(24): 4351-4357.

Kindaichi, T., Kawano, Y., Ito, T., Satoh, H. and Okabe, S. 2006. Population dynamics and in situ kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. Biotechnology and Bioengineering. **94**(6): 1111-1121.

Kindaichi, T., Okabe, S., Satoh, H. and Watanabe, Y. 2004. Effects of hydroxylamine on microbial community structure and function of autotrophic nitrifying biofilms determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **49**(11-12): 61-68.

Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Khironomos, J. N., Lee, H. and Trevors, J. T. 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods. **58**(2): 169-188.

Kohls, O., Klimant, I., Holst, G. and Kuhl, M. 1997. Development and comparision of pH microelectrodes for use in marine systems. Micro- and Nanofabricated Electro-Optical Mechanical Systems for Biomedical and Environmental Applications. **2978**: 82-91.

Li, C. 2010. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification. Master Thesis. University of Alberta. Edmonton, Canada.

Llobet-Brossa, E., Rossello-Mora, R. and Amann, R. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Applied and Environmental Microbiology. **64**(7): 2691-2696.

Lu, R. and Yu, T. 2002. Fabrication and evaluation of an oxygen microelectrode applicable to environmental engineering and science. Journal of Environmental Engineering and Science. **1**(3): 225-235.

Moter, A. and Gobel, U. B. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. Journal of Microbiological Methods. **41**(2): 85-112.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Applied and Environmental Microbiology. **59**(3): 695-700.

Nakatsu, C. H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. Soil Science Society of America Journal. **71**(2): 562-571.

Nicolaisen, M. H. and Ramsing, N. B. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. Journal of Microbiological Methods. **50**(2): 189-203.

Okabe, S., Itoh, T., Satoh, H. and Watanabe, Y. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Applied and Environmental Microbiology. **65**(11): 5107-5116.

Okabe, S., Naitoh, H., Satoh, H. and Watanabe, Y. 2002. Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes. Water Science and Technology. **46**(1-2): 233-241.

Okabe, S., Satoh, H. and Watanabe, Y. 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **65**(7): 3182-3191.

Park, J. J., Byun, I. G., Park, S. R. and Park, T. J. 2008. Nitrifying bacterial communities and its activities in aerobic biofilm reactors under different temperature conditions. Korean Journal of Chemical Engineering. **25**(6): 1448-1455.

Park, J. J., Byun, I. G., Yu, J. C., Park, S. R., Ju, D. J., Hur, S. H. and Park, T. J. 2008. Analysis of nitrifying bacterial communities in aerobic biofilm reactors with different DO conditions using molecular techniques. Water Science and Technology. **57**(12): 1889-1899.

Pellicer-Nacher, C., Sun, S. P., Lackner, S., Terada, A., Schreiber, F., Zhou, Q. and Smets, B. F. 2010. Sequential aeration of membrane-aerated biofilm reactors for high-rate autotrophic nitrogen removal: experimental demonstration. Environmental Science & Technology. **44**(19): 7628-7634.

Ramsing, N. B., Kuhl, M. and Jorgensen, B. B. 1993. Distribution of sulfate-reducing bacteria, $O_2$, and $H_2S$ in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Applied and Environmental Microbiology. **59**(11): 3840-3849.

Revsbech, N. P. 1989. An oxygen microsensor with a guard cathode. Limnology and Oceanography. **34**(2): 474-478.

Revsbech, N. P., Jorgensen, B. B., Blackburn, T. H. and Cohen, Y. 1983. Microelectrode studies of the photosynthesis and $O_2$, $H_2S$, and pH profiles of a microbial mat. Limnology and Oceanography. **28**(6): 1062-1074.

Revsbech, N. P., Nielsen, L. P., Christensen, P. B. and Sorensen, J. 1988. Combined oxygen and nitrous-oxide microsensor for denitrification studies. Applied and Environmental Microbiology. **54**(9): 2245-2249.

Santegoeds, C. M., Ferdelman, T. G., Muyzer, G. and de Beer, D. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Applied and Environmental Microbiology. **64**(10): 3731-3739.

Schramm, A., De Beer, D., Gieseke, A. and Amann, R. 2000. Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. Environmental Microbiology. **2**(6): 680-686.

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S. and Amann, R. 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira spp.* along a macroscale gradient in a nitrifying bioreactor: quantification by *in situ* hybridization and the use of microsensors. Applied and Environmental Microbiology. **65**(8): 3690-3696.

Schramm, A., de Beer, D., Wagner, M. and Amann, R. 1998. Identification and activities *in situ* of *Nitrosospira* and *Nitrospira spp.* as dominant populations in a nitrifying fluidized bed reactor. Applied and Environmental Microbiology. **64**(9): 3480-3485.

Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R. and Schleifer, K. H. 1996. Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **62**(12): 4641-4647.

Tan, S. Y. 2012. Multiple microbial processes in membrane aerated biofilms studied using microsensors. PhD Thesis. University of Alberta. Edmonton, Canada.

Throback, I. N., Enwall, K., Jarvis, A. and Hallin, S. 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology. **49**(3): 401-417.

Tsushima, I., Kindaichi, T. and Okabe, S. 2007. Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. Water Research. **41**(4): 785-794.

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y. and Gevertz, D. 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Applied and Environmental Microbiology. **62**(5): 1623-1629.

Waters, D. L. E. and Shapter, F. M. 2013. The Polymerase Chain Reaction (PCR): General Methods. Cereal Genomics: Methods and Protocols. R. J. Henry and A. Furtado. Totowa, Humana Press Inc: 65-75.

Yu, T. 2000. Stratification of microbial process and redox potential changes in biofilm. PhD Thesis. University of Cincinnati. Cincinnati, USA.

# Chapter 4 Community Structure and In Situ Activity of SRB in Municipal Wastewater Biofilm

## 4.1 Introduction

Physiochemical and ecological roles of complex microbial communities in wastewater biofilm depend on the availability of electron acceptors and other environmental and operational factors. SRB play important roles in the treatment of sulfate-enriched wastewater; sulfate can act as terminal electron acceptor utilized by SRB during dissimilatory sulfate reduction. For example, sulfate reduction might contribute up to 45% of carbon mineralization (Okabe *et al*., 2003). However, the presence of SRB could also be a concern due to the production of the highly reactive and corrosive $H_2S$, which has to be controlled in wastewater treatment systems (Bhagat *et al.,* 2004).

In conventional biofilm reactors, oxygen is usually not utilized efficiently, which might affect the treatment efficiency and increase the capital costs (Vanloosdrecht and Heijnen, 1993). Innovative technologies in wastewater treatment, such as the MABR, are overcoming these problems by demonstrating a high rate of carbon and nitrogen removal (Semmens *et al*., 2003). In an MABR, oxygen and nutrients are provided to the biofilm from opposite directions; in conventional wastewater biofilms, oxygen and nutrients are provided from the

same direction. As a result, a very different stratification in terms of substrate concentrations and bacterial activities could be established within the MAB compared to conventional wastewater biofilms. It was first reported in 1988 that nitrification, aerobic oxidation, and denitrification simultaneously occurred in a single piece of MAB (Timberlake *et al*., 1988). Since then, a number of studies and applications of MABRs in wastewater treatment have focused on organic carbon removal (Brindle *et al*., 1999), and simultaneous COD and nitrogen removal (Yamagiwa *et al*., 1994; Brindle *et al*., 1998; Semmens *et al*., 2003). These studies demonstrated that a high rate of carbon and nitrogen removal could be simultaneously achieved in a single piece of MAB.

The stratifications of nitrifiers and denitrifiers in terms of their activities and community structures within MAB have been studied (Cole *et al*., 2002; Terada *et al*., 2003; Cole *et al*., 2004; Satoh *et al*., 2004). In these studies, it was shown that aerobic ammonia-oxidizing bacteria (AOB) existed near the membrane side while denitrifiers preferred anaerobic environment near the surface of the biofilm.

Regarding the study of SRB in wastewater biofilms, several studies have demonstrated the presence of SRB and the occurrence of sulfate reduction in conventional aerobic wastewater biofilms (Kuhl and Jorgensen, 1992; Santegoeds *et al*., 1998; Okabe *et al*., 1999; Ito *et al*., 2002a; Ito *et al*., 2002b). For instance, Okabe *et al*. (1999) studied the activity and distribution of SRB in a conventional biofilm in a RBC, and found high sulfate-reducing activity present in a narrow

anaerobic zone located about 150 to 300 μm below the biofilm surface. However, MABs are quite different from conventional wastewater biofilms. As a result, it is expected that the activities and distribution of SRB in an MABR can be very different from those found in conventional biofilms.

To the authors' knowledge, a study of SRB in an MABR has been reported only recently by Tan *et al.* (2014). Simultaneous multiple microbial processes in a piece of MAB, including aerobic oxidation, nitrification, denitrification, and sulfate reduction, were observed based on a set of microsensor measurements of $O_2$, pH, ORP, $NH_4^+$, $NO_3^-$, and $H_2S$. However, this reported study did not investigate the presence, activity, and distribution of SRB in the biofilm. The present study was performed to evaluate the presence, the spatial distribution, and the *in situ* activity of SRB in the MAB using combined microsensors and molecular techniques. With the provided fundamental information of SRB in MAB, we could better evaluate how biofilm microorganisms interact with each other, and how the microbial activities within the biofilm can be optimized to improve reactor performance in practical applications.

Microsensors have been successfully used to determine *in situ* metabolic activities in microbial communities (Santegoeds *et al*., 1998; Okabe *et al*., 2003; Okabe *et al*., 2005; de la Rosa and Yu, 2006). Molecular techniques can be used to identify specific microbial populations without isolation (Amann and Kuhl, 1998; Sanz and Kochling, 2007), and have already been widely applied to

investigate the distribution of microbial communities and functional diversities in biofilms (Amann *et al*., 1992; Schramm *et al*., 1998; Xia *et al*., 2010). Molecular techniques have been successfully combined with microsensor measurements to study different metabolic processes in environmental biofilm samples (Ramsing *et al*., 1993; Schramm *et al*., 1996; Schramm *et al*., 1998; Schramm *et al*., 1999; Okabe *et al*., 1999b; Okabe and Watanabe, 2000; Okabe *et al*., 2002; Kindaichi *et al*., 2004).

In this study, the molecular techniques PCR-DGGE and FISH were combined with $H_2S$ and $O_2$ microsensor measurements to investigate the distribution of SRB and their activities in an $O_2$ based MAB. An amperometric $H_2S$ microsensor, which is superior to potentiometric sulfide microsenor because of its better performance such as more stable signal (Lewandowski and Beyenal, 2007), was employed in this study to determine $H_2S$ concentration and to predict the SRB activity within the MAB. Dissimilatory sulfite reductase subunit β (DsrB) gene based DGGE was used to study the functional diversity of SRB in the biofilm; SRB biomass distribution along the biofilm depth was investigated by *in situ* hybridization.

## 4.2 Materials and Methods

### 4.2.1 Biofilm Reactor Operation

An illustration of $O_2$ based MABR setup is shown in Figure 4-1. The reactor was constructed with acrylic board. The cover of the reactor had openings for microsensor measurements. During normal operation, the openings were blocked using rubber stoppers. The working volume of the reactor was about 0.9 L. The membrane module consisted of gas permeable dense silicone flat-sheet membranes (Model: SSP-M823, Specialty Silicone Products, Inc., USA). The reactor was inoculated with activated sludge collected from the anaerobic digester at Gold Bar Wastewater Treatment Plant in Edmonton. After start-up of the reactor, about 20 mL/min of pure $O_2$ flowed through the silicone membrane. Nitrogen gas was purged into the influent of synthetic wastewater to ensure the synthetic wastewater was $O_2$-free.

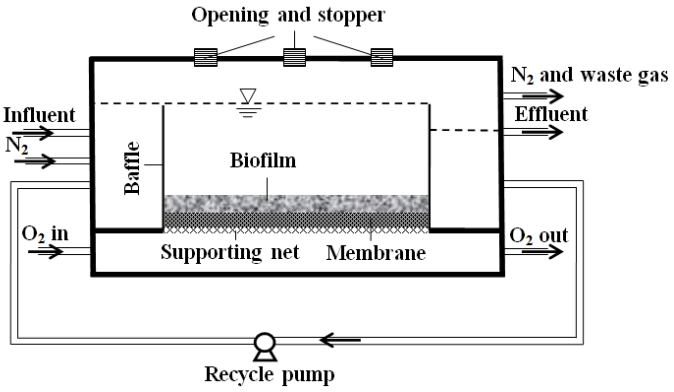



**Figure 4-1. Schematic Diagram of the Setup of the $O_2$-based MABR**

The synthetic wastewater contained 250 mg/L dextrose (COD), 5 mg/L $KH_2PO_4$, 20 mg/L $NH_4Cl$, 277.5 mg/L $Na_2SO_4$, 12.86 mg/L $MgCl_2 \cdot 6H_2O$, 2.57 mg/L $FeSO_4 \cdot 7H_2O$, 0.26 mg/L $CoCl_2 \cdot 6H_2O$, 0.77 mg/L $CaCl_2 \cdot 2H_2O$, 0.26 mg/L $CuSO_4 \cdot H_2O$, 0.26 mg/L $MnCl_2 \cdot 4H_2O$, 0.26 mg/L $ZnSO_4 \cdot 7H_2O$, and 1 mg/L yeast extract. The influent rate of synthetic wastewater was around 2.0 mL/min. The MABR was operated at 23 ± 1°C and pH 7.6 ± 0.2, with a recirculation rate of around 200 mL/min.

### 4.2.2 Standard Chemical Analysis

Bulk reactor conditions were monitored regularly. DO concentration, pH and oxidation-reduction potential (ORP) in the influent and effluent were daily measured using commercial electrodes and meters: an $O_2$ membrane electrode with a DO meter for DO measurements ($O_2$ electrode: model: Orion 97-08, Thermo Electron Corporation; model 50B, YSI Inc., USA); pH electrode with pH meter for pH measurements (pH electrode: Cat.# 13-620-108, Accumet, Fisher Scientic; pH meter: AR 15, Accumet, Fisher Scientic); an ORP electrode with a pH meter for ORP measurements (ORP electrode: Cat. 13-620-81, Accumet, Fisher Scientific; pH meter: pH meter 25, Fisher Scientific, CA), respectively. COD was analyzed using Digital Reactor Block 200 (model: DRB 200, Hatch, USA). Concentrations of $SO_4^{2-}$ in the influent and effluent were determined every one to two weeks using ion chromatography (ICS-2000, Dionex, CA). $SO_4^{2-}$

samples were filtered with 0.2 μm membrane filters and stored at 4 °C before analysis.

### 4.2.3 Microsensors and Microsensor Measurements

Combined amperometric $O_2$ microsenor with a tip diameter of about 15 μm was fabricated as described by Lu and Yu (2002). Combined amperometric $H_2S$ microsensor that was originally developed by Jeroschewski *et al*. (1996) was fabricated in this study with a tip diameter of approximately 25 μm, and the calibration curve was obtained by plotting a series dilution (0 to 500 μM) of $Na_2S$ versus current (Tan and Yu, 2007). A three-point calibration of the $O_2$ microsensor was made with $N_2$-saturated, air-saturated and pure $O_2$-saturated water.

When the MABR reached pseudo steady state, microsensor measurements of $O_2$ and $H_2S$ were made directly in the MABR under actual growth conditions. An illustration of microsensor measurement setup is shown in the following Figure 4-2.

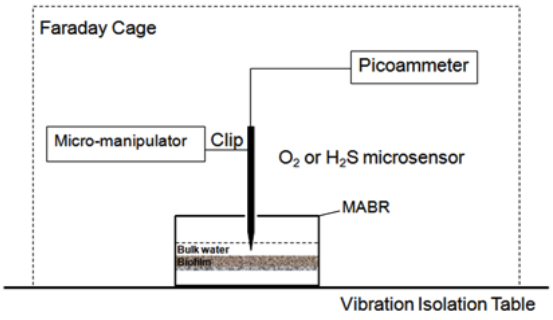



**Figure 4-2. An Illustration Diagram of Microsensor Measurement Setup**

Immediately before and after the measurements, each microsensor was calibrated with the procedure described in the above paragraph. During measurements, each microsensor was mounted on a micro-manipulator (Model M3301R, World Precision Instruments, Inc., Sarasota FL, USA). The microsensor tip was advanced into the biofilm from the openings in the cover of the reactor. When the tip touched biofilm surface, the microsensor penetrated the biofilm through the movement of the micromanipulator at intervals of 10 to 50 μm. The movement was viewed through a horizontal dissection microscope (Model: Stemi SV11, Carl Zeiss, Jena, Germany). An illuminator was used to enhance the view of movement area. A zero reading in the biofilm was the same as the reading in the $N_2$-saturated water for $O_2$ microsensor and in $H_2S$-free water for the $H_2S$ microsensor.

### 4.2.4 DNA Extraction, PCR Amplification and DGGE Analysis

DNA was extracted using the Power Soil Kit (Mo-Bio, Carlsbad, CA) following the protocol provided by the manufacture. Primer pairs of 341F with addition of a clamp on the 5' end (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') and 518R were used to amplify 16S rRNA gene fragments (Muyzer *et al*., 1993); in addition, primer pairs of AmoA-1F with addition of a GC clamp on the end of 5' (5'-CGC CGC GCG GCG GGC GGG GCG GGG GC-3') and AmoA-2R-TC targeting genes amoA (Nicolaisen and Ramsing, 2002), primers pairs of F1aCu and R3Cu with addition of a clamp

on the end of 5' (5'-GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC-3') targeting genes nirK (Throback *et al*., 2004), and primer pairs of DSR4R and DSR2060F with addition of a GC clamp on the 5' end (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG-3') (Geets *et al*., 2006) targeting genes dsrB were used to amplify gene fragments of ammonia oxidizing bacteria, denitrifiers and SRB, respectively. Amplification was performed in a 50 μL reaction system using an Authorized Thermal Cycler (Eppendorf®, Hamburg, Germany). The reaction mixture consisted of 32.5 μL water, 2 μL DNA template (approximately 35 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 1 μL of forward and 1 μL of reverse primer at 25 μM, 0.2 mM dNTP mix, 2.5% of DMSO per reaction, and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA). PCR programs for 16S rRNA gene amplification and functional genes amplification are listed in Table 4-1. Community patterns based on 16S rRNA, dsrB, nirK and amoA genes were visualized with DGGE (gels were 16 cm × 16 cm × 1 mm). The electrophoresis was performed in TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) at 60 °C. 16S rRNA gene was run on a 6.5% (w/v) polyacrylamide gel with a gradient of 30% to 60% (100% denaturants is a mixture of 7 M urea and 40% [v/v] formamide) at a constant voltage of 150 V for 8 h. DsrB gene was running on 7.5% (w/v) polyacrylamide gel with denaturing gradient from 30% to 60% at 100 V for 16 h. The nirK gene and amoA gene were run on a 7.5% (w/v) polyacrylamide gel with a denaturing gradient of 40% to 70% at 180 V for 6 h. Following electrophoresis, the gel was stained for 20 min with 5

μL ethdium bromide in 300 mL TAE buffer before being photographed by UV transillumination (Viber Lourmat, France).

**Table 4-1. PCR Program for 16S rRNA Gene and Functional Genes Amplification**

| Gene fragments | 16S rRNA gene | Functional genes amoA and dsrB | Functional genes nirK |
|---|---|---|---|
| PCR program | 94ºC, 7min<br>92ºC, 1min<br>54ºC, 1min } 35cycles<br>72ºC, 1 min<br>72ºC, 10 min | 94ºC, 5 min<br>94ºC, 45s<br>65ºC, 45s } 10 cycles<br>72ºC, 60s<br>65ºC decreased to 55ºC by 1°C every cycle.<br>94ºC, 45s<br>55ºC, 45s } 35 cycles<br>72ºC, 60s<br>72ºC, 7 min | 94ºC, 5 min<br>94ºC, 45s<br>58ºC, 30s } 10 cycles<br>72ºC, 45s<br>58ºC decreased to 48 ºC by 1°C every cycle.<br>94ºC, 45s<br>53ºC, 30s } 30 cycles<br>72ºC, 45s<br>72ºC, 7 min |

The bands were then excised from the gel and put into 50 μL Tris-HCl overnight to dissolve DNA from the gel to the solution. 2 μL of the solution was used as DNA template for subsequent amplification. PCR products were purified using the Exosap-IT clean-up kit (Amersham Biosciences, Buckinghamshire, England) and sequenced. Sequences were blasted against sequences in the nucleotide database (http://www.ncbi.nlm.nih.gov).

### 4.2.5 Sample Preparation and Cryosectioning

Biofilm sample pieces with surface areas of 5 mm × 5 mm were collected from the reactor and embedded in optimum cutting temperature (OCT) compound for at least one hour at -20 °C in order to make sure the OCT infiltrated into the biofilm. Embedded samples were placed into a microtome (Tissue-tek ® II, Cyrostat, Miles, California, USA) at -20 °C. Both vertical and horizontal sliced samples were prepared. The vertical sliced samples were subjected to *in situ* hybridization, and horizontal sliced layers were for the PCR-DGGE analysis.

Vertical sliced layers with 60 μm thickness were placed on the five wells of the six well-microscope slides, which were coated with gelatin by spreading 10 μL of 70°C heated 0.1% gelatin/0.01% chromium potassium sulfate dodecahydrate solution in each well. The slides were taken out from the microtome carefully, and air-dried overnight to make sure the sliced layers were well attached to the slides. Finally, the slides were successively dipped into 50 %, 70 % and 98 % ethanol solutions in 50 mL centrifuge tubes for dehydration, and then stored at room temperature.

Six layers horizontal to the membrane surface were sliced in the microtome. Horizontal slices were made from the substratum side (layer 1) to the biofilm/bulk water interface (layer 6); layers 1 to 5 were each 400 μm thick and layer 6 was 600 μm thick. Each sliced layer was kept in PowerBead Tubes provided in the

Power Soil DNA extraction kit (Mo-Bio, Carlsbad, CA), and total DNA extraction was processed immediately.

The samples that needed to be preserved were fixed in 4% (w/v) fresh paraformaldehyde at 4 °C for 1.5 h, then washed with phosphate buffered saline (PBS) three times, and stored in a 1:1 mixture of PBS and 96% ethanol at -20 °C (Amann *et al.,* 1990).

### 4.2.6 *In Situ* Hybridization

The sectioned biofilm slices attached to wells of microscope slides were subjected to the subsequent *in situ* hybridization and CLSM imaging. Sequences of the oligonucleotide probes used in this study are shown in Table 4-2. The final probe concentrations were 8 pmol/µL, 5 pmol/µL and 5 pmol/µL for EUB338, SRB385 and SRB385Db respectively. Hybridization buffer was composed of 5 M NaCl, 1 M Tris-HCl (pH 7.2), 10% sodium dodecyl sulfate, and 30% v/v of formamide. Hybridization was performed at 46°C for 1.5 h. The washing step was performed in 50 mL of a preheated (48°C for 15 min) solution composed of 5 M NaCl, 0.5 M EDTA, 20 mM Tris hydrochloride (pH 8) and 10% W/V sodium dodecyl sulfate. The slides were then rinsed briefly with milliQ water and air-dried. Finally, the slides were mounted with antifade reagents (Vectashield, Vector Laboratories, Burlingame, CA) and stored at -20 °C.

The FISH images were taken by CLSM on a Zeiss LSM 510 equipped with an Argon laser (488 nm) and a HeNe laser (543 nm and 633 nm). The instrument can collect 12 bit images with 4 detectors for fluorescent signals and a transmission detector for bright field images. The 2-D image was analyzed by Zeiss software. The 3-D images were processed with Imaris 6.3.1 (Bitplane, Swizerland). During optical scanning, a section of 0.8 μm thick was applied. Under a confocal microscope, all bacteria hybridized with probe EUB 338 that was labeled with 6-Fam showed green signals; while SRB, specifically hybridized with probes SRB385 and SRB 385Db that were labeled with Texas Red showed red signals. Excitation wavelengths of 6-Fam and Texas Red were 596 nm and 492 nm, respectively.

The volume of biomass was calculated using COMSTAT and briefly described by Heydorn *et al.* (2000). Total biomass was calculated based on the green signals with a threshold of 48, and biomass of SRB was calculated based on the red signals with a threshold of 180. The area examined using CLSM is 25375.5 $\mu m^2$, and the biomass calculated using COMSTAT in $\mu m^3/\mu m^2$ was then multiplied by this area and converted into volume in $\mu m^3$.

**Table 4-2. A List of 16S rRNA-Targeted Oligonucleotide Probes (FA*=30%)**

| Probe name | Target site[a] | Sequence 5'-3' | Specificity | References |
|---|---|---|---|---|
| EUB338 | 338-355 | GCTGCCTCCCGTAGGAGT | All eubacteria | (Amann *et al.,* 1990) |
| SRB385 | 385-402 | CGGCGTCGCTGCGTCAGG | SRB of the delta Proteobacteria; Several gram-positive bacteria (e.g., Clostridium spp.) | (Amann *et al*., 1992) |
| SRB385Db | 385-402 | CGGCGTTGCTGCGTCAGG | Desulfobacterales, Desulfuromonales, Syntrophobacterales, Myxococcales, other bacteria. | (Rabus *et al*., 1996) |

a Position in the 16S rRNA of *Escherichia coli* (Brosius *et al*., 1981)

* FA (%) Formamide concentration in the hybridization buffer

## 4.3 Results and Discussion

### 4.3.1 Reactor Performance

The biofilm reactor was run for over one year. The water quality of the reactor under steady state is shown in Table 4-3. In the bulk water, DO concentration was maintained at less than 0.85 mg/L, ORP was -300 ± 50 mV, and the pH value was 7.6 ± 0.2. It should be noted that DO in the bulk water was measured as less than 0.85 mg/L but not zero, which is because samples were taken from the outlet for immediate DO measurement with a commercial DO probe and a DO meter. During this sample transfer process and measurement, a small amount of oxygen in the air could potentially be dissoved in the sample solution.

**Table 4-3. Average Water Characteristics in the MABR**

| Water Characteristics | Influent | Effluent |
|---|---|---|
| DO (mg/L) | 8.6 | less than 0.85 |
| ORP (mV) | 350 | -250~-350 |
| Temp (°C) | 23 | 23 |
| pH | | 7.6 ± 0.2 |
| $SO_4^{2-}$ (mg/L) | 227 | 90 |

It is well known that the growth of SRB requires not only anaerobic conditions, but also a low reduction potential or a highly reduced environment to function efficiently (Odom and Singleton, 1993). In addition, although SRB can survive extreme pH conditions (Foti *et al.,* 2007), the optimum pH range is 5-9 (Jong and Parry, 2006). The average $SO_4^{2-}$ removal efficiency was 62%, which indicated the occurrence of sulfate reduction.

### 4.3.2 Microsensor Measurements

Compared with previous studies (Okabe *et al*., 1999; Ito *et al*., 2002), microsensor measurements for *in situ* metabolic activity in this study have been improved in that the measurements were performed under actual growth conditions in the MABR. The combined amperometric $H_2S$ and combined $O_2$ microsensors exhibited higher spatial resolution, less interference and longer life time during the measurements (Lewandowski and Beyenal, 2007). Steady-state concentration profiles of $O_2$ and $H_2S$ in the MAB were shown in Figure 4-3.

The $O_2$ concentration profile in Figure 4-3 revealed that $O_2$ penetrated from the gas permeable membrane side, and was gradually consumed and then depleted at about 685 μm below the biofilm/bulk liquid interface in the upper biofilm (near the biofilm/bulk liquid interface), indicating oxic and anoxic zone in the MAB. The oxic zone in this biofilm is thick (~1700 μm) compared to that of most wastewater biofilms; the penetration depth of oxygen in most wastewater biofilms is within 50~150 μm (Syron and Casey, 2008). The $H_2S$ concentration profile

showed that $H_2S$ production was restricted to the upper layer (285 µm below the biofilm/bulk liquid interface) of the anoxic zone.

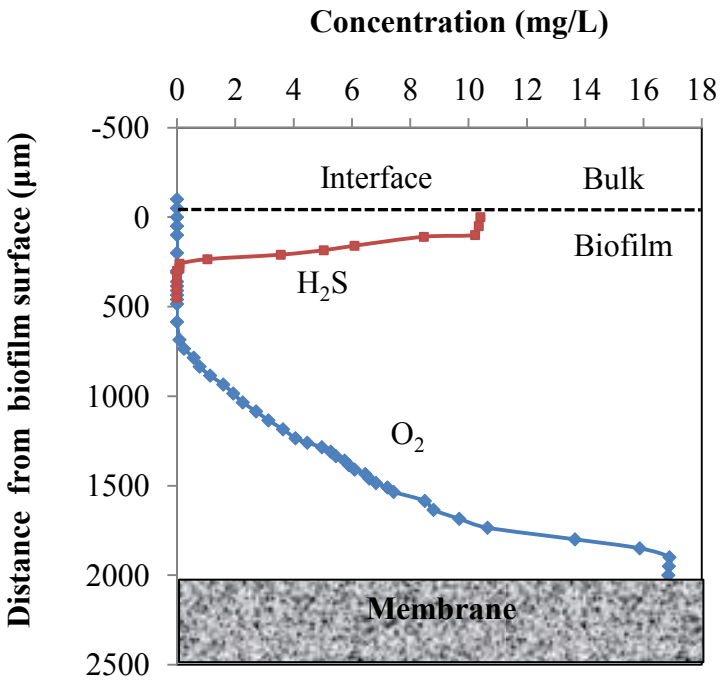



**Figure 4-3. $O_2$ and $H_2S$ Profiles in an $O_2$-based MAB (Biofilm/Bulk Liquid Interface is at 0 µm)**

### 4.3.3 Community Structure of SRB

DsrB gene (gene encoding β subunit of DSR) based DGGE was employed to explore the functional diversity of SRB. The dissimilatory sulfate reductase enzyme catalyzes sulfate reduction and is essential for all known SRB, and therefore it is ideal for profiling SRB biodiversity (Wagner *et al.,* 1998). The

DGGE fingerprints are shown in Figure 4-4. Separation of dsrB gene amplicons in a piece of biofilm with 5 mm × 5 mm surface area is shown in Figure 4-4 (A). The multiple bands indicated the functional diversity of SRB communities in the MAB. According to the Basic Local Alignment Search Tool (BLAST) results, the nucleotide sequence similarities between the detected genes and genes in nucleotide database were 85% to 96%. Sequences of band 1 to band 9 showed similarities to EF065002.1, AB061536.1, EU717109.1, AF273034, AF418194.1, FJ648437.1, EU426866.1, EF065059.1, and GQ324675.1. Band 2 and band 4 represent *Desulfovibrio*, and sequences of band 5 and band 6 showed similarity to *Desulfacinum* and *Desulfotomaculum*, respectively. Other bands were uncultured bacterium isolate DGGE gel band dsrB gene. The principal SRB *Desulfovibrio* and *Desulfotomaculum* in WWTPs were also detected in the MAB studied here. Although most bands represented uncultured bacterium, which was probably due to the limited bacteria information for specific functional genes in the NCBI database, the functional gene based DGGE analysis provided evidence of functional diversity and information about physiological dynamics for complex multispecies biofilms.

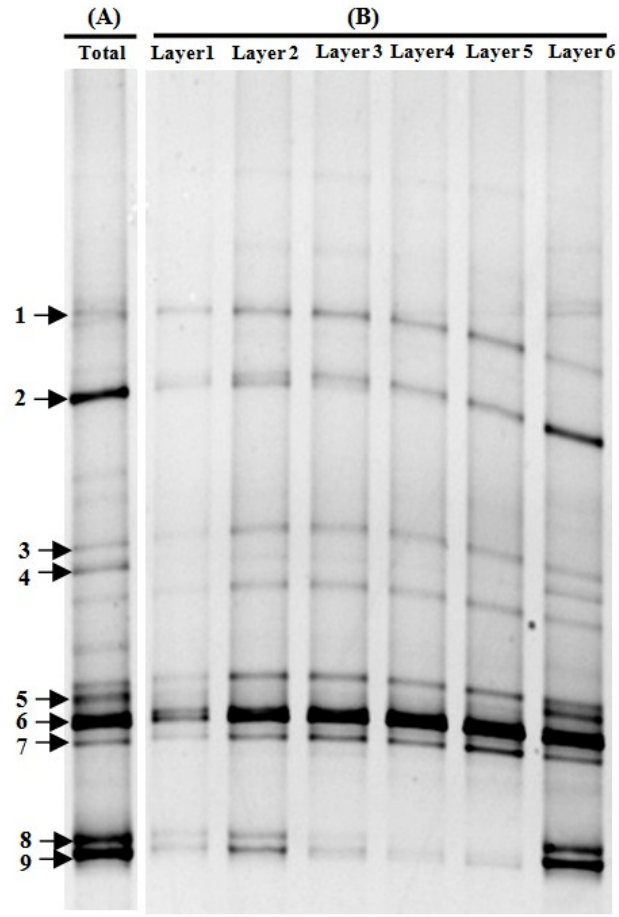



**Figure 4-4. DsrB-based DGGE Fingerprints of SRB Microbial Communities in an MAB. (A): dsrB Gene Amplicons from the Whole Biofilm; (B): dsrB Gene Amplicons from Sliced Biofilm Layers. Horizontal Sliced Layer 1 to Layer 6 is from the Substratum Side to Biofilm/Bulk Water Intersurface (Layers 1-5: 400 µm Thick, Respectively; Layer 6: 600 µm Thick).**

The DGGE fingerprints of the dsrB gene amplicons from horizontally sliced biofilm (layer 1 to layer 6) are shown in Figure 4-4 (B). It was obvious that the DGGE bands showed differences in terms of band number and intensity for different layers, especially for layer 1 (substratum surface of biofilm) and layer 6

(biofilm/bulk water interface). The DGGE bands were less in number and lower in intensity for layer 1 than layer 6, indicating that layer 6 harbored a higher diversity of SRB than layer 1.

### 4.3.4 Visualization and Spatial Distribution of SRB

In a vertical sliced biofilm section with 60 μm thickness, the biofilm morphology and biovolume of SRB were observed and calculated, respectively. The spatial organization of eukaryotes and SRB cells within the MAB is shown in Figure 4-5. Within the sliced biofilm section, the CLSM scanned 12 layers (area=112 μm × 225 μm) along the biofilm from the surface at the bulk water interface to the bottom (substratum). Spatial variations of SRB can be directly visualized in the 3-D reconstructed serial sections shown in Figure 4-6. The biovolume of SRB at each layer was calculated, and the vertical distributions of SRB biomass are shown in Figure 4-7.

Figure 4-5 shows a heterogeneous organization in the biofilm. The microorganisms are surrounded with void spaces and channels, which allow the flux of substrates and metabolic products.

Within the scanned 12 layers of the 2700 μm thick biofilm (Figure 4-7), the SRB biovolume in the upper six layers near the biofilm/bulk water interface was 0.09 $\mu m^3/\mu m^2$, which was much higher than the 0.01 $\mu m^3/\mu m^2$ in the deeper six layers near the substratum. The distribution of SRB biomass along the biofilm depth can be attributed to a higher $O_2$ concentration deeper in the biofilm (low SRB biovolume) and a lower $O_2$ concentration zone in upper biofilm (high SRB

biovolume). Also, the spatial organization of SRB within the MAB might be controlled by many other factors such as the nutrition gradients, active chemotactic (cells mobility to or away from substrates, chemicals, etc.) movements leading to reorganization of biomass (Tolker-Nielsen and Molin 2000).

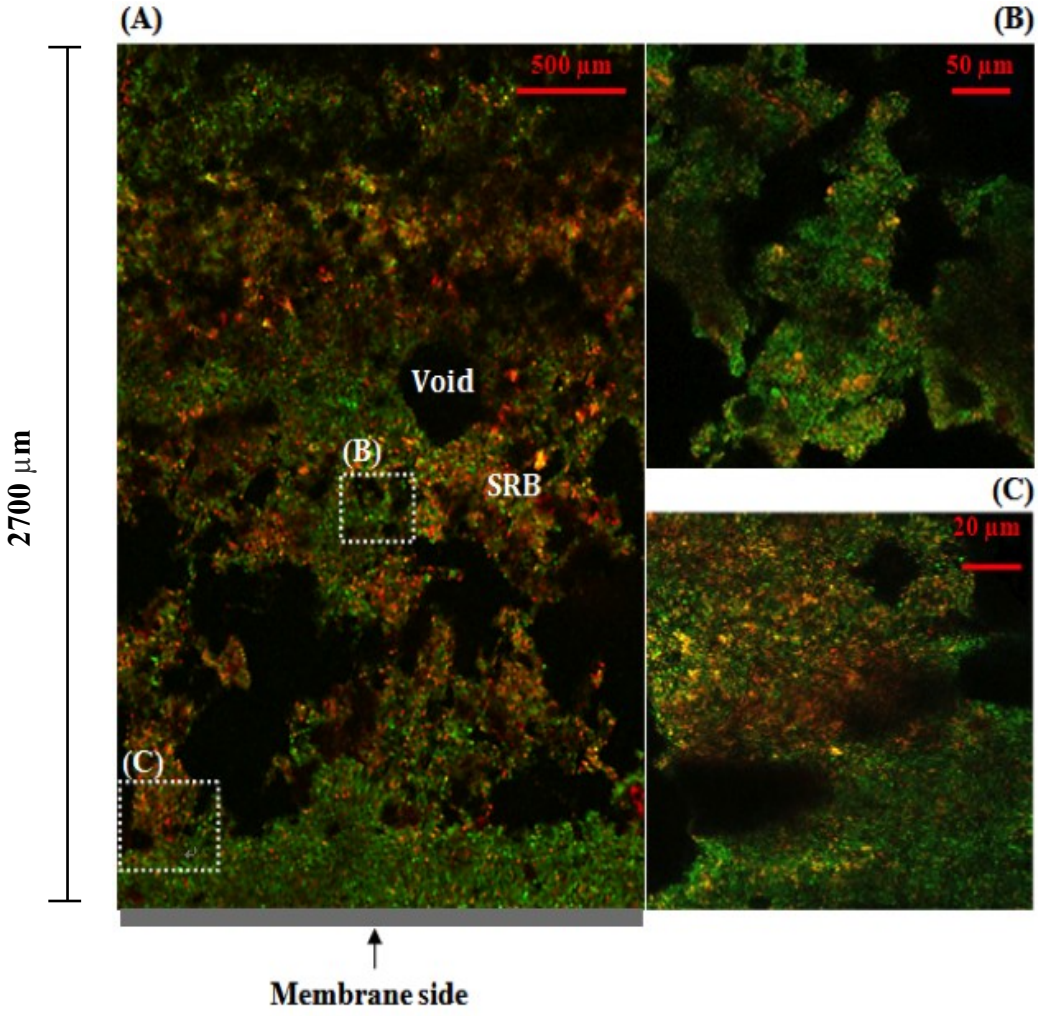
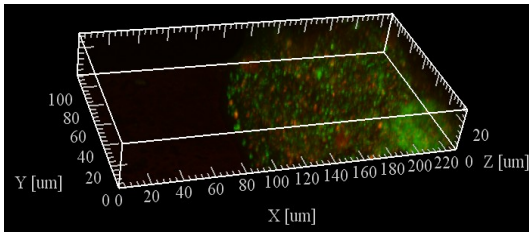
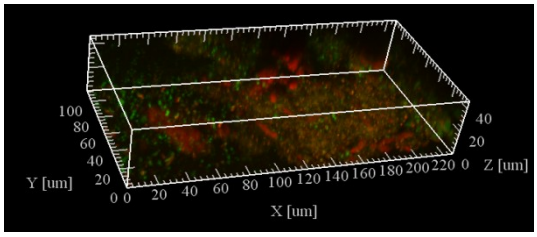



**Figure 4-5. In Situ Hybridization of a Vertical Section of an MAB with a 6-Fam Green-Labeled EUB 338 Probe and Texas Red-Labeled SRB385 Plus SRB385Db Probes. Image (A): Vertical Section of Biofilm; Images of (B) and (C) are from the Enclosed Boxes of Image (A).**

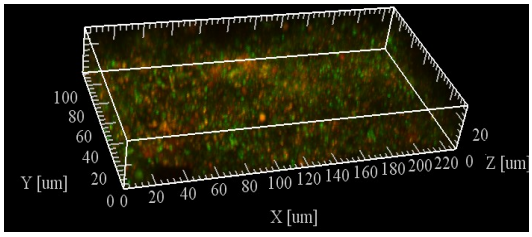
**Layer 1**



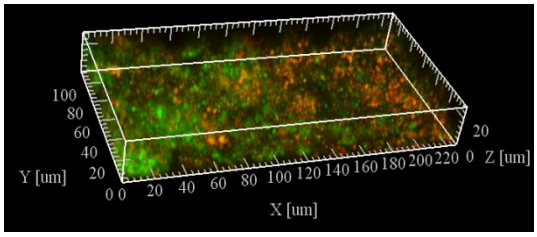

**Layer 2**



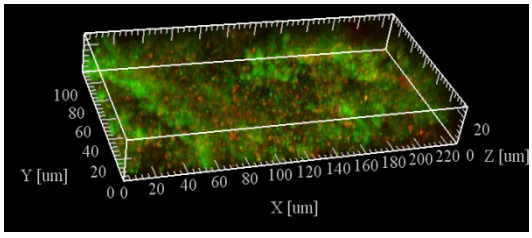

**Layer 3**




**Layer 4**




**Layer 5**




**Layer 6**

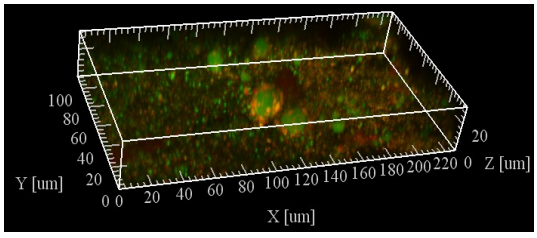
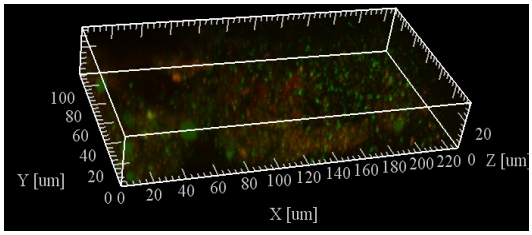
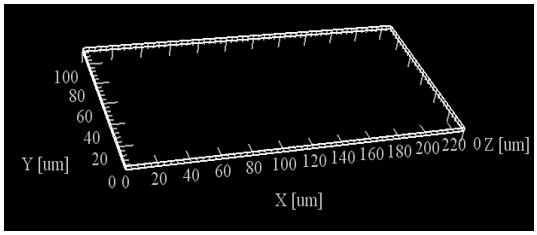



**Figure 4-6. The Spatial Distribution of SRB in Reconstructed Three-Dimensional Images. Layer 1 to Layer 12 Represent Layers from Biofilm Surface to Membrane Side.**

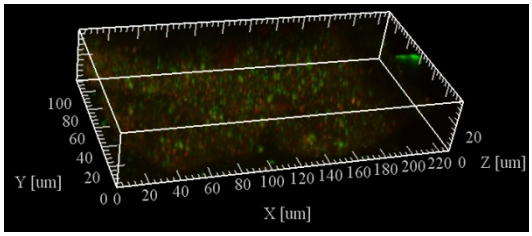
**Layer 7**



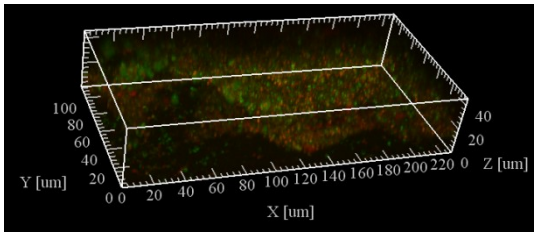

**Layer 8**



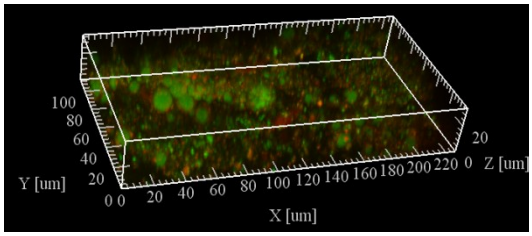

**Layer 9**




**Layer 10**




**Layer 11**




**Layer 12**

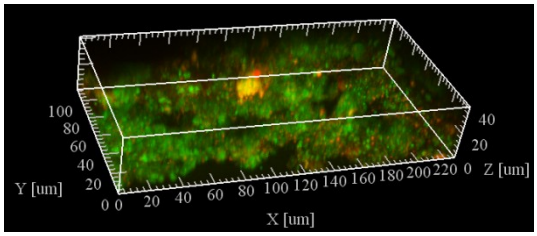



**Figure 4-6 (Cont'). The Spatial Distribution of SRB in Reconstructed Three-Dimensional Images. Layer 1 to Layer 12 Represents Layers from Biofilm Surface to Membrane Side.**

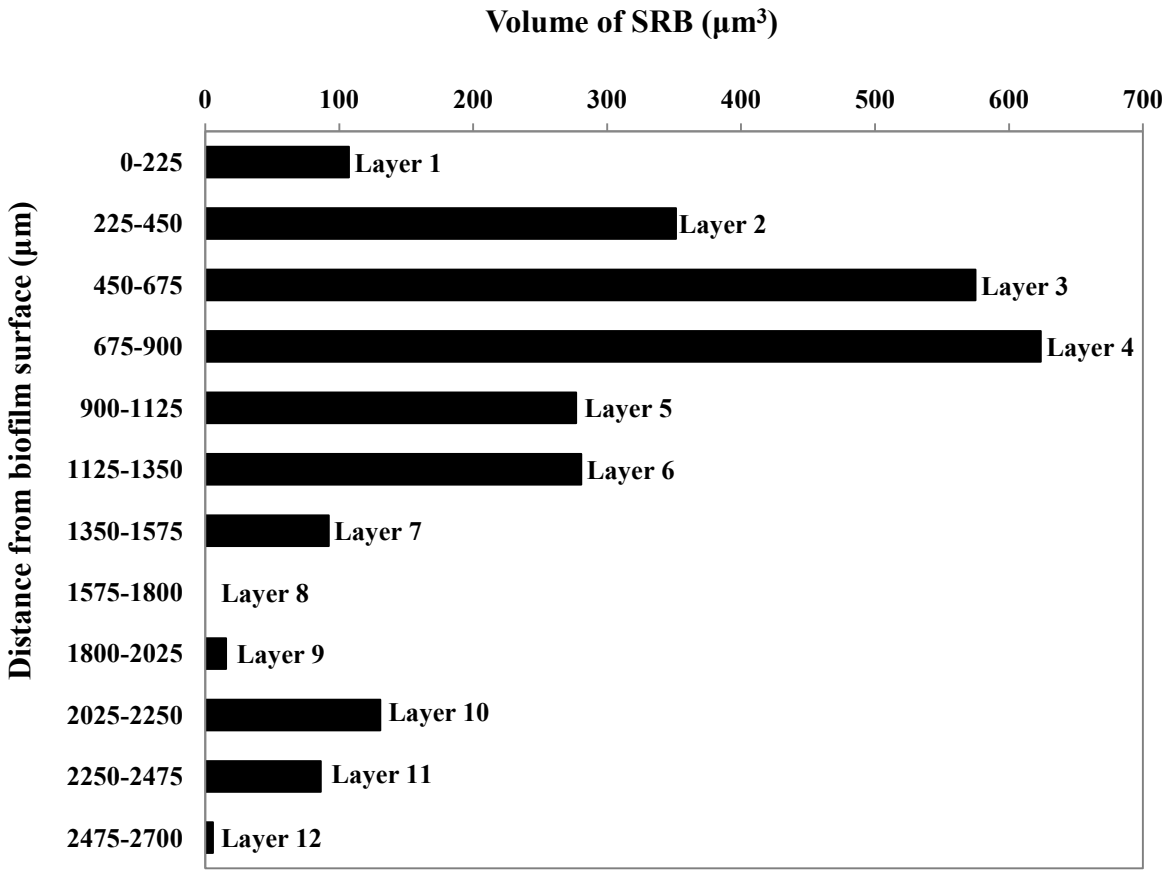



**Figure 4-7. The Vertical Distributions of SRB Biovolume in the MAB in an $O_2$-based MABR**

### 4.3.5 Vertical Distribution and Activity of SRB

SRB biomass was evident across almost the whole depth of biofilm (layer 1 to layer 12), but was more prevalent in the upper layers (near the membrane/bulk water interface) than in the deeper layers (Figure 4-7). $O_2$ and $H_2S$ profiles (Figure 4-3) suggest SRB activity was only present in part of anoxic zone. There is a tendency that SRB and the most active production of $H_2S$ predominantly existed in the anaerobic layer near the biofilm / bulk water intersurface.

Based on the $O_2$ profile in Figure 4-3 and the distributions of SRB biomass in Figure 4-7, there was an overlap of the oxic zone with the presence of SRB cells. We found SRB was present at all depths of the MAB. Previous studies have shown that SRB can respire aerobically. Dilling and Cypionka (1990) mentioned that various strains of SRB such as *D. propionicus*, *Desulfococcus multivorans*, *D. autotrophicum*, and several strains of *Desulfovibrio* species could not only tolerate $O_2$ but could consume $O_2$ and generate ATP in this process; it has also been reported that some SRB can perform disimilatory reduction of nitrate or nitrite to ammonia as a sole energy conserving process (Barton and Fauque, 2009). Alternatively, the SRB cells detected in the oxic zone might be dead. The presence of SRB at all depths in the oxic and anoxic zones of biofilm that was grown in RBCs has been observed (Okabe *et al*., 1999). Santegoeds *et al*. (1998) also found a significant portion of SRB present in the oxic zone of a wastewater biofilm, and *Desulfovibrio* species were the main SRB members in it. Based on this information, it is expected that SRB could be present in the oxic

microenvironment within the MAB. The low $H_2S$ concentration (Figure 4-3) in a region where SRB were shown to be present (layers 3 to 6 in Figure 4-7) could be explained by slow SRB metabolic activity or limited sulfate availability (Beyenal and Lewandowski, 2001). Alternatively, produced $H_2S$ or sulfur compounds might be re-oxidized, converted to sulfate and other intermediates, or react with metals and form precipitates. For example, SRB species *Desulfobulbus* spp. can oxidize reduced sulfur compounds to sulfate using $O_2$ or $NO_3^-$ as the electron acceptor (Dilling and Cypionka, 1990; Dannenberg *et al*., 1992), and species *Desulfovibrio desulfurican* can oxidize $S^0$ to sulfate with oxidized metals such as Mn (IV) as electron acceptors (Lovley and Phillips, 1994).

### 4.3.6 Impact of Oxygen Condition on the SRB Activity

Experiments were conducted in two phases (P1 and P2). During P1, oxygen was supplied at a flow rate of 20 $mL \cdot min^{-1}$; the DO concentration detected in the bulk water was 2.2 mg/L. The biofilm was drenched with bulk water (DO concentration 2.2 ± 0.28 mg/L) for several months. During P2, oxygen was supplied at a flow rate of 10 $mL \cdot min^{-1}$; the bulk water DO concentration was reduced from 2.2 mg/L to 0.64 mg/L for the subsequent 20 days, and then remained at 0.61 ± 0.3 mg/L for five consecutive months of operation. At the end of each phase of operation, at least three biofilm samples with 3 mm × 3 mm surface area were taken from different locations in the reactor without sacrificing the membrane. The collected biofilm samples were kept in PowerBead Tubes provided in the

Power Soil DNA extraction kit (Mo-Bio, Carlsbad, CA), and DNA extraction was processed immediately.

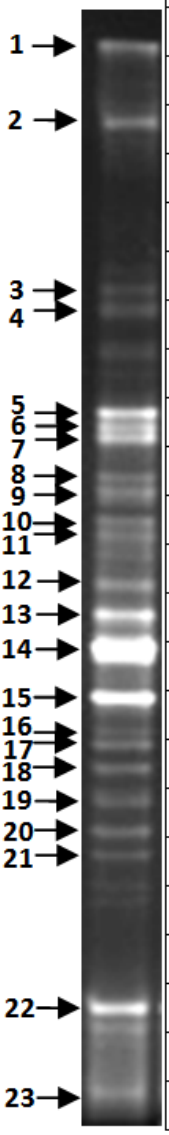
During P1, universal 16S rRNA gene primers were used to track SRB, denitrifiers and nitrifiers within the MAB. 16S rRNA based gene-DGGE fingerprints (Figure 4-8) and phylogenetic analysis (Figure 4-9) indicated that a diverse microbial community existed in the biofilm, as represented by the 28 bands displayed on the gel.

Twenty three of the 28 bands were sequenced successfully. The 23 identified strains fell into seven phylums, including *Proteobacteria* (with subdivisions of *Alpha-, Beta-* and *Gamma-Proteobacteria*), *Bacteroidetes*, *Chlorobi*, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *and Nitrospira*. Among these microorganisms, *Proteobacteria* occupied a large proportion of these microorganisms, including *Rhodocyclaceae*, *Denitratisoma*, *Nitrosomonas*, *Thiobacillus*, *Roseobacter*, *Methylophilus* and *Methylococcales*. The results obtained here were in accordance with previous findings in wastewater systems. It has been found that *Proteobacteria* amounted to 60-70% of all bacteria in conventional activated sludge systems (Wagner and Loy, 2002).

Four types of nitrifiers, i.e., *Nitrosospira*, *Nitrosomonas*, *Methylophilus*, and *Nitrospira*, were detected in the MAB. *Nitrosospira*, *Nitrosomonas*, *and Methylophilus* are AOB known to be responsible for transforming ammonia to nitrite, and *Nitrospira* are responsible for transferring nitrite to nitrate (Gerardi, 2006). *Nitrospira*, *Nitrosospira*, and *Nitrosomonas* are frequently detected in

wastewater treatment plants (WWTPs) (Daims *et al*., 2001; Wang *et al*., 2010). In addition, we detected a large diversity of denitrifiers or bacteria that could be involved in denitrification activity which have been reported previously (Doi and Shioi, 1991; Baltzis *et al.,* 1993; Lim *et al.,* 2005; Smith *et al.,* 2005; Baytshtok *et al.,* 2008), such as *Roseobacter*, *Methyloversatilis*, *Rhodocyclus*, *Denitratisoma.sp*, *Pseudomonas,* and *Firmicutes*. *Pseudomonas* has been reported to be the dominant denitrifiers involved in denitrification in WWTPs (Drysdale *et al.,* 1999).

*Malonomonas*, which is phylogenetically related to *Desulfuromonales* (Kolb *et al.,* 1998) belonging to an SRB order within the *Proteobacteria* (Garrity *et al.,* 2004), was detected in the study. In addition, four types of sulfur oxidizing bacteria (SOB), i.e., *Thiobacillus.sp*, *Limnobacter.sp*, *Chlorobi*, and *Thiorhodospira*, were detected. *Thiobacillus.sp* is responsible for oxidation of hydrogen sulfide to sulfuric acid (Ravichandra *et al.,* 2007). *Limnobacter* is responsible for thiosulfate oxidizing activity. *Chlorobi*, also named as *green sulfur bacteria*, are anaerobes that could oxidize sulfur compounds. *Thiobacillus* is one type of *non-filamentous* bacteria found in wastewater systems (Gerardi, 2006).




| Band | Taxonomy | Accession | Identity |
|---|---|---|---|
| 1 | Rhodocyclaceae bacterium | GU472572.1 | 98% |
| 2 | Firmicutes bacterium | EF665859.1 | 100% |
| 3 | Acidobacteria bacterium | GQ383885.1 | 100% |
| 4 | Methylococcales bacterium | FJ037358.1 | 96% |
| 5 | Actinobacterium | FJ568860.1 | 96% |
| 6 | Pseudomonas | EU919222.1 | 94% |
| 7 | Malonomonas sp. | GQ421131.1 | 94% |
| 8 | Uncultured bacterium | HQ703586.1 | 87% |
| 9 | Limnobacter sp. | HQ836391 | 90% |
| 10 | Chlorobi group bacterium | AY118152.1 | 93% |
| 11 | Rhodocyclaceae bacterium | AY687926.1 | 97% |
| 12 | Denitratisoma sp. | AB542411.1 | 98% |
| 13 | Nitrosomonas | JF506034.1 | 95% |
| 14 | Denitratisoma sp. | HM769664 | 96% |
| 15 | Thiobacillus sp. | FJ933365.1 | 99% |
| 16 | Chlorobi bacterium | AY118152.1 | 93% |
| 17 | Thiobacillus sp. | FJ480830.1 | 88% |
| 18 | Cytophagales bacterium | GQ471878.1 | 88% |
| 19 | Rhodospirillales bacterium | HM447689.1 | 100% |
| 20 | Nitrospira sp. | JF703520.1 | 89% |
| 21 | Methylophilus sp. | JF808846.1 | 96% |
| 22 | Chlorobi bacterium | EU266920.1 | 99% |
| 23 | Roseobacter sp. | DQ479380.1 | 94% |

**Figure 4-8. Universal 16S rRNA Gene Primer (GC-341F/518R) Based DGGE Fingerprints. Taxonomy, Sequence Number, and Top Hit Identities are Shown in the Table.**

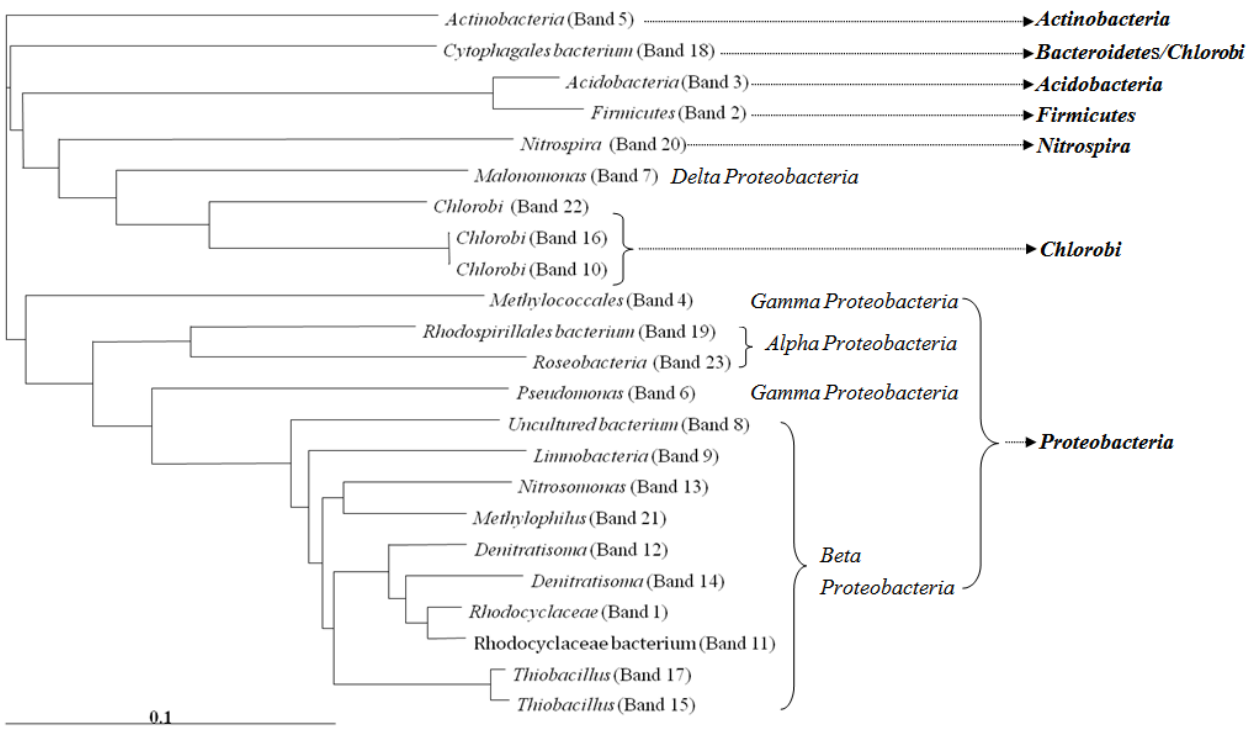



**Figure 4-9. Phylogenetic Tree Based on 16S rRNA Gene Sequences of Bacterial Communities in an MABR. The Scale Bar Represents 0.1 Substitutions Per Nucleotide Position. The Tree is Based on Distance Matrix Analysis and a Neighbor-Joining Method. Clustal X and Treeview Software Were Used for Analysis.**

It should be noted that the PCR-DGGE technique has its limitations. In the study, five out of 28 bands failed to be sequenced. The complex environmental biofilm sample, which harbors a large diversity of species, may have hampered band separation (Miletto *et al.,* 2007). Previously, researchers have reported possible biases in PCR amplification of the 16S rRNA gene (von Wintzingerode *et al.,* 1997). For example, from a DNA mixture, one set of primers may preferentially amplify the 16S rRNA gene of one species, while another primer pair would preferentially amplify the 16S rRNA gene of another species (Hansen *et al.,* 1998). Therefore, to better understand the functional diversities and dynamics of microbial communities in the MAB, further tests on specific functional genes of dsrB, nirK and amoA, which encode subunit of proteins that catalyze respiration processes accordingly, were performed.

DGGE band patterns and relative intensities varied with changes in oxygen concentrations in the bulk liquid in contact with the biofilm. As shown in Figure 4-10, when the detected DO concentration was high (P1: DO = 2.2 ± 0.28 mg/L), the DGGE bands were low in number and intensity, suggesting low SRB activity. When the bulk oxygen concentration was less than 1 mg/L (P2), the DGGE bands increased in number and intensity. Similar trends were observed for denitrifiers. For AOB band patterns, there were no obvious differences of band numbers and intensities when the biofilm was exposed under high and low bulk oxygen concentrations.

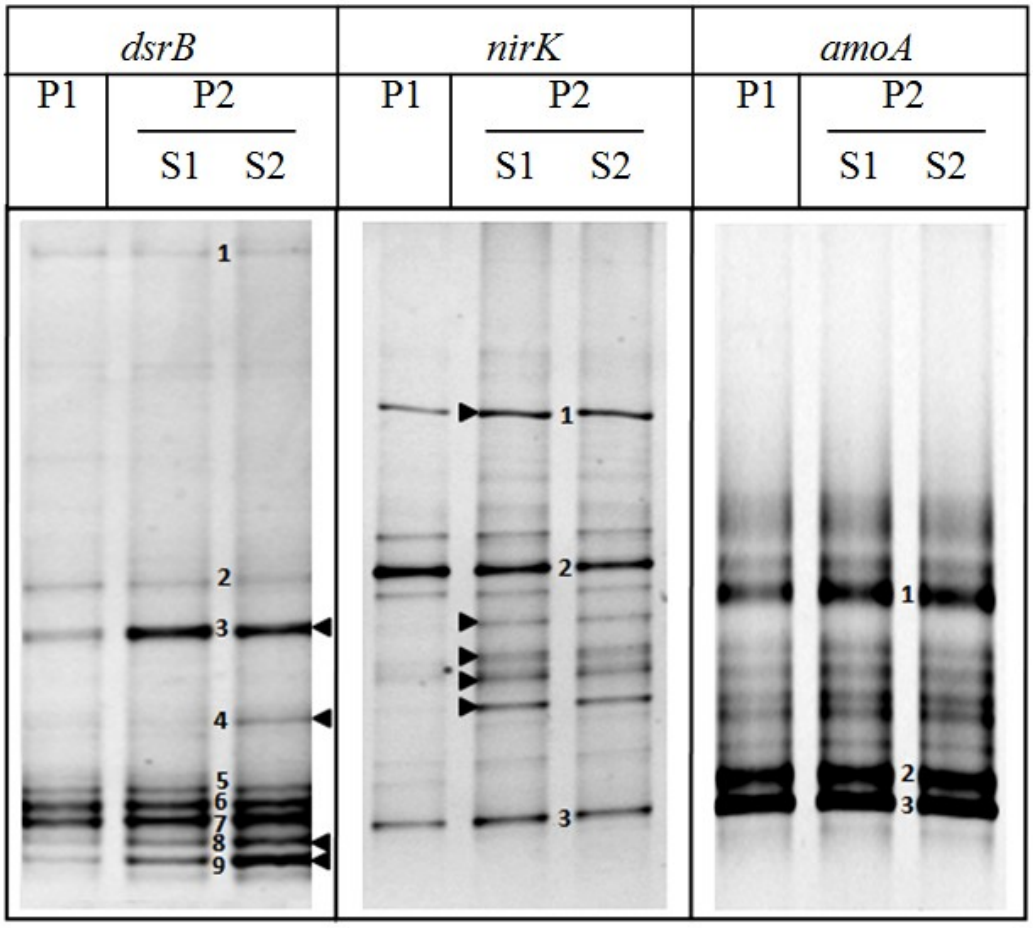



**Figure 4-10. Gene Specific Primers Based DGGE Fingerprints: S1 and S2 Represented Different DNA Samples Taken from Reactor During Phase (P2). The Black Triangles Indicate Bands that Showed Differences in Number or Intensity.**

The results showed that anaerobic bacteria (nirK and dsrB) were more sensitive than aerobic bacteria (amoA) to DO concentration in the bulk liquid. The presence and function of anaerobic bacteria including denitrifiers and SRB within the biofilm could be inhibited under a higher bulk DO condition. In

addition, we have observed sloughing of biofilm when the bulk DO was at around 2 mg/L; the anaerobic microorganisms near the surface of the biofilm might be sheared away. These factors both contributed to a lower functional diversity of denitrifiers and SRB during this phase (P1). No obvious differences of band numbers and intensities were observed for AOB when the biofilm was exposed to different bulk DO, which indicated that oxygen might not be a limiting factor for AOB. In addition, based on previous assumptions of microorganisms' stratification within MAB (Schramm *et al*., 2000; Cole *et al*., 2002; Hibiya *et al*., 2003; Cole *et al*., 2004; Matsumoto *et al*., 2007), nitrifying bacteria exist near the membrane side, while denitrifying bacteria and SRB prefer anaerobic zone and exist near the biofilm-bulk liquid interface. Thus, the anaerobic bacteria in the outer zone of biofilm may create a protective barrier for AOB in the inner zone.

It should be noted that before P1, the reactor was already running for over one year with almost zero oxygen in the bulk water. Therefore, we believe that although the reactor was running with bulk DO as 2 mg/L for several months in P1, there were anaerobic denitrifiers and SRB present in the oxic biofilm. During P2, we found that these anaerobic bacteria present in the MAB were stimulated and reactivated. Comparing the two samples collected during P2, no substantial differences in terms of band numbers and intensities were observed except that one more band (band 4) appeared on the dsrB gene gel for S2, which indicated that during P2 these biofilm microorganisms had already adapted to the reactor conditions and could play their role in the metabolic function.

## 4.4 Conclusions and Environmental Implications

The comprehensive analysis in this study indicated that results from DGGE and FISH in terms of SRB community structures corresponded well with that from microsensor measurements in terms of their metabolic functions in biofilm. SRB preferred more anaerobic microenvironment and were more active in the anaerobic zone within the $O_2$ based MAB. In addition, the co-existence of AOB, denitrifiers and SRB in the single piece of MAB based on DGGE analysis of functional genes of amoA, nirK, dsrB was confirmed; furthermore, the oxygen concentration in bulk water had an impact on the microbial community behavior, particularly on anaerobic bacteria, including SRB and denitrifiers. Under different DO concentrations in the bulk liquid, the DGGE fingerprint pattern of SRB and denitrifiers varied in band number and band intensity, which indirectly reflected microbial activities.

The information obtained here expands the fundamental knowledge of the multiple microbial communities co-existing in one piece of membrane aerated biofilm, and is expected to help modify biofilm models and suggest conditions for MABR operation that account for the behavior of microbial populations in the biofilm.

## References

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science. **7**(2): 123-138.

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: II. A review of emerging technologies. Journal of Environmental Engineering and Science. **7**(5): 499-524.

Amann, R., Fuchs, B. M. and Behrens, S. 2001. The identification of microorganisms by fluorescence in situ hybridisation. Current Opinion in Biotechnology. **12**(3): 231-236.

Amann, R. and Kuhl, M. 1998. In situ methods for assessment of microorganisms and their activities. Current Opinion in Microbiology. **1**(3): 352-358.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. 1990. Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology. **56**(6): 1919-1925.

Amann, R. I., Krumholz, L. and Stahl, D. A. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental-studies in microbiology. Journal of Bacteriology. **172**(2): 762-770.

Amann, R. I., Stromley, J., Devereux, R., Key, R. and Stahl, D. A. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms Applied and Environmental Microbiology. **58**(2): 614-623.

An, D. S., Brown, D., Chatterjee, I., Dong, X. L., Ramos-Padron, E., Wilson, S., Bordenave, S., Caffrey, S. M., Gieg, L. M., Sensen, C. W. and Voordouw, G. 2013. Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. Genome. **56**(10): 612-618.

Aoi, Y. 2002. In situ identification of microorganisms in biofilm communities. Journal of Bioscience and Bioengineering. **94**(6): 552-556.

Aoi, Y., Miyoshi, T., Okamoto, T., Tsuneda, S., Hirata, A., Kitayama, A. and Nagamune, T. 2000. Microbial ecology of nitrifying bacteria in wastewater treatment process examined by fluorescence in situ hybridization. Journal of Bioscience and Bioengineering. **90**(3): 234-240.

Baltzis, B. C., Lewandowski, G. A. and Wang, J. H. 1993. Denitrification with a strain of *pseudomonas denitrificans* - fundamental kinetic studies and their implications for bioreactor design. Abstracts of Papers of the American Chemical Society. **205**: 26-BIOT.

Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology. **68**: 41-98.

Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology. **68**: 41-98.

Baytshtok, V., Kim, S., Yu, R., Park, H. and Chandran, K. 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. Water Science and Technology. **58**(2): 359-365.

Beyenal, H. and Lewandowski, Z. 2001. Mass-transport dynamics, activity, and structure of sulfate-reducing biofilms. AIChE Journal. **47**(7): 1689-1697.

Bhagat, M., Burgess, J. E., Antunes, A. P. M., Whiteley, C. G. and Duncan, J. R. 2004. Precipitation of mixed metal residues from wastewater utilising biogenic sulphide. Minerals Engineering. **17**(7-8): 925-932.

Bordenave, S., Kostenko, V., Dutkoski, M., Grigoryan, A., Martinuzzi, R. J. and Voordouw, G. 2010. Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings. Chemosphere. **81**(5): 663-668.

Brindle, K. and Stephenson, T. 1996. The application of membrane biological reactors for the treatment of wastewaters. Biotechnology and Bioengineering. **49**(6): 601-610.

Brindle, K., Stephenson, T. and Semmens, M. J. 1998. Nitrification and oxygen utilisation in a membrane aeration bioreactor. Journal of Membrane Science. **144**(1-2): 197-209.

Brindle, K., Stephenson, T. and Semmens, M. J. 1999. Pilot-plant treatment of a high-strength brewery wastewater using a membrane-aeration bioreactor. Water Environment Research. **71**(6): 1197-1204.

Brosius, J., Dull, T. J., Sleeter, D. D. and Noller, H. F. 1981. Gene organization and primary structure of a ribosomal-RNA operon from escherichia-coli. Journal of Molecular Biology. **148**(2): 107-127.

Caughill, D. L., Morgenstern, N. R. and Scott, J. D. 1993. Geotechnics of nonsegregating oil sands tailings. Canadian Geotechnical Journal. **30**(5): 801-811.

Chalaturnyk, R. J., Scott, J. D. and Ozum, B. 2002. Management of oil sands tailings. Petroleum Science and Technology. **20**(9-10): 1025-1046.

Cole, A. C., Semmens, M. J. and LaPara, T. M. 2004. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. Applied and Environmental Microbiology. **70**(4): 1982-1989.

Cole, A. C., Shanahan, J. W., Semmens, M. J. and LaPara, T. M. 2002. Preliminary studies on the microbial community structure of membrane-aerated biofilms treating municipal wastewater. Desalination. **146**(1-3): 421-426.

Cote, P., Bersillon, J. L., Huyard, A. and Faup, G. 1988. Bubble-free aeration using membranes - process analysis. Journal Water Pollution Control Federation. **60**(11): 1986-1992.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. and Wagner, M. 2001. In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. Applied and Environmental Microbiology. **67**(11): 5273-5284.

Dannenberg, S., Kroder, M., Dilling, W. and Cypionka, H. 1992. Oxidation of $H_2$, organic compounds and inorganic sulfur compounds coupled to reduction of $O_2$ or nitrate by sulfate-reducing bacteria. Archives of Microbiology. **158**(2): 93-99.

de la Rosa, C. and Yu, T. 2006. Development of an automation system to evaluate the three-dimensional oxygen distribution in wastewater biofilms using microsensors. Sensors and Actuators B-Chemical. **113**(1): 47-54.

DeBeer, D., Schramm, A., Santegoeds, C. M. and Kuhl, M. 1997. A nitrite microsensor for profiling environmental biofilms. Applied and Environmental Microbiology. **63**(3): 973-977.

Debeer, D., Stoodley, P., Roe, F. and Lewandowski, Z. 1994. Effects of biofilm structures on oxygen distribution and mass-transport. Biotechnology and Bioengineering. **43**(11): 1131-1138.

Debeer, D. and Sweerts, J. P. R. 1989. Measurement of nitrate gradients with an ion-selective microelectrode. Analytica Chimica Acta. **219**(2): 351-356.

Debeer, D. and Vandenheuvel, J. C. 1988. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin. Talanta. **35**(9): 728-730.

Dilling, W. and Cypionka, H. 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiology Letters. **71**(1-2): 123-127.

Doi, M. and Shioi, Y. 1991. Enhancement of denitrifying activity in cells of *roseobacter-denitrificans* grown aerobically in the light. Plant and Cell Physiology. **32**(3): 365-370.

Drysdale, G. D., Kasan, H. C. and Bux, F. 1999. Denitrification by heterotrophic bacteria during activated sludge treatment. Water Sa. **25**(3): 357-362.

Ebert, A. and Brune, A. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite Reticulitermes flavipes (Kollar). Applied and Environmental Microbiology. **63**(10): 4039-4046.

Eckert, W. F., Masliyah, J. H., Gray, M. R. and Fedorak, P. M. 1996. Prediction of sedimentation and consolidation of fine tails. AIChE Journal. **42**(4): 960-972.

Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., van der Meer, J. R. and Zehnder, A. J. B. 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Archives of Microbiology. **175**(3): 198-207.

Essila, N. J., Semmens, M. J. and Voller, V. R. 2000. Modeling biofilms on gas-permeable supports: Concentration and activity profiles. Journal of Environmental Engineering-Asce. **126**(3): 250-257.

Fedorak, P. M., Coy, D. L., Dudas, M. J., Simpson, M. J., Renneberg, A. J. and MacKinnon, M. D. 2003. Microbially-mediated fugitive gas production from oil sands tailings and increased tailings densification rates. Journal of Environmental Engineering and Science. **2**(3): 199-211.

Fedorak, P. M., Coy, D. L., Salloum, M. J. and Dudas, M. J. 2002. Methanogenic potential of tailings samples from oil sands extraction plants. Canadian Journal of Microbiology. **48**(1): 21-33.

Ferris, M. J., Nold, S. C., Revsbech, N. P. and Ward, D. M. 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. Applied and Environmental Microbiology. **63**(4): 1367-1374.

Foght, J. M., Fedorak, P. M. and Westlake, D. W. S. 1985. Microbial content and metabolic activities in the Syncrude tailings pond. Aostra Journal of Research. **1**:139-146.

Foti, M., Sorokin, D. Y., Lomans, B., Mussman, M., Zacharova, E. E., Pimenov, N. V., Kuenen, J. G. and Muyzer, G. 2007. Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. Applied and Environmental Microbiology. **73**(7): 2093-2100.

Garrity, G. M., Bell, J. A. and Lilburn, T. G. 2004. Taxonomic outline of procaryotes. 2nd edition, Springer, Berlin Heidelberg New York. 132.

Geets, J., Borrernans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D. and Vanbroekhoven, K. 2006. DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. Journal of Microbiological Methods. **66**(2): 194-205.

Gerardi, M. H. 2006. Wastewater Bacteria. United States John Wiley & Sons, Pennsylvania.

Golby, S., Ceri, H., Gieg, L. M., Chatterjee, I., Marques, L. L. R. and Turner, R. J. 2011. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. Fems Microbiology Ecology. **79**(1): 240-250.

Guo, C. (2009). Rapid densification of the oil sands mature fine tailings (MFT) by microbial activity. PhD Thesis, University of Alberta. Edmonton, Canada.

Hall-Stoodley, L., Costerton, J. W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology. **2**(2): 95-108.

Hansen, M. C., Tolker-Nielsen, T., Givskov, M. and Molin, S. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiology Ecology. **26**(2): 141-149.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. 1996. Real time quantitative PCR. Genome Research. **6**(10): 986-994.

Herman, D. C., Fedorak, P. M., Mackinnon, M. D. and Costerton, J. W. 1994. Biodegradation of naphthenic acids by microbial-populations indigenous to oil sands tailings. Canadian Journal of Microbiology. **40**(6): 467-477.

Hibiya, K., Terada, A., Tsuneda, S. and Hirata, A. 2003. Simultaneous nitrification and denitrification by controlling vertical and horizontal microenvironment in a membrane-aerated biofilm reactor. Journal of Biotechnology. **100**(1): 23-32.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology. **46**(10): 927-937.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research. **36**(11): 2843-2855.

Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. Genome Biology. **3**(2).

Ibrahim, H. T., He, Q., Al-Rekabi, W. S. and Yang, Q. 2012. Improvements in biofilm processes for wastewater treatment. Pakistan Journal of Nutrition. **11**(8): 610-636.

Ito, T., Nielsen, J. L., Okabe, S., Watanabe, Y. and Nielsen, P. H. 2002. Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Applied and Environmental Microbiology. **68**(1): 356-364.

Ito, T., Okabe, S., Satoh, H. and Watanabe, Y. 2002. Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. Applied and Environmental Microbiology. **68**(3): 1392-1402.

Jeroschewski, P., Steuckart, C. and Kuhl, M. 1996. An amperometric microsensor for the determination of $H_2S$ in aquatic environments. Analytical Chemistry. **68**(24): 4351-4357.

Jong, T. and Parry, D. L. 2006. Microbial sulfate reduction under sequentially acidic conditions in an upflow anaerobic packed bed bioreactor. Water Research. **40**(13): 2561-2571.

Jorgensen, B. B. 1982. Mineralization of organic matter in the sea bed - the role of sulfate reduction. Nature. **296**(5858): 643-645.

Kindaichi, T., Kawano, Y., Ito, T., Satoh, H. and Okabe, S. 2006. Population dynamics and in situ kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. Biotechnology and Bioengineering. **94**(6): 1111-1121.

Kindaichi, T., Okabe, S., Satoh, H. and Watanabe, Y. 2004. Effects of hydroxylamine on microbial community structure and function of autotrophic nitrifying biofilms determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **49**(11-12): 61-68.

Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Khironomos, J. N., Lee, H. and Trevors, J. T. 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods. **58**(2): 169-188.

Kohls, O., Klimant, I., Holst, G. and Kuhl, M. 1997. Development and comparision of pH microoptodes for use in marine systems. Micro- and Nanofabricated Electro-Optical Mechanical Systems for Biomedical and Environmental Applications. **2978**: 82-91.

Kolb, S., Seeliger, S., Springer, N., Ludwig, W. and Schink, B. 1998. The fermenting bacterium Malonomonas rubra is phylogenetically related to sulfur-reducing bacteria and contains a c-type cytochrome similar to those of sulfur and sulfate reducers. Systematic and Applied Microbiology. **21**(3): 340-345.

Kuhl, M. and Jorgensen, B. B. 1992. Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. . Applied and Environmental Microbiology. **58**(4): 1164-1174.

Lewandowski, Z. and Beyenal, H. 2007. Fundamentals of Biofilm Research. New York, CRC press.

Li, C. 2010. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification. Master Thesis, University of Alberta. Edmonton, Canada.

Li, J. and Bishop, P. L. 2004. Time course observations of nitrifying biofilm development using microelectrodes. Journal of Environmental Engineering and Science. **3**(6): 523-528.

Lim, Y. W., Lee, S. A., Kim, S. B., Yong, H. Y., Yeon, S. H., Park, Y. K., Jeong, D. W. and Park, J. S. 2005. Diversity of denitrifying bacteria isolated from Daejeon sewage treatment plant. Journal of Microbiology. **43**(5): 383-390.

List, B. R. and Lord, E. R. F. 1997. Syncrude's tailings management practices from research to implementation. Cim Bulletin. **90**(1010): 39-44.

Llobet-Brossa, E., Rossello-Mora, R. and Amann, R. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Applied and Environmental Microbiology. **64**(7): 2691-2696.

Lovley, D. R. and Phillips, E. J. P. 1994. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Applied and Environmental Microbiology. **60**(7): 2394-2399.

Lu, R. and Yu, T. 2002. Fabrication and evaluation of an oxygen microelectrode applicable to environmental engineering and science. Journal of Environmental Engineering and Science. **1**(3): 225-235.

Matsumoto, S., Terada, A., Aoi, Y., Tsuneda, S., Alpkvist, E., Picioreanu, C. and van Loosdrecht, M. C. M. 2007. Experimental and simulation analysis of community structure of nitrifying bacteria in a membrane-aerated biofilm. Water Science and Technology. **55**(8-9): 283-290.

Miletto, M., Bodelier, P. L. E. and Laanbroek, H. J. 2007. Improved PCR-DGGE for high resolution diversity screening of complex sulfate-reducing prokaryotic communities in soils and sediments. Journal of Microbiological Methods. **70**(1): 103-111.

Minz, D., Flax, J. L., Green, S. J., Muyzer, G., Cohen, Y., Wagner, M., Rittmann, B. E. and Stahl, D. A. 1999. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis

of dissimilatory sulfite reductase genes. Applied and Environmental Microbiology. **65**(10): 4666-4671.

Moter, A. and Gobel, U. B. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. Journal of Microbiological Methods. **41**(2): 85-112.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Applied and Environmental Microbiology. **59**(3): 695-700.

Nakatsu, C. H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. Soil Science Society of America Journal. **71**(2): 562-571.

Nicolaisen, M. H. and Ramsing, N. B. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. Journal of Microbiological Methods. **50**(2): 189-203.

Norsker, N. H., Nielsen, P. H. and Hvitvedjacobsen, T. 1995. Influence of oxygen on biofilm growth and potential sulfate reduction in gravity sewer biofilm. Water Science and Technology. **31**(7): 159-167.

Odom, J. M. and Singleton, R. J. 1993. The sulfate-reducing bacteria:contemporary perspectives. New York, Springer-Verlag.

Okabe, S., Ito, T. and Satoh, H. 2003. Sulfate reducing bacterial community structure and their contribution to carbon mineralization in a wastewater biofilm growing under microaerophilic conditions. Applied Microbiology and Biotechnology. **63**(3): 322-334.

Okabe, S., Ito, T., Sugita, K. and Satoh, H. 2005. Succession of internal sulfur cycles and sulfur-oxidizing bacterial communities in microaerophilic wastewater biofilms. Applied and Environmental Microbiology. **71**(5): 2520-2529.

Okabe, S., Itoh, T., Satoh, H. and Watanabe, Y. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Applied and Environmental Microbiology. **65**(11): 5107-5116.

Okabe, S., Matsuda, T., Satoh, H., Itoh, T. and Watanabe, Y. 1998. Sulfate reduction and sulfide oxidation in aerobic mixed population biofilms. Water Science And Technology. **37**(4-5): 131-138.

Okabe, S., Naitoh, H., Satoh, H. and Watanabe, Y. 2002. Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes. Water Science and Technology. **46**(1-2): 233-241.

Okabe, S., Satoh, H. and Watanabe, Y. 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **65**(7): 3182-3191.

Okabe, S., Satoh, H. and Watanabe, Y. 1999b. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **65**(7): 3182-3191.

Okabe, S. and Watanabe, Y. 2000. Structure and function of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **42**(12): 21-32.

Park, J. J., Byun, I. G., Park, S. R. and Park, T. J. 2008. Nitrifying bacterial communities and its activities in aerobic biofilm reactors under different temperature conditions. Korean Journal of Chemical Engineering. **25**(6): 1448-1455.

Park, J. J., Byun, I. G., Yu, J. C., Park, S. R., Ju, D. J., Hur, S. H. and Park, T. J. 2008. Analysis of nitrifying bacterial communities in aerobic biofilm reactors with different DO conditions using molecular techniques. Water Science and Technology. **57**(12): 1889-1899.

Pellicer-Nacher, C., Sun, S. P., Lackner, S., Terada, A., Schreiber, F., Zhou, Q. and Smets, B. F. 2010. Sequential aeration of membrane-aerated biofilm reactors for high-rate autotrophic nitrogen removal: experimental demonstration. Environmental Science & Technology. **44**(19): 7628-7634.

Penner, T. J. and Foght, J. M. 2010. Mature fine tailings from oil sands processing harbour diverse methanogenic communities. Canadian Journal of Microbiology. **56**(6): 459-470.

Pol, L. W. H., Lens, P. N. L., Stams, A. J. M. and Lettinga, G. 1998. Anaerobic treatment of sulphate-rich wastewaters. Biodegradation. **9**(3-4): 213-224.

Postgate, J. R. 1984. The sulphate-reducing bacteria. Cambridge, UK, Cambridge University Press.

Powter, C. B., Biggar, K.W., Silva, M.J., McKenna, G.T and Scordo, E.B. 2010. Review of oil sands tailings technology options. The 14th International Conference on Tailings and Mine Waste: 381-391.

Quagraine, E. K., Peterson, H. G. and Headley, J. V. 2005. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering. **40**(3): 685-722.

Rabus, R., Fukui, M., Wilkes, H. and Widdel, F. 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Applied and Environmental Microbiology. **62**(10): 3605-3613.

Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I. M., Dong, X., Sensen, C. W., Fournier, J., Voordouw, G. and Gieg, L. M. 2011. Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond. Environmental Science & Technology. **45**(2): 439-446.

Ramsing, N. B., Kuhl, M. and Jorgensen, B. B. 1993. Distribution of sulfate-reducing bacteria, $O_2$, and $H_2S$ in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Applied and Environmental Microbiology. **59**(11): 3840-3849.

Ravichandra, P., Mugeraya, G., Rao, A. G., Ramakrishna, M. and Jetty, A. 2007. Isolation of *Thiobacillus sp* from aerobic sludge of distillery and dairy effluent treatment plants and its sulfide oxidation activity at different concentrations. Journal of Environmental Biology. **28**(4): 819-823.

Revsbech, N. P. 1989. An oxygen microsensor with a guard cathode. Limnology and Oceanography. **34**(2): 474-478.

Revsbech, N. P. and Jorgensen, B. B. 1986. Microelectrodes - their use in microbial ecology. Advances in Microbial Ecology. **9**: 293-352.

Revsbech, N. P., Jorgensen, B. B., Blackburn, T. H. and Cohen, Y. 1983. Microelectrode studies of the photosynthesis and $O_2$, $H_2S$, and pH profiles of a microbial mat. Limnology and Oceanography. **28**(6): 1062-1074.

Revsbech, N. P., Nielsen, L. P., Christensen, P. B. and Sorensen, J. 1988. Combined oxygen and nitrous-oxide microsensor for denitrification studies. Applied and Environmental Microbiology. **54**(9): 2245-2249.

Salloum, M. J., Dudas, M. J. and Fedorak, P. M. 2002. Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Management & Research. **20**(2): 162-171.

Santegoeds, C. M., Damgaard, L. R., Hesselink, C., Zopfi, J., Lens, P., Muyzer, G. and de Beer, D. 1999. Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. Applied and Environmental Microbiology. **65**(10): 4618-4629.

Santegoeds, C. M., Ferdelman, T. G., Muyzer, G. and de Beer, D. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Applied and Environmental Microbiology. **64**(10): 3731-3739.

Sanz, J. L. and Kochling, T. 2007. Molecular biology techniques used in wastewater treatment: An overview. Process Biochemistry. **42**(2): 119-133.

Sass, A. M., Eschemann, A., Kuhl, M., Thar, R., Sass, H. and Cypionka, H. 2002. Growth and chemosensory behavior of sulfate-reducing bacteria in oxygen-sulfide gradients. FEMS Microbiology Ecology. **40**(1): 47-54.

Sass, H., Cypionka, H. and Babenzien, H. D. 1997. Vertical distribution of sulfate-reducing bacteria at the oxic-anoxic interface in sediments of the oligotrophic Lake Stechlin. FEMS Microbiology Ecology. **22**(3): 245-255.

Satoh, H., Ono, H., Rulin, B., Kamo, J., Okabe, S. and Fukushi, K. I. 2004. Macroscale and microscale analyses of nitrification and denitrification in biofilms attached on membrane aerated biofilm reactors. Water Research. **38**(6): 1633-1641.

Schramm, A., De Beer, D., Gieseke, A. and Amann, R. 2000. Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. Environmental Microbiology. **2**(6): 680-686.

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S. and Amann, R. 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira spp.* along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. Applied and Environmental Microbiology. **65**(8): 3690-3696.

Schramm, A., de Beer, D., Wagner, M. and Amann, R. 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira spp.* as dominant populations in a nitrifying fluidized bed reactor. Applied and Environmental Microbiology. **64**(9): 3480-3485.

Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R. and Schleifer, K. H. 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **62**(12): 4641-4647.

Schulze, E.-D. and Mooney, H. A. 1993. Biodiversity and ecosystem function, Springer-Verlag.

Semmens, M. J., Dahm, K., Shanahan, J. and Christianson, A. 2003. COD and nitrogen removal by biofilms growing on gas permeable membranes. Water Research. **37**(18): 4343-4350.

Siddique, T., Penner, T., Klassen, J., Nesbo, C. and Foght, J. M. 2012. Microbial communities involved in methane production from hydrocarbons in oil sands tailings. Environmental Science & Technology. **46**(17): 9802-9810.

Siddique, T., Penner, T., Semple, K. and Foght, J. M. 2010. Anaerobic biodegradation of longer-chain n-Alkanes coupled to methane production in oil sands tailings. Environmental Science & Technology. **45**(13): 5892-5899.

Smith, R. L., Buckwalter, S. P., Repert, D. A. and Miller, D. N. 2005. Small-scale, hydrogen-oxidizing-denitrifying bioredctor for treatment of nitrate-pontaminated drinking water. Water Research. **39**(10): 2014-2023.

Syron, E. and Casey, E. 2008. Membrane-aerated biofilms for high rate biotreatment: Performance appraisal, engineering principles, scale-up, and development requirements. Environmental Science & Technology. **42**(6): 1833-1844.

Tan, S. Y., Yu, T. and Shi, H. C. 2014. Microsensor determination of multiple microbial processes in an oxygen-based membrane aerated biofilm. Water Science and Technology. **69**(5): 909-914.

Tan, S. Y. 2012. Multiple microbial processes in membrane aerated biofilms studied using microsensors. PhD Thesis, University of Alberta. Edmonton, Canada.

Tan, S. Y. and Yu, T. 2007. Fabrication of an amperometric $H_2S$ microsensor and its application in wastewater biofilms. Proceedings of International Workshop on Monitoring and Sensors for Water Pollution Control, Beijing, China.

Terada, A., Hibiya, K., Nagai, J., Tsuneda, S. and Hirata, A. 2003. Nitrogen removal characteristics and biofilm analysis of a membrane-aerated biofilm reactor applicable to high-strength nitrogenous wastewater treatment. Journal of Bioscience and Bioengineering. **95**(2): 170-178.

Throback, I. N., Enwall, K., Jarvis, A. and Hallin, S. 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology. **49**(3): 401-417.

Timberlake, D. L., Strand, S. E. and Williamson, K. J. 1988. Combined aerobic heterotrophic oxidation, nitrification and denitrification in a permeable support biofilm. Water Research. **22**(12): 1513-1517.

Tolker-Nielsen, T. and Molin, S. 2000. Spatial organization of microbial biofilm communities. Microbial Ecology. **40**(2): 75-84.

Tsushima, I., Kindaichi, T. and Okabe, S. 2007. Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. Water Research. **41**(4): 785-794.

Vanloosdrecht, M. C. M. and Heijnen, S. J. 1993. Biofilm bioreactors for wastewater treatment. Trends in Biotechnology. **11**(4): 117-121.

von Wintzingerode, F., Gobel, U. B. and Stackebrandt, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews. **21**(3): 213-229.

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y. and Gevertz, D. 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Applied and Environmental Microbiology. **62**(5): 1623-1629.

Wagner, M. and Loy, A. 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology. **13**(3): 218-227.

Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A. and Stahl, D. A. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. Journal of Bacteriology. **180**(11): 2975-2982.

Wang, X. H., Wen, X. H., Criddle, C., Wells, G., Zhang, J. and Zhao, Y. 2010. Community analysis of ammonia-oxidizing bacteria in activated sludge of eight wastewater treatment systems. Journal of Environmental Sciences-China. **22**(4): 627-634.

Waters, D. L. E. and Shapter, F. M. The Polymerase Chain Reaction (PCR): General Methods. Cereal Genomics: Methods and Protocols. R. J. Henry and A. Furtado. Totowa, Humana Press Inc: 65-75.

Xia, X., Zhang, X., Feng, H. and Zhao, L. 2010. Diversity and quantification analysis of functional genes in a lab scale denitrifying quinoline-degrading bioreactor. Weishengwu Xuebao. **50**(12): 1613-1618.

Yamagiwa, K., Ohkawa, A. and Hirasa, O. 1994. Simultaneous organic-carbon removal and nitrification by biofilm formed on oxygen enrichment membrane. Journal of Chemical Engineering of Japan. **27**(5): 638-643.

Yamagiwa, K., Ohkawa, A. and Hirasa, O. 1994. Simultaneous organic-carbon removal and nitrification by biofilm formed on oxygen enrichment memrane. . Journal of Chemical Engineering of Japan. **27**(5): 638-643.

Yu, T. 2000. Stratification of microbial process and redox potential changes in biofilm. PhD Thesis, University of Cincinnati. Cincinnati, USA.

Yu, T. and Bishop, P. L. 2001. Stratification and oxidation-reduction potential change in an aerobic and sulfate-reducing biofilm studied using microelectrodes. Water Environment Research. **73**(3): 368-373.

Zhang, T. C. and Bishop, P. L. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. Water Environment Research. **68**(7): 1107-1115.

# Chapter 5 Community Structure and In Situ Activity of SRB in Mature Fine Tailings

## 5.1 Introduction

Oil sands tailings ponds in Northern Alberta receive and store wastes from the bitumen extraction process, and contain large amounts of recalcitrant and toxic organics. The current total volume of the fine tailings has exceeded 700 $m^3$, and this volume keeps increasing as mining operations proceed because each cubic meter of mined oil sands uses 3 $m^3$ of water. One of the problems in oil sands tailing ponds is the very slow settlement of fine clay particles. The fine clay particles that stay in suspension in tailings ponds are called mature fine tailings (MFT). It has been estimated that the densification of the fine clay would need over a hundred years (Eckert *et al*., 1996). Technologies being applied to solve the tailings densification problem include physical mechanical processes, chemical amendments, natural processes such as freeze-thaw, and in situ biological treatments (Powter, 2010).

Over the past years, there have been big changes related to the presence of microorganisms and their activities in tailing ponds. It has been indicated that the tailings are not just sitting in tailing ponds but are microbiologically active. Management of MFT has lately turned toward biological treatment technology. Researchers are interested to find whether microorganisms endogenous in MFT

can be utilized to aid tailings densification. Foght *et al*. (1985) detected both aerobic and anaerobic microbes present in Syncrude Canada's Mildred Lake Settling Basin (MLSB) (Foght *et al*., 1985; Li, 2010). However, the activities of the existing microorganisms were very slow and had not been noticed until the early 1990s when gas bubbles were observed at the surface of Syncrude's MLSB tailing ponds. Holowenko *et al*. (2000) reported that 60 % - 80 % of the observed gas flux to the atmosphere across the surface area of MLSB was methane. It has been estimated that a daily flux of 12 g $CH_4$ $m^{-2}$ was produced in a single pond, evidence of a very active methanoAZgenesis in the pond. Several studies investigated the relationship between methanogenesis and MFT densification. Fedorak *et al*. (2003) demonstrated that the MFT densification rate can be significantly accelerated due to the produced $CH_4$ gas by microbial activity of methane-producing archea, known as methanogens. Guo (2009) studied the influence of microbial activity on rapid MFT densification. It was found that water drainage volumes from MFT was improved due to the increase of microbial activity and the resulting gas generation.

In some experiments tailing were mixed with gypsum ($CaSO_4 \cdot 2H_2O$) to yield "consolidated" or "composite" tailings (CT) which would accelerate the densification rate of the solids (List and Lord, 1997). As a result, the addition of $SO_4^{2-}$ would facilitate the growth of SRB and sulfate reduction process in MFT. During the biological sulfate reduction process, $H_2S$ could be generated. The produced toxic $H_2S$ itself is one of the concerns (Barton and Fauque, 2009); in

addition, the $H_2S$ promotes corrosion of facilities (Pol *et al*., 1998), and the produced $H_2S$ might transport toxic organics such as NAs from MFT to cap water. All of these concerns will hinder the efficiency of landscape remediation of oil sands tailing ponds.

Up to now, most studies have focused on the sulfate inhibition of methanogenesis (Holowenko *et al*., 2000; Salloum *et al*., 2002; Ramos-Padron *et al*., 2011). To the best of our knowledge, there is little information regarding the effect of gypsum on the community characteristics and the in situ activity of SRB in MFT. And the in situ $H_2S$ data is missing in oil sands tailing ponds. Microsensors allow for the measurements of chemical variables with high spatial resolution in microbial environment (Revsbech and Jorgensen, 1986), while molecular techniques provide a way to investigate the presence and functional diversity of specific populations without isolation (Sanz and Kochling, 2007). This study represents the first attempt to apply combined technologies in MFT microbial environment in order to investigate the effects of gypsum on the *in situ* microbial activity and functional diversity of SRB.

## 5.2 Material and Methods

### 5.2.1 Experimental Setup

Two settling columns (column A and column B) were constructed for the development of microbial stratified MFT. A schematic drawing of a settling column is shown in Figure 5-1.

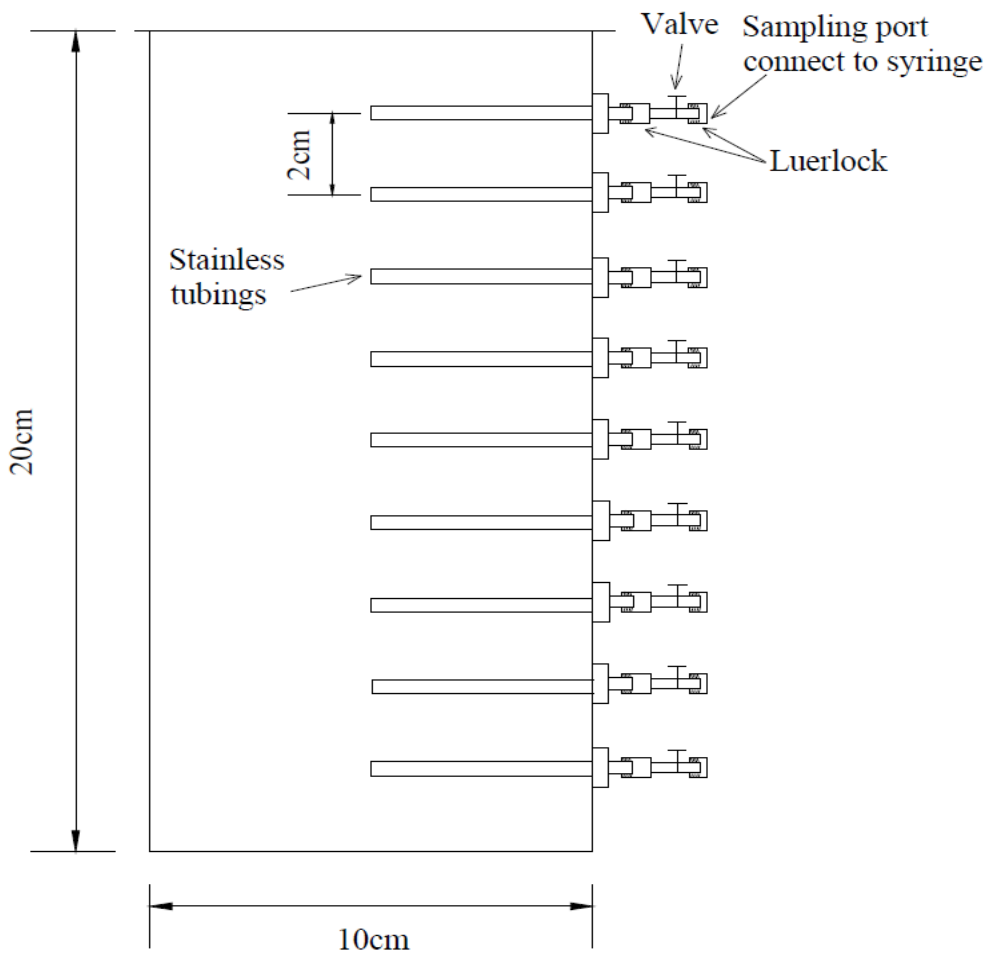



**Figure 5-1. Schematic Drawing of Settling Column**

The columns were made from acrylic material. The cylinder column was 10 cm in diameter and 25 cm in height, had a total volume of around 2 L, and contained nine sampling ports on its wall. The sampling ports were constructed with luer locks connected with stainless steel tubing extended into the center of column. At sampling time, the valve would be turned on, and a 5 mL syringe

would be connected to the luer lock to take samples. The columns were filled with a mixture of 1300 mL MFT and 200 mL OSPW, and one of the columns was amended with 1.5 g gypsum in order to facilitate the growth of SRB. MFT and OSPW samples were provided by an oil sands company in Fort McMurray, Alberta, Canada. The mixture in the column was allowed to settle for eight months. After eight months' settling, six samples were taken from sampling ports #1, #2, #3, #4, #5, #6 (shown in Figure 5-2) from column A and column B, respectively

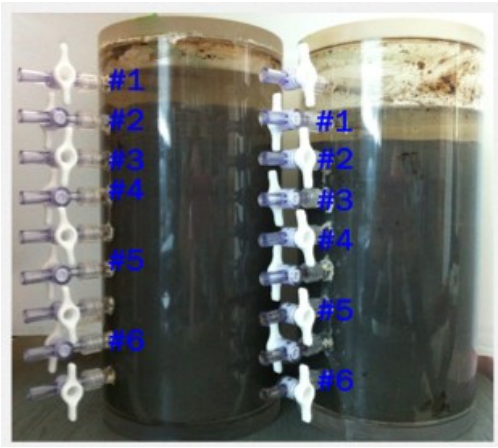



**Figure 5-2. Photos of Settling Columns after Eight Months**

### 5.2.2 $H_2S$ and $O_2$ Microsensors Measurement Methods

Combined amperometric $H_2S$ microsensors ($H_2S$-100) with a tip diameter of 100 μm and combined amperometric $O_2$ microsensors (OX-50) with a tip

diameter of 50 μm were purchased from Unisense (http://www.unisense.com), Denmark. Before measurement, the $H_2S$ microsensor was evaluated by creating a five-point calibration curve using a dilution series (0, 100 μM, 400 μM, 2500 μM, and 10000 μM) of $Na_2S$. The calibration curve is shown in Figure 5-3; while the $O_2$ microsensor was polarized and calibrated in water according to the method described by a previous study (Tan, 2012). The calibration was conducted with nitrogen gas (0% oxygen, DO: 0 $mg \cdot L^{-1}$) and compressed air (21% oxygen, DO: 8.36 $mg \cdot L^{-1}$). During measurements, the microsensor was mounted on a micromanipulator (Model M3301R, World Precision Instruments, Inc., Sarasota FL, USA) so that the tip of the microsensor was positioned at the interface of the cap water and MFT. The tip of the $H_2S$ microsensor was advanced into the MFT through 1 mm movements, while the $O_2$ microsensor was advanced into the MFT through 50 to 100 μm movements of the micromanipulator. Figure 5-4 shows an illustration of the microsensor setup.

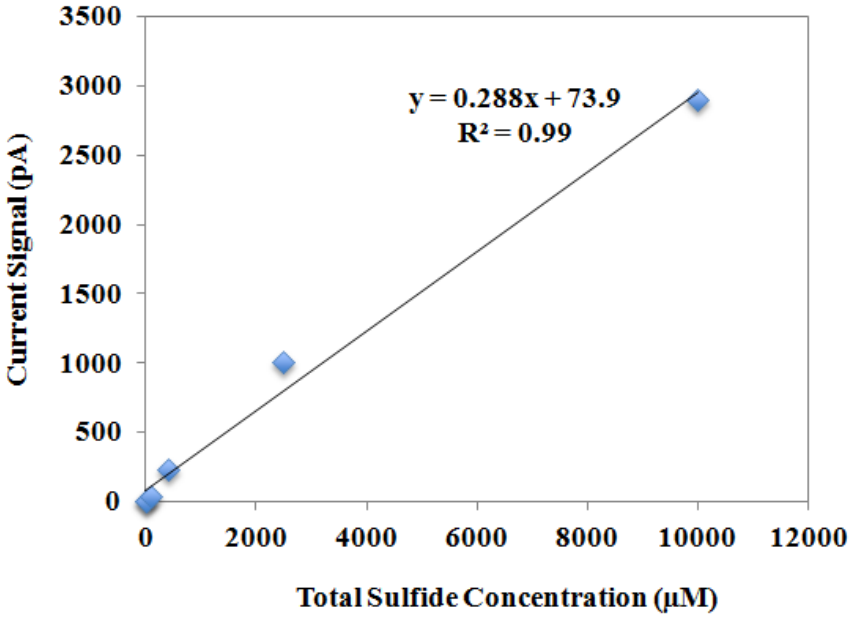



**Figure 5-3. Calibration Curve for $H_2S$ Microsensor**

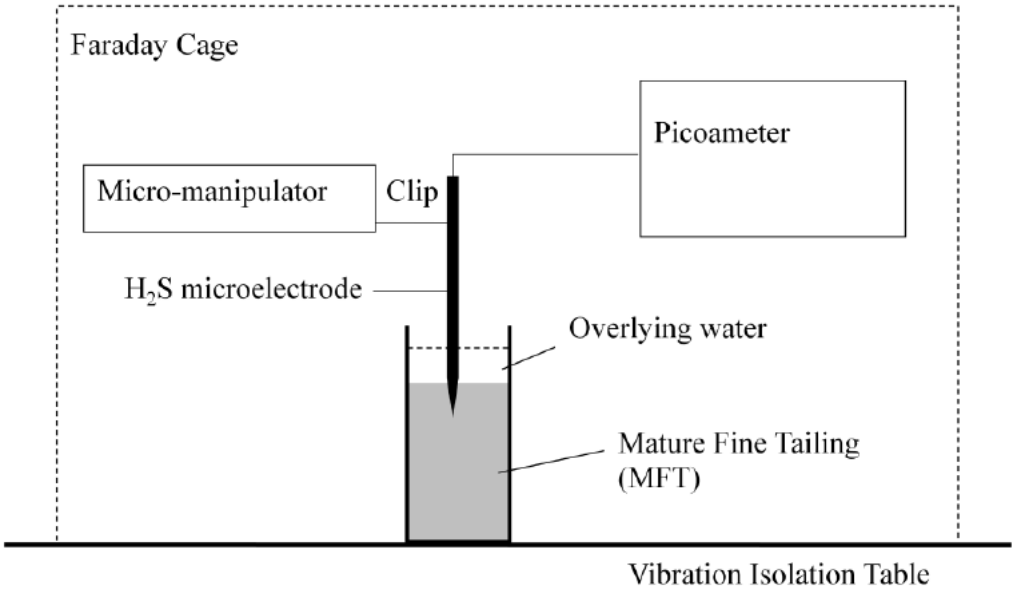



**Figure 5-4. Illustration of Microsensor Measurement Setup**

### 5.2.3 DsrB Genes Based DGGE

Following microsensor measurement, the MFT samples taken from the sampling ports were subjected to DNA extraction and molecular biology analysis to investigate the functional diversity of SRB in the MFT microbial environments. For each sample, DNA was extracted using the Power Soil Kit (Mo-Bio, Carlsbad, CA) following the protocol provided by the manufacture. A set of primers (dsr4R: 5'-GTGTAGCAGTTACCGCA-3' and dsr2060F: 5'-CAACATCGTYCACCAGGG-3') targeting the dsrB genes were used to amplify gene fragments of SRB.

Amplifications were performed in a total 50 μL volume reaction mixture that consisted of 2 μL DNA template (approximately 35 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 1 μL of each primer at 25 μM, 0.2 mM dNTP mix, and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA). DsrB gene fragments were amplified using the following PCR program: initial denature of the DNA at 94 ºC for 5 min, followed by 10 cycles of 45 s at 94 ºC, 45 s at 65 ºC and 1 min at 72 ºC. The annealing temperature was decreased by 1 °C every cycle from 65 ºC to 55 ºC, then 35 cycles of 45 s at 94 ºC, 45 s at 55 ºC, and 1 min at 72 ºC. The final extension was 7 min at 72 ºC.

DGGE was performed in a Bio-Rad D-code system. The amplified products were separated with DGGE on a 16 cm × 16 cm, and 1 mm thick gel in TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) buffer at 60 °C. To separate dsrB gene amplicons, 7.5% (w/v) polyacrylamide gel with denaturing gradient

from 0% to 80% was run at 200 V for 6 h. Gels were visualized with ethydium bromide (EB) and captured with a UV transilluminator imaging system (Viber Lourmat, France). The DGGE electrophoresis was performed in TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) at 60 °C.

### 5.2.4 Real-time qPCR of dsrB Gene

Real-time qPCR amplification and detection were performed in a CFX 96-well PCR System with a C1000 Thermal cycler (Bio-Rad Laboratories, Inc.). The amplification program was the same as it for PCR amplification mentioned above for dsrB gene amplification. Each 20 μL qPCR mixture contained 10 μL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 10 pmol of each forward and reverse primer, and 2 μL of DNA template.

Standard curves were constructed with serial dilutions of known amounts of dsrB gene, which was amplified with dsr4R/dsr2060F primers from a pure culture of *Desulfovibrio desulfuricans* and quantified with a NanoDrop spectrophotometer. Serial dilutions covered a range of 8 orders of magnitude of template copies per assay ($10^2$ to $10^9$). The $r^2$ range of the standard curves obtained by the real-time PCR measurements was 0.996. The qPCR measurements were done in triplicate. Using the standard curve, we determined the dsrB gene copy numbers of MFT samples.

## 5.3 Results and Discussion

### 5.3.1 MFT Settlement

When the settling began, the interfaces of the overlying water and sediment for the two columns were at the same level. After eight months' settling, it can be seen from Figure 5-2 that the interface of the overlying water and sediment for the gypsum amended MFT column was lower than that of unamended MFT column. Moreover, much more gas-generated void space (marked with yellow circles) was observed in the gypsum amended MFT column than in the unamended MFT column shown in Figure 5-5.

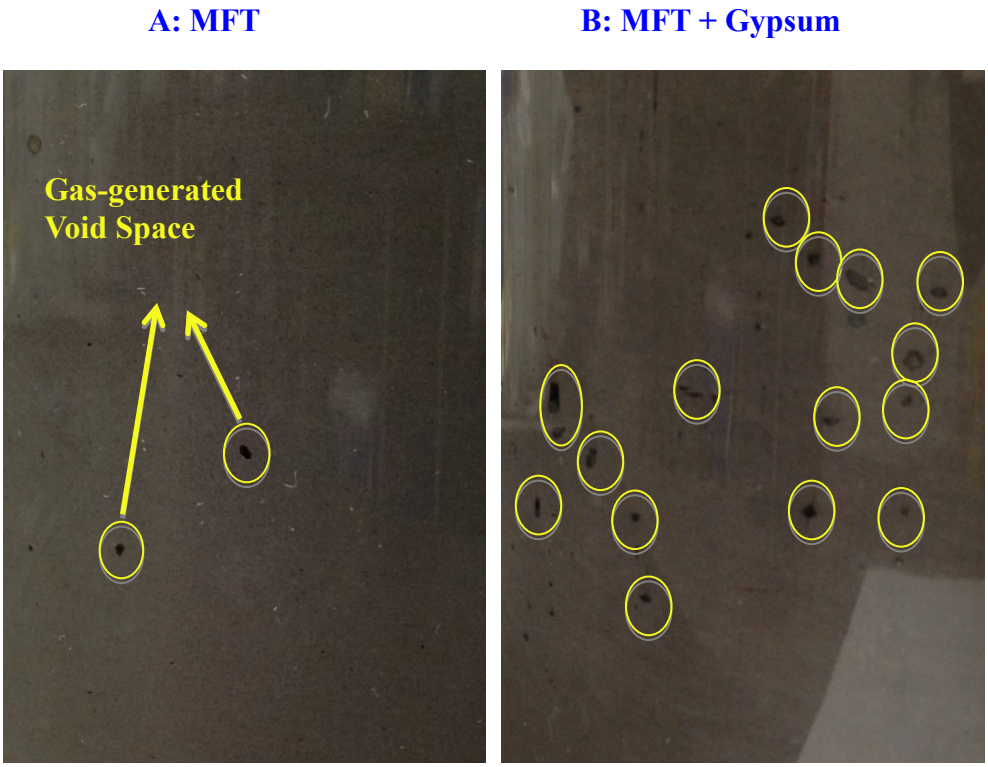



**Figure 5-5. Observed Gas-generated Void Space**

Although the mechanisms of the settlement of tailings are not thoroughly understood due to the comprehensive physical, chemical and microbial activities involved during settling, results observed in this study were comparable to previous research findings. First of all, the lowered interface of overlying water and sediment in the gypsum amended MFT column indicated that the addition of gypsum to the MFT accelerated the densification rate of tailings and released more water. In industry, gypsum as a coagulant was mixed with MFT and coarse sands to generate composite/consolidated tailings (CT) ponds, where, over time it will become more non-segregating and water will be released (Caughill *et al.,* 1993); furthermore, the observed gas bubbles trapped in the gypsum amended MFT were considered as products of microbial activity. Previous studies have demonstrated that microbial activity accelerates the sedimentation rate by producing gas, which creates gas channels and allows water release through pore pressures in deeper tailings (Fedorak *et al*., 2003; Guo, 2009). Microbes can absorb to the surface of fine clays, resulting in solid aggregations and subsequently enhanced sedimentation.

### 5.3.2 In Situ Activity of SRB

The profiles of $O_2$ and $H_2S$ as a function of depth are plotted in Figure 5-6 and Figure 5-7. The DO concentrations in bulk water in both columns were around 4.7 mg/L, and was gradually consumed and then depleted from the surface to the deeper zone. The $O_2$ concentration profile in Figure 5-6 reveals that $O_2$ in MFT without added gypsum was depleted at 1700 μm from the interface of bulk

water and MFT sediment; while the $O_2$ in MFT with added gypsum was depleted at 3200 μm from the interface of bulk water and MFT sediment. Results indicated the oxic and anoxic zone in these two column samples, and the oxic zone is a very shallow layer near the interface of overlying water and sediment. In the meantime, the $O_2$ profile in gypsum amended MFT was lifted in the upper side compared to the $O_2$ profile in MFT, indicating that more of the $O_2$ in gypsum amended MFT was consumed than that in MFT without gypsum, which might attributed to a greater consumption of $O_2$ due to the re-oxidation of produced $H_2S$ in gypsum amended MFT.

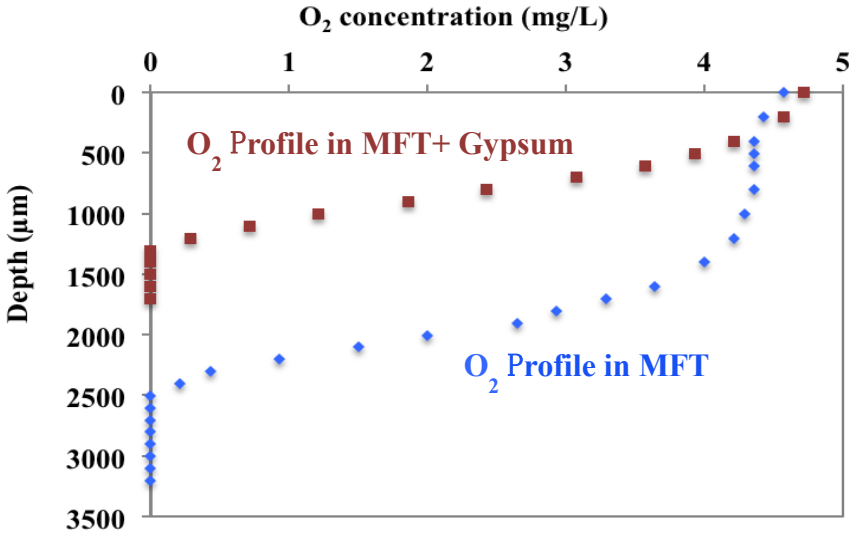



**Figure 5-6. Microsensor Profiles of $O_2$ in MFT and in Gypsum-amended MFT**

The $H_2S$ profiles along the depth of the two samples were used to indicate the *in situ* sulfate reduction activity. As displayed in Figure 5-7, the $H_2S$

concentration gradually increased along the depth of the column, which indicated that a higher sulfate reduction activity occurred in the deeper zones of both samples. And apparently, much more $H_2S$ was detected in the gypsum amended MFT sample than in the unamended sample.

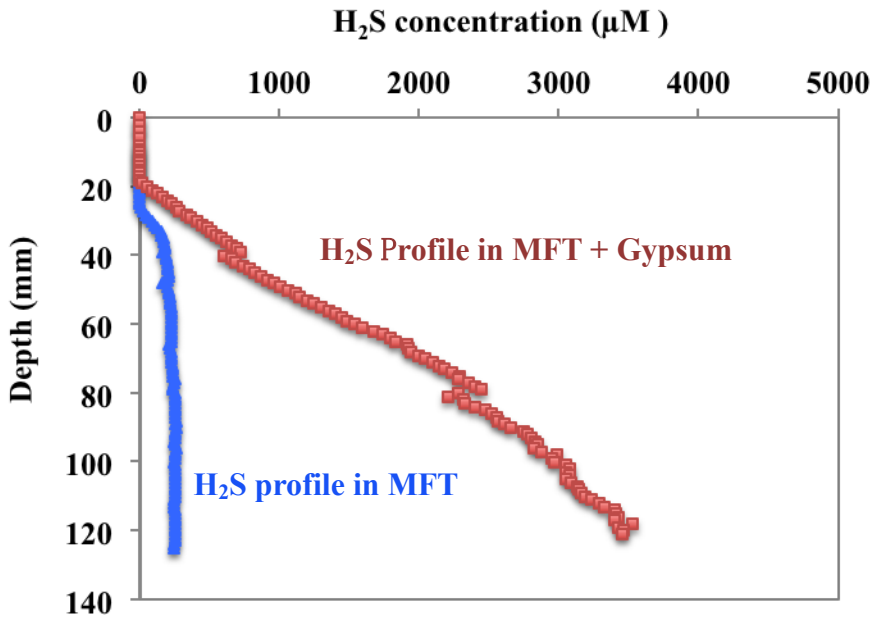



**Figure 5-7. Profile of $H_2S$ in MFT and in Gypsum Amended MFT (0 mm Indicated the Interface Between MFT and Water Cap)**

Results indicate that the addition of gypsum stimulated the *in situ* activity of SRB. Moreover, it should be noted that no $H_2S$ was detected in the surface zone of the tailings (from the interface of tailings and overlying water at 0 mm in the Y axis to around 20 mm below the interface), $O_2$ and $H_2S$ gradients did not overlap within the sediment in this zone, which indicated that no $H_2S$ was detected

although the sediment appears in anoxic condition based on $O_2$ concentration profile. Firstly, it should be considered that the oil sands mining tailing waste contain a variety of organics and heavy metals; there is a substantial opportunity for sulfur oxidative and reductive reactions in the tailing samples. Reacting with metals could deplete the produced $H_2S$ and form precipitates as metal sulfides. In addition, based on previous report that some SRB species such as *Desulfobulbus spp.* can oxidize reduced sulfur compounds to sulfate using $O_2$ or $NO_3^-$ as the electron acceptor (Dilling and Cypionka, 1990; Dannenberg *et al*., 1992); it is also possible that the produced $H_2S$ might be re-oxidized, converted to sulfate and other intermediates with the availability of oxygen or nitrate near the interface of overlying water and sediment.

### 5.3.3 Functional Diversity and Vertical Distribution of SRB

DGGE fingerprints of PCR-amplified dsrB genes in MFT and gypsum amended MFT are shown in Figure 5-8. The multiple bands indicates that diverse SRB were present in both MFT and gypsum amended MFT. As shown in left side of Figure 5-8, for MFT column, the DGGE bands showed differences in terms of band numbers and intensities for samples from the six sampling ports; moreover, the sample from port #1 has the lowest band number and intensity, suggesting a lower SRB activity there as compared to the deeper zones within the sediment of the tailings. Similar trends can be observed for the gypsum amended MFT column. In addition, the intensity of the bands can be used to estimate the amount of the bacteria: the more intense the band, the more bacteria are estimated to be present.

Comparing the bands intensity for MFT and gypsum amended MFT, it can be estimated that more SRB existed in the gypsum amended MFT.

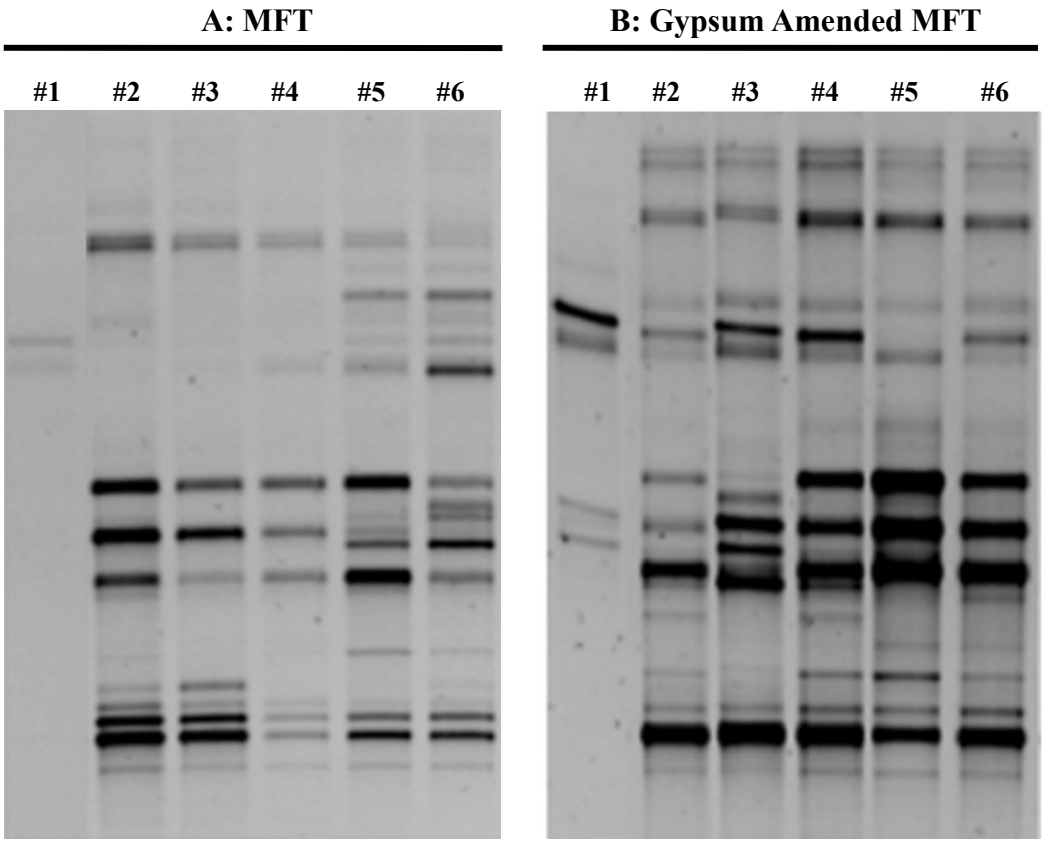
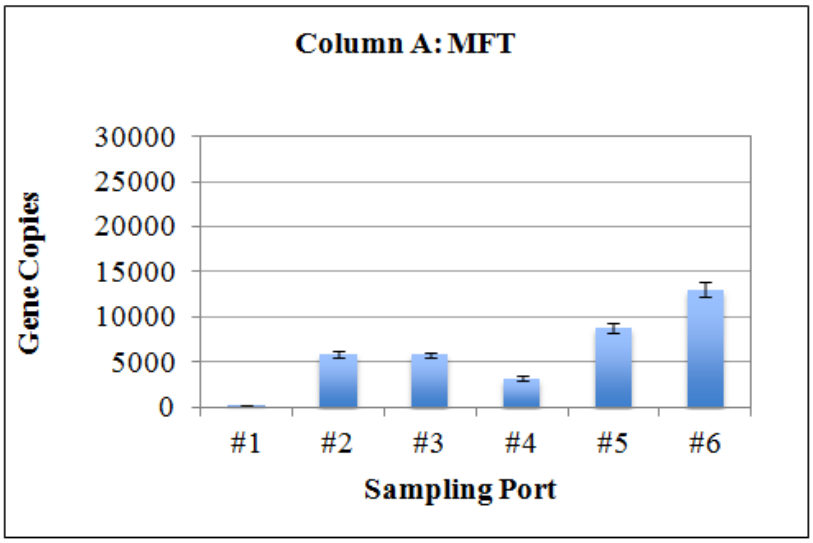



**Figure 5-8. DsrB Genes-based DGGE Analysis of Functional Diversity of SRB in MFT and Gypsum Amended MFT**

The abundance of dsrB gene was then detected by quantitative real-time PCR for samples at different depths from both columns. The qPCR results were shown in Figure 5-9. The SRB abundance indicated clear differences in terms of

dsrB gene copies between samples in MFT and gypsum amended MFT, and also between samples at different depths for each column.



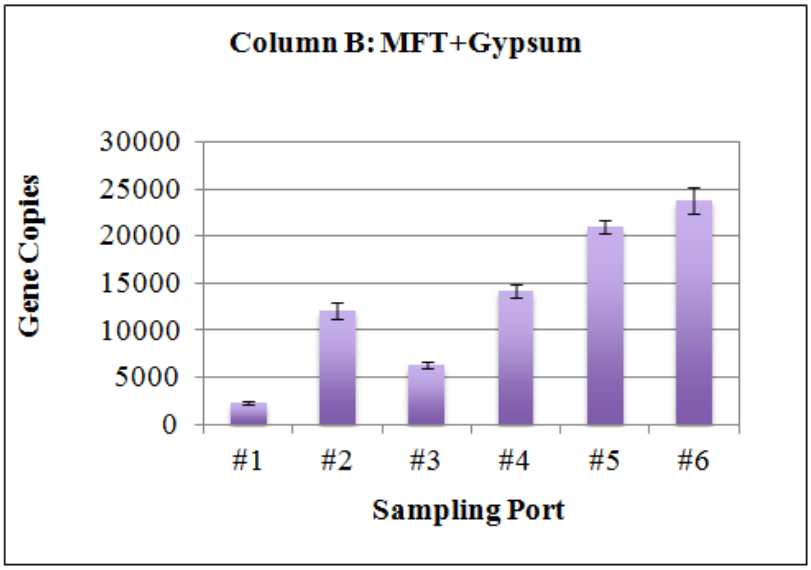




**Figure 5-9. qPCR of dsrB Gene in MFT and Gypsum Amended MFT**

Results indicated that the amendment of gypsum stimulated the growth of SRB; furthermore, the deeper in the column, the greater abundance of SRB except

for samples from port #2 and port #3 in MFT column and the sample from port #2 in the gypsum amended MFT column. It is noticed that these sampling ports were near or within the oxic-anoxic interface. Similar results have been obtained in previous studies (Sass *et al.,* 1997; Sass *et al.,* 2002). It was suggested in these studies that different SRB populations could exist in this interface zone compared to those SRB populations present in deeper zone. The SRB populations in the interface zone might have capacity of aerobic respiration and grow even by utilizing oxygen. Moreover, the high abundance of SRB in these zones might be attributed to more sulfate available. Sulfate has been profiled as a function of depth in tailing ponds (Holowenko *et al*., 2000; Ramos-Padron *et al*., 2011), and it was indicated that the sulfate concentration in the MFT was depth dependent. It was mentioned in both studies that the sulfate concentration was highest at the pond surface near interface of surface water and tailing sediment, and then dropped with increasing depth. This relatively higher sulfate-containing zone presumably would provide more sources for SRB growth.

## 5.4 Conclusions

The overall agreement in the vertical distribution and microbial *in situ* activity of SRB supported the validity of the combined microsensor measurement and molecular biology approaches in complex MFT microbial environments. Based on the combined techniques, we observed that: 1) more $H_2S$ was produced in gypsum amended MFT than that in unamended MFT, indicating a higher sulfate reduction activity in gypsum amended MFT; 2) diversity of SRB were

present in both columns: higher diversity of SRB exist in gypsum amended MFT; 3) the deeper the more active of SRB and the more $H_2S$ produced. The studies expand the fundamental knowledge on the community functions and microbial activities of SRB in MFT, and provide information for further considerations on how gypsum might influence site reclamation plans of MFT.

## References


Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology: 41-98.

Caughill, D. L., Morgenstern, N. R. and Scott, J. D. 1993. Geotechnics of nonsegregating oil sands tailings. Canadian Geotechnical Journal. **30**(5): 801-811.

Dannenberg, S., Kroder, M., Dilling, W. and Cypionka, H. 1992. Oxidation of $H_2$, organic compounds and inorganic sulfur compounds coupled to reduction of $O_2$ or nitrate by sulfate-reducing bacteria. Archives of Microbiology. **158**(2): 93-99.

Dilling, W. and Cypionka, H. 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiology Letters. **71**(1-2): 123-127.

Eckert, W. F., Masliyah, J. H., Gray, M. R. and Fedorak, P. M. 1996. Prediction of sedimentation and consolidation of fine tails. AIChE Journal. **42**(4): 960-972.

Fedorak, P. M., Coy, D. L., Dudas, M. J., Simpson, M. J., Renneberg, A. J. and MacKinnon, M. D. 2003. Microbially-mediated fugitive gas production from oil sands tailings and increased tailings densification rates. Journal of Environmental Engineering and Science. **2**(3): 199-211.

Foght, J. M., Fedorak, P. M. and Westlake, D. W. S. 1985. Microbial content and metabolic activities in the Syncrude tailings pond, Aostra Journal of Research.**1**: 139-146.

Guo, C. (2009). Rapid densification of the oil sands mature fine tailings (MFT) by microbial activity. PhD Thesis, University of Alberta. Edmonton, Canada.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology. **46**(10): 927-937.

Li, C. 2010. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification. Master Thesis. University of Alberta. Edmonton. Canada.

List, B. R. and Lord, E. R. F. 1997. Syncrude's tailings management practices from research to implementation. CIM Bulletin. **90**(1010): 39-44.

Pol, L. W. H., Lens, P. N. L., Stams, A. J. M. and Lettinga, G. 1998. Anaerobic treatment of sulphate-rich wastewaters. Biodegradation. **9**(3-4): 213-224.

Powter, C. B., Biggar, K.W., Silva, M.J., McKenna, G.T and Scordo, E.B. 2010. Review of oil sands tailings technology options. The 14th International Conference on Tailings and Mine Waste: 381-391.

Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I. M., Dong, X., Sensen, C. W., Fournier, J., Voordouw, G. and Gieg, L. M. 2011. Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond. Environmental Science & Technology. **45**(2): 439-446.

Revsbech, N. P. and Jorgensen, B. B. 1986. Microelectrodes - their use in microbial ecology. Advances in Microbial Ecology. **9**: 293-352.

Salloum, M. J., Dudas, M. J. and Fedorak, P. M. 2002. Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Management & Research. **20**(2): 162-171.

Sanz, J. L. and Kochling, T. 2007. Molecular biology techniques used in wastewater treatment: An overview. Process Biochemistry. **42**(2): 119-133.

Sass, A. M., Eschemann, A., Kuhl, M., Thar, R., Sass, H. and Cypionka, H. 2002. Growth and chemosensory behavior of sulfate-reducing bacteria in oxygen-sulfide gradients. FEMS Microbiology Ecology. **40**(1): 47-54.

Sass, H., Cypionka, H. and Babenzien, H. D. 1997. Vertical distribution of sulfate-reducing bacteria at the oxic-anoxic interface in sediments of the oligotrophic Lake Stechlin. FEMS Microbiology Ecology. **22**(3): 245-255.

Tan, S. Y. 2012. Multiple microbial processes in membrane aerated biofilms studied using microsensors. PhD Thesis. University of Alberta. Edmonton, Canada.

# Chapter 6 Community Structure and In Situ Activity of SRB in Oil Sands Process-affected Wastewater Biofilm

## 6.1 Introduction

Bitumen extraction from each cubic meter of the Alberta oil sands requires up to three cubic meters of water, and produces about four cubic meters of slurry wastes consisting mainly of sand, clay, water, unrecovered bitumen, and dissolved inorganic and organic compounds (Fedorak et al., 2002). Under the zero discharge policy of the Alberta government, the slurry wastes have to be stored in tailings ponds onsite. The oil sands process-affected wastewater (OSPW) released from the densification of the slurry wastes is recycled to the bitumen extraction operation. Continuous recycling of tailings pond water concentrates the organic and inorganic compounds in the water (Allen, 2008). The volume of slurry wastes in the oil sands mining operation are continuously accumulating, and it is estimated that over 1 billion $m^3$ of tailings pond water will be produced by 2025 (Quagraine *et al*., 2005). Consequently, treatment of OSPW is a critical challenge for the oil sands industry.

The biofilm technology, as a conventional biological wastewater treatment technology, exhibits considerable advantages such as environmental-friendliness, cost-effectiveness, and strong adaptability to wastewater quality compared to other biological treatments. The multispecies biofilms contain a high cell density

and are metabolically active; therefore their potential for in situ bioremediation of OSPW has been investigated. Studies have characterized the microorganisms involved in the biodegradation of pollutants in oil sands tailings. For instance, Siddique *et al*. (2012) found increased proportions of *Clostridiales* and *Syntrophobacterales* involved in methanogenic degradation of short-chain n-alkanes ($C_6$-$C_{10}$) and BTEX (benzene, toluene, ethylbenzene, and xylenes). An *et al*. (2013) found that *Proteobacteria*, *Euryarchaeota*, *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *and Bacteroidetes* were involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond based on a pyrosequencing survey of 16S rRNA amplicons from 10 samples obtained from different depths.

Golby *et al.* (2011) demonstrated that oil sands' indigenous microorganisms could form a biofilm in the Calgary Biofilm Device (CBD). It was revealed that the biofilm with 1-3 cells layers thickness contain dominant species belonging to *Pseudomonas*, *Thauera*, *Hydrogenophaga*, *Rhodoferax*, and *Acidovorax*. A variety of biofilm reactors have proved useful for treating OSPW. Islam *et al*. (2014) evaluated the treatment of OSPW using a fluidized bed biofilm reactor (FBBR) with granular activated carbon (GAC) as support media. The biofilm with 30-40 μm thickness after 120 day's treatment has a diverse community including *Polaromonas jejuensis*, *Algoriphagus sp*., *Chelatococcus sp*. and *Methylobacterium fujisawaense*. Choi *et al*. (2014) incubated 20-30 μm thickness biofilms in 28 days for treating OSPW. It was revealed that the reactor biofilms

contained *Flavobacterium, Rhizosphere soil bacteria*, *Rhizobium, Azoarcus, Stigmatella aurantiaca, Sulfuritaleahydrogenivorans* and *Actino-bacterium,*

Despite the successful development and characterization of biofilms for OSPW treatment, it should be noted that the biofilms in these studies were thin and were no more than 50 μm after a short period's culture. However, typical wastewater biofilms can be as thick as 1000 μm (Li and Bishop, 2004). Within a thicker biofilm, oxic and anoxic zones could be formed simultaneously, and anaerobic bacteria would develop their microenvironment and play significant roles within the biofilm. Anaerobic SRB in the biofilm is a point of interest for designing and operating biofilm reactors, as it would utilize sulfate as a terminal electron acceptor for the degradation of organic compounds and generating hydrogen sulfide ($H_2S$) during the biological sulfate reduction process. The production of $H_2S$ brings odor problems and may hinder the efficiency of biofilm reactor operation. To the best of our knowledge, the development and the characteristics of biofilm in OSPW are far from clear, and no studies have evaluated the potential of SRB development and function in OSPW biofilms. Therefore, it is necessary to investigate the presence, functional diversity and activity of SRB within the biofilm, in order to promote the beneficial use of biofilms treating sulfate containing OSPW.

Microsensors are innovative experimental tools for the study of complex structure and functions of biofilms. They can quantify local chemistries and determine in situ bacterial activity inside biofilms with a high spatial resolution

without destruction of biofilm samples; while molecular techniques provide a way to investigate the presence and functional diversity of specific populations without isolation.

The aim of this study was to develop a thicker biofilm in an engineered biofilm reactor and to investigate the presence, activity and functional diversity of SRB in the biofilm. The dissimilatory sulfite reductase subunit B (dsrB) gene based PCR-DGGE and qPCR were used to study the functional diversity of SRB; while $H_2S$, pH, ORP and $O_2$ microsensor measurements were employed to obtain chemical profiles for predicting the activity of SRB and their microenvironment conditions within the biofilm. The obtained information will ultimately guide the rational optimization of biofilm reactor design, further promoting effective biological wastewater treatment for OSPW.

## 6.2 Material and Methods

### 6.2.1 Biofilm Samples

Two identical 1 L glass jars (Catalog No: 02-912-313, Fisher Scientific) were used as reactor vessels. A schematic drawing of a reactor is shown in Figure 6-1. The reactor is a vessel 9 cm in diameter and 15 cm in height. Four bio-carriers for biofilm attachment were strung together on each of four rods which were fastened at one end to the lid of the bioreactor. Two of the rods held bio-carriers that are commonly used in moving bed biofilm reactors (MBBRs), and two rods held modified MBBR bio-carriers. The modified biocarriers were

prepared by trimming the inside of a normal MBBR bio-carrier and inserting a flat substratum with more roughness on one side. Both reactors were run at room temperature on a shaker with a speed of 150 r.p.m.

One reactor (R1) held 600 mL raw OSPW; the other reactor (R2) held 300 mL OSPW supplemented with 300 mL external growth medium R2B containing 0.525 g/L peptone, 0.35 g/L yeast extract, 0.35 g/L dextrose, 0.35 g/L starch, 0.21 g/L $K_2HPO_4$, 0.21 g/L sodium pyruvate, 0.175 g/L tryptone, and 0.0168 g/L $MgSO_4$. Reactor operation was started by immersion in the water of two biocarriers that had been stored in tailings sludge for over one year; under shaking condition, the microorganisms attached to the biocarriers will transfer to the other two biocarriers in the reactor and form a biofilm layer.

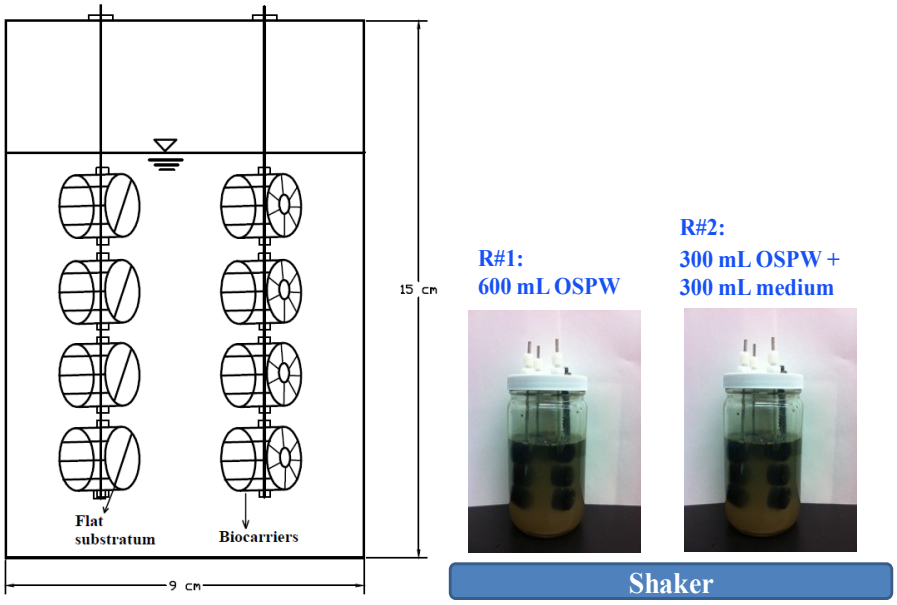



**Figure 6-1. Schematic Diagram and Photos of OSPW Biofilm Reactor**

### 6.2.2 Chemical Analysis

Before draining the water from the reactors, 50 mL samples were filtered with 0.22 μm membrane and preserved at 4 ℃ for chemical analysis. The chemical parameters including COD, sulfate, ammonium, nitrate and total nitrogen were determined. Data are provided in Appendix B.

### 6.2.3 $H_2S$, $O_2$, pH, and ORP Microsensors Measurement

Amperometric microsensors, including an $H_2S$ ($H_2S$-50) microsensor with tip diameter of 50 μm and $O_2$ (OX-50) microsensor with a tip diameter of 50 μm, were purchased from Unisense, Denmark (http://www.unisense.com). $H_2S$ and $O_2$ microsensors were calibrated before measurement as described in Tan and Yu 2007. A five-point calibration (Figure 6-2) of the $H_2S$ microsensor was performed in a $Na_2S$ dilution series (0–10 mM) of according to the method mentioned in Tan (2012). The $O_2$ microsensor was polarized and calibrated in water according to the method mentioned in Tan and Yu (2007). The calibration was conducted with nitrogen gas (0 % oxygen, DO: 0 $mg \cdot L^{-1}$) and compressed air (21 % oxygen, DO: 8.36 $mg \cdot L^{-1}$).

Potentiometric ORP and pH microsensors were fabricated, calibrated and applied according to the procedure reported in Yu (2000). An Ag/AgCl microreference electrode (Cat. # MI-409, Microelectrodes Inc., USA) was used as the reference electrode. Figure 6-3 shows the response curves of the ORP

microsensor against standard and reference solutions. Figure 6-4 shows pH microsensor calibration curves at pH 5, pH 6, pH 7, and pH 8.

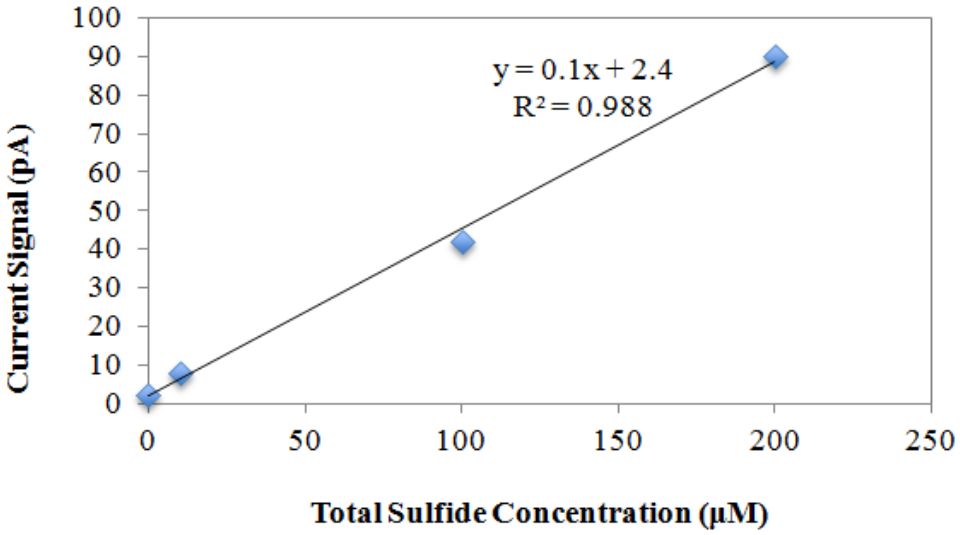



**Figure 6-2. Calibration Curves of $H_2S$ Microsensor**

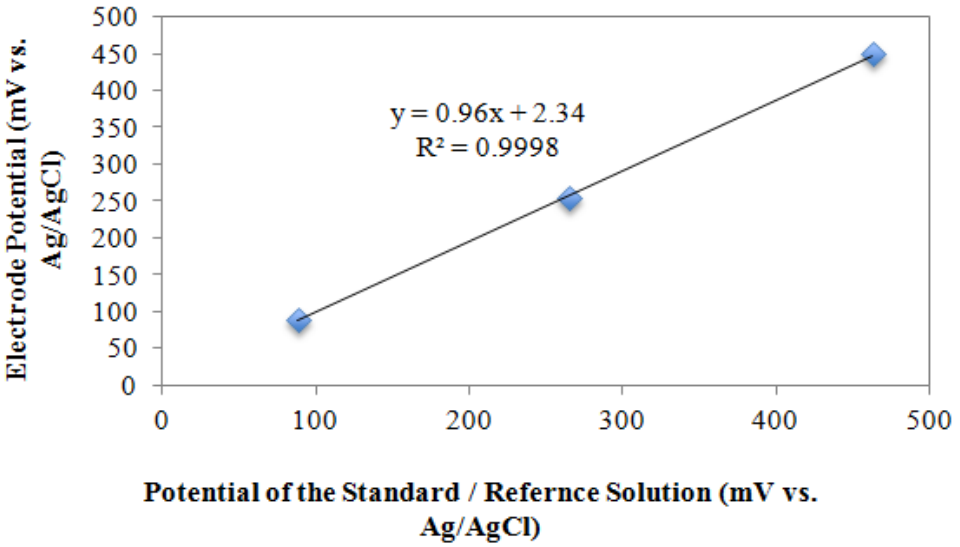



**Figure 6-3. Potential Response of ORP Microsensor in Standard Solutions**

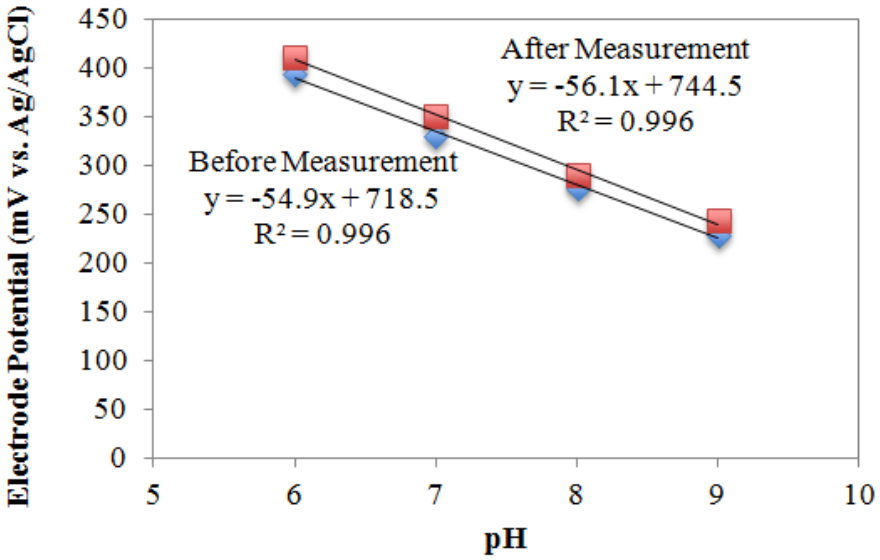



**Figure 6-4. Calibration Curves of pH Microsensor**

Immediately before microsensors measurements, the flat substratum biocarrier was taken out of the reactor and put into a culture plate. 50 mL of water from the reactor was slowly poured into the culture plate to fully immerse the biofilm in the water. The biofilm was immediately subjected to microsensors measurements.

During amperometric $H_2S$ and $O_2$ microsensors measurements, the microsenor was mounted on a micro-manipulator (Model M3301R, World Precision Instruments, Inc., Sarasota FL, USA), and the tip of the microsensor was positioned just above the interface between the bulk water and the biofilm surface. The microsensor tip was then advanced into the biofilm in steps of 50 µm to 100 µm by operating the micro-manipulator. The signal was recorded by a picoammeter (PA2000, Unisense, Denmark).

During potentiometric ORP and pH microsensor measurements, the reference electrode tip was dipped into the water in the culture plate, and the microsensor tip was positioned just above the interface between the bulk water and the biofilm surface. The potential signals were read from an electrochemical analyzer (Model 600B, CH Instruments).

### 6.2.4 Nucleic Acid Extraction and dsrB Gene Based PCR-DGGE

Genomic DNA of biofilm bacteria growing on the biocarrier were extracted using the PowerSoil Kit (Mo-Bio, Carlsbad, CA) following the protocol provided by the manufacturer. Approximately 0.5 g of wet biofilm sample was taken from each reactor (R1 and R2). A set of primers (dsr4R: 5'-GTGTAGCAGTTACCGCA-3' and dsr2060F: 5'-CAACATCGTYCACCAGGG-3') targeting the dsrB gene were used to amplify gene fragments of sulfate reducing bacteria (SRB).

Amplifications were performed in a total 50 μL volume reaction mixture that consisted of 2 μL DNA template (approximately 35 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 1 μL of each primer at 25 μM, 0.2 mM dNTP mix, 2.5% of DMSO per reaction, and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA). DsrB gene fragments were amplified using a temperature gradient touchdown PCR program: initial denature of the DNA at 94 ºC for 5 min, followed by 10 cycles of 45 s at 94 ºC, 45 s at 65 ºC and 1 min at 72 ºC. The annealing temperature was decreased by 1 °C every cycle from 65 ºC to 55 ºC, then 35 cycles of 45 s at 94 ºC, 45 s at 55 ºC, and 1 min at 72 ºC. The final extension was 7 min at 72 ºC.

DGGE was performed in a Bio-Rad D-code system. The amplified products were separated with DGGE on a 16 cm × 16 cm, and 1 mm thick gel in TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) buffer at 60 °C. To separate dsrB gene amplicons, 7.5% (w/v) polyacrylamide gel with denaturing gradient from 0% to 80% was run at 200 V for 6 h. Gels were visualized with SYBR Green I nucleic acid gel stain (1:10000 dilution), and captured with a UV transilluminator imaging system (Viber Lourmat, France).

### 6.2.5 qPCR of the dsrB Gene

Real-time qPCR amplification and detection of the dsrB gene were performed in a CFX 96-well PCR System with a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). The 20-µl qPCR mixtures contained 10 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 10 pmol of each forward and reverse primer, and 2 µL of DNA template. The amplification program was the same as in PCR amplification for dsrB gene amplification.

Standard curves were constructed with serial dilutions of known amounts of dsrB gene, which was amplified with dsr4R/dsr2060F primers from a pure culture of *Desulfovibrio desulfuricans* and quantified with a NanoDrop spectrophotometer. Serial dilutions covered a range of 8 orders of magnitude of template copies per assay ($10^2$ to $10^9$). The $r^2$ range of the standard curves obtained by the real-time PCR measurements was 0.992. The qPCR

measurements were done in triplicate. Using the standard curve, we determined the dsrB gene copy numbers of OSPW biofilm samples.

## 6.3 Results and Discussion

### 6.3.1 Reactor Operation and Biofilm Development

Reactor 1 (R1) and Reactor 2 (R2) were operated in semi-batch scale. Water in R1 and R2 was drained off and replaced with new water four to five times a month at the initial stage, and once a month at the last stage of the reactor operation. After around six months operation, attached cells were observed spread over the biocarrier surfaces in R1 (Figure 6-5); while a relatively thicker biofilm was formed on the surfaces of the biocarriers in R2.


**Figure 6-5. Photos of Biofilm Formed in Biocarriers in Reactor 1 and Reactor 2**

To some extent, the removal of COD, sulfate and ammonia was observed in R2, although the design of this reactor was to develop stratified biofilm to study SRB instead of improving the treatment performance. For example, during the

last months' continuous reactor operation, a maximum removal rate (7 %) of COD was observed in R1, while nitrogen and sulfate were almost constant. In R2, COD was reduced to 68 mg/L, total nitrogen decreased from 92 mg/L to 45 mg/L, and sulfate concentration reduced from 104 mg/L to 84 mg/L. These monitored parameters indicated the occurrence of simultaneous microbial processes of nitrification, denitrification, and sulfate reduction within the OSPW biofilm.

### 6.3.2 Microbial Activity of SRB in the OSPW Biofilm

Figure 6-6 shows profiles of $O_2$, $H_2S$, pH, and ORP measured using microsensors in the 6-month's biofilm in R2. The $O_2$ concentration profile revealed that the $O_2$ concentration was highest near the biofilm surface, and gradually decreased to zero near the bottom of the biofilm. $O_2$ penetrated about 800 μm below the bulk water-biofilm interface in a 1000 μm thick biofilm, and a shallow anoxic zone with around 200 μm thickness was formed in the deeper parts of the biofilm. The production of $H_2S$ in the deep anoxic zone in the biofilm was detected. A gradual increase in $H_2S$ production was observed from around 750 μm to 1000 μm below the water-biofilm interface, revealing in situ sulfate reduction in the deeper zone of the biofilm. Along the entire biofilm depth, the pH changed slightly within 0.3 units.

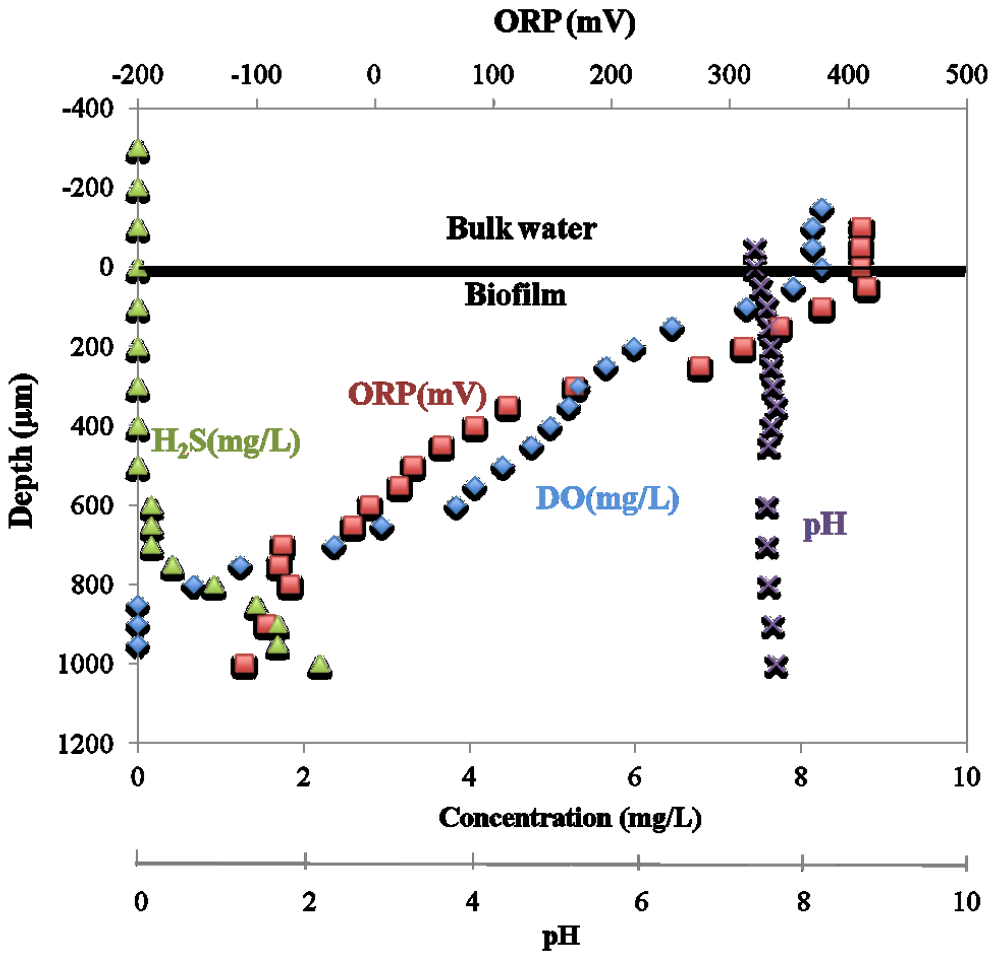



**Figure 6-6. Profiles of $O_2$, pH, ORP, and $H_2S$ in a Piece of OSPW Biofilm. The Interface Between the Biofilm and Bulk Liquid is Indicated by Depth of 0 µm.**

The ORP profile indicates that the redox potential gradually decreased from the surface to the bottom of the biofilm. The initial redox potential near the interface of bulk water and biofilm was around + 410 mV; it decreased to a negative value at a depth of 550 µm and was -110 mV at 1000 µm below the interface. Multiple microbial and chemical activities in a complex multispecies microbial environment will determine the redox potential in the

microenvironment and, conversely, the redox potential in the microenvironment will affect the microbial species and related microbial chemical processes. A redox potential lower than -100 mV is one of the specific environmental requirements for SRB activity (Postgate, 1984). The anoxic condition and redox potential environment developed in the OSPW biofilm made it possible for the growth and function of SRB.

### 6.3.3 Functional Diversity of SRB

Biofilm samples were taken from R2 at 1 month, 2 months, 3 months, 4 months, 5 months, and 6 months to check the development of the SRB community within the biofilm. No band was apparent on an agrose gel after amplification of dsrB gene fragments in 1 month and 2 month biofilm samples. SRB were first detected in biofilm samples taken at 3 months and were present in subsequent 4 month, 5 month, and 6 month samples. DGGE of amplified dsrB gene fragments was performed for biofilm samples taken after 3, 4, 5, and 6 months of bioreactor operation to investigate SRB communities at different stages during biofilm development (Figure 6-7).

The DGGE fingerprint shows that band numbers increased with the time of reactor operation. At 4 months, only one band can be seen on the DGGE gel; after one month of biofilm development, an additional band (band A) appeared. In biofilm samples at 5 months and 6 months, four more bands (band B, C, D, E) can be seen on the DGGE fingerprint, and the band patterns for biofilm samples at 5

months and 6 months remained the same. The multiple bands that appeared on the gel indicated that diverse SRB were developed in the OSPW biofilm.

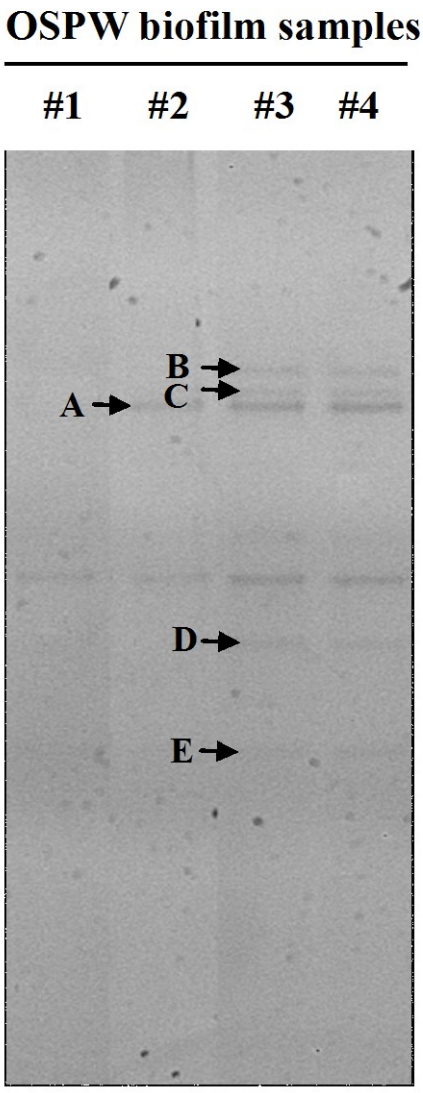



**Figure 6-7. DsrB Gene Based DGGE Fingerprints（#1, #2, #3, #4 Represent the Biofilm Samples Taken at 3 Months, 4 Months, 5 Months, and 6 Months).**

Functional dsrB genes were quantified by quantitative real-time PCR in the four samples (Figure 6-8). The abundance of the amplified dsrB gene of SRB was initially low and gradually increased with time; the abundance of the amplified dsrB gene of SRB in biofilm samples at 5 months and 6 months was around five times the abundance in samples taken at 3 months and 4 months. The results obtained here corresponded well with the functional diversity and in situ activity detected by DGGE and microsensors.

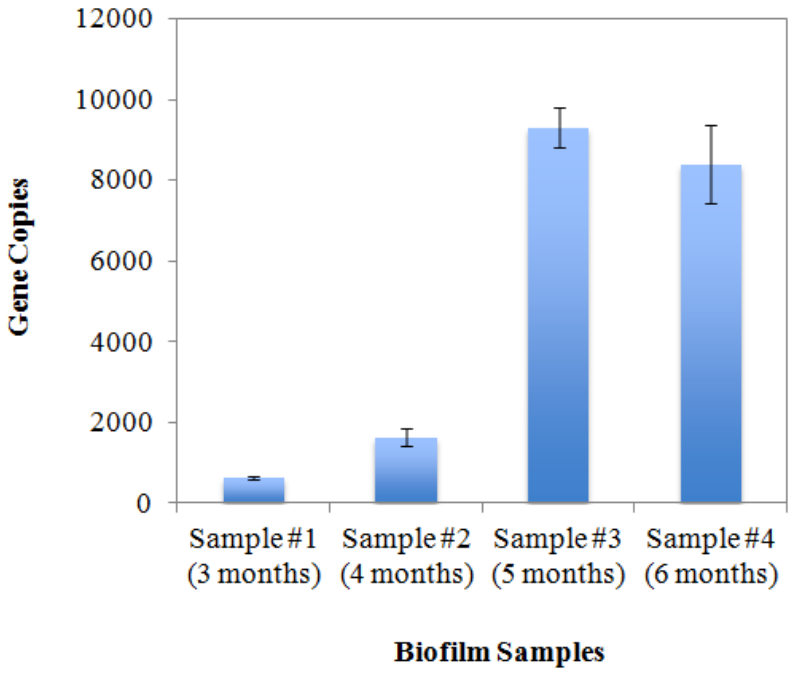



**Figure 6-8. Real-time qPCR of dsrB Genes Amplified from Samples（#1, #2, #3, #4 Represent the Biofilm Samples Taken at 3 Months, 4 Months, 5 Months, and 6 Months).**

## 6.4 Conclusions

Multispecies biofilms were successfully developed on engineered biocarriers. The biofilm cultured in OSPW supplemented with growth medium was capable of simultaneous removal of COD, sulfate, and nitrogen. SRB activity in the deep anoxic zone in the stratified biofilm indicated that the biofilm was potentially capable of managing sulfate-enriched OSPW.

## References

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science. **7**(2): 123-138.

An, D. S., Brown, D., Chatterjee, I., Dong, X. L., Ramos-Padron, E., Wilson, S., Bordenave, S., Caffrey, S. M., Gieg, L. M., Sensen, C. W. and Voordouw, G. 2013. Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. Genome. **56**(10): 612-618.

Choi, J., Hwang, G., Gamal El-Din, M., Liu, Y. 2014. Effect of reactor configuration and microbial characteristics on biofilm reactors for oil sands process-affected water treatment. International Biodeterioration & Biodegradation. **89**: 74-81.

Fedorak, P. M., Coy, D. L., Salloum, M. J. and Dudas, M. J. 2002. Methanogenic potential of tailings samples from oil sands extraction plants. Canadian Journal of Microbiology. **48**(1): 21-33.

Golby, S., Ceri, H., Gieg, L. M., Chatterjee, I., Marques, L. L. R. and Turner, R. J. 2011. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. FEMS Microbiology Ecology. **79**(1): 240-250.

Islam, S.; Dong, T.; Sheng, Z.; Zhang, Y.; Liu, Y. and Gamal El-Din, M. 2014. Microbial community structure and operational performance of a fluidized bed biofilm reactor treating oil sands process-affected water. International Biodeterioration & Biodegradation. **91**: 111–118.

Li, J. and Bishop, P. L. 2004. Time course observations of nitrifying biofilm development using microelectrodes. Journal of Environmental Engineering and Science. **3**(6): 523-528.

Postgate, J. R. 1984. The sulphate-reducing bacteria. Cambridge, UK, Cambridge University Press.

Quagraine, E. K., Peterson, H. G. and Headley, J. V. 2005. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering. **40**(3): 685-722.

Siddique, T., Penner, T., Klassen, J., Nesbo, C. and Foght, J. M. 2012. Microbial Communities Involved in Methane Production from Hydrocarbons in Oil Sands Tailings. Environmental Science & Technology. **46**(17): 9802-9810.

Tan, S. Y. and Yu, T. 2007. Fabrication of an amperometric $H_2S$ microsensor and its application in wastewater biofilms. Proceedings of International Workshop on Monitoring and Sensors for Water Pollution Control, Beijing, China.

Yu, T. 2000. Stratification of microbial process and redox potential changes in biofilm. PhD Thesis. University of Cincinnati. Cincinnati, USA.

# Chapter 7 Conclusions and Recommendations

Three different samples—membrane aerated biofilm (MAB), oil sands process-affected water (OSPW) biofilm and mature fine tailings (MFT)—were studied as three examples of the complex microbial environments of the community structure and microbial activity of sulfate reducing bacteria (SRB). The presence, in situ activity, and functional diversity of the SRB in these complex microbial environments were investigated based on combined technologies of microsensor measurements and molecular biology analysis. The obtained information will ultimately guide the rational optimization of biofilm reactors design and oil sands tailings reclamation plans.

In the MAB sample, the presence of SRB was evidenced co-existing with ammonia oxidizing bacteria (AOB) and denitrifiers. SRB preferred anaerobic microenvironment and were more active in the anaerobic zone within the $O_2$ based MAB. In the OSPW biofilm sample, SRB was detected in the biofilm with a thickness of around 1000 μm, and $H_2S$ was observed in the deeper anoxic zone from around 750 μm to 1000 μm below the interface, revealing in situ sulfate reduction in the deeper zone of the stratified biofilm. In the MFT sample, a higher diversity of SRB was present and more $H_2S$ was produced in gypsum amended MFT than in unamended MFT, and a higher sulfate reduction activity in gypsum

amended MFT than in unamended MFT was indicated; in addition, more $H_2S$ was produced in the deeper regions of the MFT samples.

With the provided fundamental information on SRB in MAB and in OSPW biofilms, we could better evaluate how microorganisms within the biofilm would interact with each other, and how the microbial activities within the biofilm can be optimized to improve reactor performance in practical applications. And the effort on the studies of the fundamental knowledge on the presence, the community structure and *in situ* activities of SRB in MFT would provide information and suggest further assess upon the long-term plan for reclaiming oil sands tailings.

This is the first study that evaluated the presence, *in situ* activity, and the vertical abundance distribution of SRB in MAB, in OSPW biofilm and in MFT microenvironments. The general overall agreement in the community structure and microbial activity of SRB supported the validity and utility of the combined microsensor measurement and molecular biology approaches to better elucidate the microbial dynamics in complex microbial environments.

However, tedious work was involved when developing and applying the combined techniques of microsensor measurement and molecular biology methods in the complex microbial environments, the following future research works are strongly recommended:

1. More microsensors are required to be developed:

In this study, $H_2S$ microsensor was applied to study the *in situ* activity of sulfate reduction. In order to better explain the sulfate reduction process, sulfate microsensor is recommended to be developed. The direct measurement of sulfate combined with $H_2S$ profile would provide more detailed information on sulfur cycle within the microbial environments.

Regarding the study of sulfate reduction in MFT, the methane profile is important to study the impact of SRB microbial activity on methanogenesis. Unfortunately, the development of methane microsensor is quite challenging. A M.Sc student in our research group is currently developing methane microsensor.

2. More information on the community structure and microbial activity of other microorganisms are recommended:

The present study was focused on the community structure and microbial activity of SRB in the three complex microbial environments. In order to investigate how microorganisms interact with each other in these microbial environments, and how the microbial activities within the microbial environments can be optimized to improve reactor performance and MFT recalmation in practical applications, the community structure and microbial activity of other microorganisms such as nitrifiers, denitrifiers, and methanogens are also important. However, the author could only perform and focus on the experiments targeted to SRB. Providing chemical profiles (nitrate, ammonium, nitrite) and community structures of nitrifiers, denitrifiers and methanogens would provide a

more comprehensive picture of multiple microbial processes within complex microbial environments.

3. Microsensor measurements improvement in MFT column:

In the study of SRB in MFT, $H_2S$, $O_2$, and ORP microsensor measurements were performed. It can be noted from $H_2S$ profiles that there was a small drift at depths of around 40 mm and 80 mm, and unfortunately, our obtained ORP data drifted a lot at these depths whthin the MFT, which might be attributed to the technology limitation during microsensor measurement. Usually, microsensor measurements were applied in microenvironments such as in biofilms which are only several millimeters thick. In the MFT column in this case, the measured depth was around 15 mm; therefore, specific elongated microsensors were needed; however, the maximum step that the micromanipulator could realize is around 40 mm, the manipulator has to be put back and the microsensor was re-positioned after each 40 mm depth measurement. During this process, errors and other external interferences might be introduced, and hence affect the measurement results. It was difficult to ignore these limitations with current microsensor technology, an improved microsensor and manipulator setup need to be developed or Field Microprofiling System from Unisense might be applied in order to obtain chemical profiles within MFT samples.

## References

Alleman, J. E., Veil, J. A. and Canaday, J. T. 1982. Scanning electron-microscope evaluation of rotating biological contactor biofilm. Water Research. **16**(5): 543-550.

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science. **7**(2): 123-138.

Allen, E. W. 2008. Process water treatment in Canada's oil sands industry: II. A review of emerging technologies. Journal of Environmental Engineering and Science. **7**(5): 499-524.

Amann, R., Fuchs, B. M. and Behrens, S. 2001. The identification of microorganisms by fluorescence in situ hybridisation. Current Opinion in Biotechnology. **12**(3): 231-236.

Amann, R. and Kuhl, M. 1998. In situ methods for assessment of microorganisms and their activities. Current Opinion in Microbiology. **1**(3): 352-358.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. 1990. Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology. **56**(6): 1919-1925.

Amann, R. I., Krumholz, L. and Stahl, D. A. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental-studies in microbiology. Journal of Bacteriology. **172**(2): 762-770.

Amann, R. I., Stromley, J., Devereux, R., Key, R. and Stahl, D. A. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms Applied and Environmental Microbiology. **58**(2): 614-623.

An, D. S., Brown, D., Chatterjee, I., Dong, X. L., Ramos-Padron, E., Wilson, S., Bordenave, S., Caffrey, S. M., Gieg, L. M., Sensen, C. W. and Voordouw, G. 2013. Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. Genome. **56**(10): 612-618.

Aoi, Y. 2002. In situ identification of microorganisms in biofilm communities. Journal of Bioscience and Bioengineering. **94**(6): 552-556.

Aoi, Y., Miyoshi, T., Okamoto, T., Tsuneda, S., Hirata, A., Kitayama, A. and Nagamune, T. 2000. Microbial ecology of nitrifying bacteria in wastewater treatment process examined by fluorescence in situ hybridization. Journal of Bioscience and Bioengineering. **90**(3): 234-240.

Baltzis, B. C., Lewandowski, G. A. and Wang, J. H. 1993. Denitrification with a strain of *pseudomonas denitrificans* - fundamental kinetic studies and their implications for bioreactor design. Abstracts of Papers of the American Chemical Society. **205**: 26-BIOT.

Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology. **68**: 41-98.

Barton, L. L. and Fauque, G. D. 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology. **68**: 41-98.

Baytshtok, V., Kim, S., Yu, R., Park, H. and Chandran, K. 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. Water Science and Technology. **58**(2): 359-365.

Beyenal, H. and Lewandowski, Z. 2001. Mass-transport dynamics, activity, and structure of sulfate-reducing biofilms. AIChE Journal. **47**(7): 1689-1697.

Bhagat, M., Burgess, J. E., Antunes, A. P. M., Whiteley, C. G. and Duncan, J. R. 2004. Precipitation of mixed metal residues from wastewater utilising biogenic sulphide. Minerals Engineering. **17**(7-8): 925-932.

Bordenave, S., Kostenko, V., Dutkoski, M., Grigoryan, A., Martinuzzi, R. J. and Voordouw, G. 2010. Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings. Chemosphere. **81**(5): 663-668.

Brindle, K. and Stephenson, T. 1996. The application of membrane biological reactors for the treatment of wastewaters. Biotechnology and Bioengineering. **49**(6): 601-610.

Brindle, K., Stephenson, T. and Semmens, M. J. 1998. Nitrification and oxygen utilisation in a membrane aeration bioreactor. Journal of Membrane Science. **144**(1-2): 197-209.

Brindle, K., Stephenson, T. and Semmens, M. J. 1999. Pilot-plant treatment of a high-strength brewery wastewater using a membrane-aeration bioreactor. Water Environment Research. **71**(6): 1197-1204.

Brosius, J., Dull, T. J., Sleeter, D. D. and Noller, H. F. 1981. Gene organization and primary structure of a ribosomal-RNA operon from escherichia-coli. Journal of Molecular Biology. **148**(2): 107-127.

Caughill, D. L., Morgenstern, N. R. and Scott, J. D. 1993. Geotechnics of nonsegregating oil sands tailings. Canadian Geotechnical Journal. **30**(5): 801-811.

Chalaturnyk, R. J., Scott, J. D. and Ozum, B. 2002. Management of oil sands tailings. Petroleum Science and Technology. **20**(9-10): 1025-1046.

Choi, J., Hwang, G., Gamal El-Din, M., Liu, Y. 2014. Effect of reactor configuration and microbial characteristics on biofilm reactors for oil sands process-affected water treatment. International Biodeterioration & Biodegradation. **89**: 74-81.

Cole, A. C., Semmens, M. J. and LaPara, T. M. 2004. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. Applied and Environmental Microbiology. **70**(4): 1982-1989.

Cole, A. C., Shanahan, J. W., Semmens, M. J. and LaPara, T. M. 2002. Preliminary studies on the microbial community structure of membrane-aerated biofilms treating municipal wastewater. Desalination. **146**(1-3): 421-426.

Cote, P., Bersillon, J. L., Huyard, A. and Faup, G. 1988. Bubble-free aeration using membranes - process analysis. Journal Water Pollution Control Federation. **60**(11): 1986-1992.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. and Wagner, M. 2001. In situ characterization of Nitrospira-like nitrite oxidizing bacteria active in wastewater treatment plants. Applied and Environmental Microbiology. **67**(11): 5273-5284.

Dannenberg, S., Kroder, M., Dilling, W. and Cypionka, H. 1992. Oxidation of $H_2$, organic compounds and inorganic sulfur compounds coupled to reduction of $O_2$ or nitrate by sulfate-reducing bacteria. Archives of Microbiology. **158**(2): 93-99.

de la Rosa, C. and Yu, T. 2006. Development of an automation system to evaluate the three-dimensional oxygen distribution in wastewater biofilms using microsensors. Sensors and Actuators B-Chemical. **113**(1): 47-54.

deBeer, D., Schramm, A., Santegoeds, C. M. and Kuhl, M. 1997. A nitrite microsensor for profiling environmental biofilms. Applied and Environmental Microbiology. **63**(3): 973-977.

Debeer, D., Stoodley, P., Roe, F. and Lewandowski, Z. 1994. Effects of biofilm structures on oxygen distribution and mass-transport. Biotechnology and Bioengineering. **43**(11): 1131-1138.

Debeer, D. and Sweerts, J. P. R. 1989. Measurement of nitrate gradients with an ion-selective microelectrode. Analytica Chimica Acta. **219**(2): 351-356.

Debeer, D. and Vandenheuvel, J. C. 1988. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin. Talanta. **35**(9): 728-730.

Dilling, W. and Cypionka, H. 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiology Letters. **71**(1-2): 123-127.

Doi, M. and Shioi, Y. 1991. Enhancement of denitrifying activity in cells of *roseobacter-denitrificans* grown aerobically in the light. Plant and Cell Physiology. **32**(3): 365-370.

Drysdale, G. D., Kasan, H. C. and Bux, F. 1999. Denitrification by heterotrophic bacteria during activated sludge treatment. Water Sa. **25**(3): 357-362.

Ebert, A. and Brune, A. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower

termite Reticulitermes flavipes (Kollar). Applied and Environmental Microbiology. **63**(10): 4039-4046.

Eckert, W. F., Masliyah, J. H., Gray, M. R. and Fedorak, P. M. 1996. Prediction of sedimentation and consolidation of fine tails. AIChE Journal. **42**(4): 960-972.

Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., van der Meer, J. R. and Zehnder, A. J. B. 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Archives of Microbiology. **175**(3): 198-207.

Essila, N. J., Semmens, M. J. and Voller, V. R. 2000. Modeling biofilms on gas-permeable supports: Concentration and activity profiles. Journal of Environmental Engineering-Asce. **126**(3): 250-257.

Fedorak, P. M., Coy, D. L., Dudas, M. J., Simpson, M. J., Renneberg, A. J. and MacKinnon, M. D. 2003. Microbially-mediated fugitive gas production from oil sands tailings and increased tailings densification rates. Journal of Environmental Engineering and Science. **2**(3): 199-211.

Fedorak, P. M., Coy, D. L., Salloum, M. J. and Dudas, M. J. 2002. Methanogenic potential of tailings samples from oil sands extraction plants. Canadian Journal of Microbiology. **48**(1): 21-33.

Ferris, M. J., Nold, S. C., Revsbech, N. P. and Ward, D. M. 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. Applied and Environmental Microbiology. **63**(4): 1367-1374.

Foght, J. M., Fedorak, P. M. and Westlake, D. W. S. 1985. Microbial content and metabolic activities in the Syncrude tailings pond. Aostra Journal of Research. **1**:139-146.

Foti, M., Sorokin, D. Y., Lomans, B., Mussman, M., Zacharova, E. E., Pimenov, N. V., Kuenen, J. G. and Muyzer, G. 2007. Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. Applied and Environmental Microbiology. **73**(7): 2093-2100.

Garrity, G. M., Bell, J. A. and Lilburn, T. G. 2004. Taxonomic outline of procaryotes. 2nd edition, Springer, Berlin Heidelberg New York. 132.

Geets, J., Borrernans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D. and Vanbroekhoven, K. 2006. DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. Journal of Microbiological Methods. **66**(2): 194-205.

Gerardi, M. H. 2006. Wastewater Bacteria. United States John Wiley & Sons, Pennsylvania.

Golby, S., Ceri, H., Gieg, L. M., Chatterjee, I., Marques, L. L. R. and Turner, R. J. 2011. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. Fems Microbiology Ecology. **79**(1): 240-250.

Guo, C. (2009). Rapid densification of the oil sands mature fine tailings (MFT) by microbial activity. PhD Thesis, University of Alberta. Edmonton, Canada.

Hall-Stoodley, L., Costerton, J. W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology. **2**(2): 95-108.

Hansen, M. C., Tolker-Nielsen, T., Givskov, M. and Molin, S. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiology Ecology. **26**(2): 141-149.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. 1996. Real time quantitative PCR. Genome Research. **6**(10): 986-994.

Herman, D. C., Fedorak, P. M., Mackinnon, M. D. and Costerton, J. W. 1994. Biodegradation of naphthenic acids by microbial-populations indigenous to oil sands tailings. Canadian Journal of Microbiology. **40**(6): 467-477.

Hibiya, K., Terada, A., Tsuneda, S. and Hirata, A. 2003. Simultaneous nitrification and denitrification by controlling vertical and horizontal

microenvironment in a membrane-aerated biofilm reactor. Journal of Biotechnology. **100**(1): 23-32.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology. **46**(10): 927-937.

Holowenko, F. M., MacKinnon, M. D. and Fedorak, P. M. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research. **36**(11): 2843-2855.

Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. Genome Biology. **3**(2).

Ibrahim, H. T., He, Q., Al-Rekabi, W. S. and Yang, Q. 2012. Improvements in biofilm processes for wastewater treatment. Pakistan Journal of Nutrition. **11**(8): 610-636.

Islam, S.; Dong, T.; Sheng, Z.; Zhang, Y.; Liu, Y. and Gamal El-Din, M. 2014. Microbial community structure and operational performance of a fluidized bed biofilm reactor treating oil sands process-affected water. International Biodeterioration & Biodegradation. **91**: 111–118.

Ito, T., Nielsen, J. L., Okabe, S., Watanabe, Y. and Nielsen, P. H. 2002. Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining

microautoradiography and fluorescent in situ hybridization. Applied and Environmental Microbiology. **68**(1): 356-364.

Ito, T., Okabe, S., Satoh, H. and Watanabe, Y. 2002. Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. Applied and Environmental Microbiology. **68**(3): 1392-1402.

Jeroschewski, P., Steuckart, C. and Kuhl, M. 1996. An amperometric microsensor for the determination of $H_2S$ in aquatic environments. Analytical Chemistry. **68**(24): 4351-4357.

Jong, T. and Parry, D. L. 2006. Microbial sulfate reduction under sequentially acidic conditions in an upflow anaerobic packed bed bioreactor. Water Research. **40**(13): 2561-2571.

Jorgensen, B. B. 1982. Mineralization of organic matter in the sea bed - the role of sulfate reduction. Nature. **296**(5858): 643-645.

Kindaichi, T., Kawano, Y., Ito, T., Satoh, H. and Okabe, S. 2006. Population dynamics and in situ kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. Biotechnology and Bioengineering. **94**(6): 1111-1121.

Kindaichi, T., Okabe, S., Satoh, H. and Watanabe, Y. 2004. Effects of hydroxylamine on microbial community structure and function of autotrophic

nitrifying biofilms determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **49**(11-12): 61-68.

Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Khironomos, J. N., Lee, H. and Trevors, J. T. 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods. **58**(2): 169-188.

Kohls, O., Klimant, I., Holst, G. and Kuhl, M. 1997. Development and comparision of pH microoptodes for use in marine systems. Micro- and Nanofabricated Electro-Optical Mechanical Systems for Biomedical and Environmental Applications. **2978**: 82-91.

Kolb, S., Seeliger, S., Springer, N., Ludwig, W. and Schink, B. 1998. The fermenting bacterium Malonomonas rubra is phylogenetically related to sulfur-reducing bacteria and contains a c-type cytochrome similar to those of sulfur and sulfate reducers. Systematic and Applied Microbiology. **21**(3): 340-345.

Kuhl, M. and Jorgensen, B. B. 1992. Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. . Applied and Environmental Microbiology. **58**(4): 1164-1174.

Lewandowski, Z. and Beyenal, H. 2007. Fundamentals of Biofilm Research. New York, CRC press.

Li, C. 2010. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification. Master Thesis, University of Alberta. Edmonton, Canada.

Li, J. and Bishop, P. L. 2004. Time course observations of nitrifying biofilm development using microelectrodes. Journal of Environmental Engineering and Science. **3**(6): 523-528.

Lim, Y. W., Lee, S. A., Kim, S. B., Yong, H. Y., Yeon, S. H., Park, Y. K., Jeong, D. W. and Park, J. S. 2005. Diversity of denitrifying bacteria isolated from Daejeon sewage treatment plant. Journal of Microbiology. **43**(5): 383-390.

List, B. R. and Lord, E. R. F. 1997. Syncrude's tailings management practices from research to implementation. Cim Bulletin. **90**(1010): 39-44.

Llobet-Brossa, E., Rossello-Mora, R. and Amann, R. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Applied and Environmental Microbiology. **64**(7): 2691-2696.

Lovley, D. R. and Phillips, E. J. P. 1994. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Applied and Environmental Microbiology. **60**(7): 2394-2399.

Lu, R. and Yu, T. 2002. Fabrication and evaluation of an oxygen microelectrode applicable to environmental engineering and science. Journal of Environmental Engineering and Science. **1**(3): 225-235.

Matsumoto, S., Terada, A., Aoi, Y., Tsuneda, S., Alpkvist, E., Picioreanu, C. and van Loosdrecht, M. C. M. 2007. Experimental and simulation analysis of community structure of nitrifying bacteria in a membrane-aerated biofilm. Water Science and Technology. **55**(8-9): 283-290.

Miletto, M., Bodelier, P. L. E. and Laanbroek, H. J. 2007. Improved PCR-DGGE for high resolution diversity screening of complex sulfate-reducing prokaryotic communities in soils and sediments. Journal of Microbiological Methods. **70**(1): 103-111.

Minz, D., Flax, J. L., Green, S. J., Muyzer, G., Cohen, Y., Wagner, M., Rittmann, B. E. and Stahl, D. A. 1999. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. Applied and Environmental Microbiology. **65**(10): 4666-4671.

Moter, A. and Gobel, U. B. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. Journal of Microbiological Methods. **41**(2): 85-112.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Applied and Environmental Microbiology. **59**(3): 695-700.

Nakatsu, C. H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. Soil Science Society of America Journal. **71**(2): 562-571.

Nicolaisen, M. H. and Ramsing, N. B. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. Journal of Microbiological Methods. **50**(2): 189-203.

Norsker, N. H., Nielsen, P. H. and Hvitvedjacobsen, T. 1995. Influence of oxygen on biofilm growth and potential sulfate reduction in gravity sewer biofilm. Water Science and Technology. **31**(7): 159-167.

Odom, J. M. and Singleton, R. J. 1993. The sulfate-reducing bacteria:contemporary perspectives. New York, Springer-Verlag.

Okabe, S., Ito, T. and Satoh, H. 2003. Sulfate reducing bacterial community structure and their contribution to carbon mineralization in a wastewater biofilm growing under microaerophilic conditions. Applied Microbiology and Biotechnology. **63**(3): 322-334.

Okabe, S., Ito, T., Sugita, K. and Satoh, H. 2005. Succession of internal sulfur cycles and sulfur-oxidizing bacterial communities in microaerophilic wastewater biofilms. Applied and Environmental Microbiology. **71**(5): 2520-2529.

Okabe, S., Itoh, T., Satoh, H. and Watanabe, Y. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Applied and Environmental Microbiology. **65**(11): 5107-5116.

Okabe, S., Matsuda, T., Satoh, H., Itoh, T. and Watanabe, Y. 1998. Sulfate reduction and sulfide oxidation in aerobic mixed population biofilms. Water Science And Technology. **37**(4-5): 131-138.

Okabe, S., Naitoh, H., Satoh, H. and Watanabe, Y. 2002. Structure and function of nitrifying biofilms as determined by molecular techniques and the use of microelectrodes. Water Science and Technology. **46**(1-2): 233-241.

Okabe, S., Satoh, H. and Watanabe, Y. 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **65**(7): 3182-3191.

Okabe, S., Satoh, H. and Watanabe, Y. 1999b. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **65**(7): 3182-3191.

Okabe, S. and Watanabe, Y. 2000. Structure and function of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Water Science and Technology. **42**(12): 21-32.

Park, J. J., Byun, I. G., Park, S. R. and Park, T. J. 2008. Nitrifying bacterial communities and its activities in aerobic biofilm reactors under different

temperature conditions. Korean Journal of Chemical Engineering. **25**(6): 1448-1455.

Park, J. J., Byun, I. G., Yu, J. C., Park, S. R., Ju, D. J., Hur, S. H. and Park, T. J. 2008. Analysis of nitrifying bacterial communities in aerobic biofilm reactors with different DO conditions using molecular techniques. Water Science and Technology. **57**(12): 1889-1899.

Pellicer-Nacher, C., Sun, S. P., Lackner, S., Terada, A., Schreiber, F., Zhou, Q. and Smets, B. F. 2010. Sequential aeration of membrane-aerated biofilm reactors for high-rate autotrophic nitrogen removal: experimental demonstration. Environmental Science & Technology. **44**(19): 7628-7634.

Penner, T. J. and Foght, J. M. 2010. Mature fine tailings from oil sands processing harbour diverse methanogenic communities. Canadian Journal of Microbiology. **56**(6): 459-470.

Pol, L. W. H., Lens, P. N. L., Stams, A. J. M. and Lettinga, G. 1998. Anaerobic treatment of sulphate-rich wastewaters. Biodegradation. **9**(3-4): 213-224.

Postgate, J. R. 1984. The sulphate-reducing bacteria. Cambridge, UK, Cambridge University Press.

Powter, C. B., Biggar, K.W., Silva, M.J., McKenna, G.T and Scordo, E.B. 2010. Review of oil sands tailings technology options. The 14th International Conference on Tailings and Mine Waste: 381-391.

Quagraine, E. K., Peterson, H. G. and Headley, J. V. 2005. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering. **40**(3): 685-722.

Rabus, R., Fukui, M., Wilkes, H. and Widdel, F. 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Applied and Environmental Microbiology. **62**(10): 3605-3613.

Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I. M., Dong, X., Sensen, C. W., Fournier, J., Voordouw, G. and Gieg, L. M. 2011. Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond. Environmental Science & Technology. **45**(2): 439-446.

Ramsing, N. B., Kuhl, M. and Jorgensen, B. B. 1993. Distribution of sulfate-reducing bacteria, $O_2$, and $H_2S$ in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Applied and Environmental Microbiology. **59**(11): 3840-3849.

Ravichandra, P., Mugeraya, G., Rao, A. G., Ramakrishna, M. and Jetty, A. 2007. Isolation of *Thiobacillus sp* from aerobic sludge of distillery and dairy effluent treatment plants and its sulfide oxidation activity at different concentrations. Journal of Environmental Biology. **28**(4): 819-823.

Revsbech, N. P. 1989. An oxygen microsensor with a guard cathode. Limnology and Oceanography. **34**(2): 474-478.

Revsbech, N. P. and Jorgensen, B. B. 1986. Microelectrodes - their use in microbial ecology. Advances in Microbial Ecology. **9**: 293-352.

Revsbech, N. P., Jorgensen, B. B., Blackburn, T. H. and Cohen, Y. 1983. Microelectrode studies of the photosynthesis and $O_2$, $H_2S$, and pH profiles of a microbial mat. Limnology and Oceanography. **28**(6): 1062-1074.

Revsbech, N. P., Nielsen, L. P., Christensen, P. B. and Sorensen, J. 1988. Combined oxygen and nitrous-oxide microsensor for denitrification studies. Applied and Environmental Microbiology. **54**(9): 2245-2249.

Salloum, M. J., Dudas, M. J. and Fedorak, P. M. 2002. Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Management & Research. **20**(2): 162-171.

Santegoeds, C. M., Damgaard, L. R., Hesselink, C., Zopfi, J., Lens, P., Muyzer, G. and de Beer, D. 1999. Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and

molecular analyses. Applied and Environmental Microbiology. **65**(10): 4618-4629.

Santegoeds, C. M., Ferdelman, T. G., Muyzer, G. and de Beer, D. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Applied and Environmental Microbiology. **64**(10): 3731-3739.

Sanz, J. L. and Kochling, T. 2007. Molecular biology techniques used in wastewater treatment: An overview. Process Biochemistry. **42**(2): 119-133.

Sass, A. M., Eschemann, A., Kuhl, M., Thar, R., Sass, H. and Cypionka, H. 2002. Growth and chemosensory behavior of sulfate-reducing bacteria in oxygen-sulfide gradients. FEMS Microbiology Ecology. **40**(1): 47-54.

Sass, H., Cypionka, H. and Babenzien, H. D. 1997. Vertical distribution of sulfate-reducing bacteria at the oxic-anoxic interface in sediments of the oligotrophic Lake Stechlin. FEMS Microbiology Ecology. **22**(3): 245-255.

Satoh, H., Ono, H., Rulin, B., Kamo, J., Okabe, S. and Fukushi, K. I. 2004. Macroscale and microscale analyses of nitrification and denitrification in biofilms attached on membrane aerated biofilm reactors. Water Research. **38**(6): 1633-1641.

Schramm, A., De Beer, D., Gieseke, A. and Amann, R. 2000. Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. Environmental Microbiology. **2**(6): 680-686.

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S. and Amann, R. 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira spp.* along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. Applied and Environmental Microbiology. **65**(8): 3690-3696.

Schramm, A., de Beer, D., Wagner, M. and Amann, R. 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira spp.* as dominant populations in a nitrifying fluidized bed reactor. Applied and Environmental Microbiology. **64**(9): 3480-3485.

Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R. and Schleifer, K. H. 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Applied and Environmental Microbiology. **62**(12): 4641-4647.

Schulze, E. D. and Mooney, H. A. 1993. Biodiversity and ecosystem function, Springer-Verlag.

Semmens, M. J., Dahm, K., Shanahan, J. and Christianson, A. 2003. COD and nitrogen removal by biofilms growing on gas permeable membranes. Water Research. **37**(18): 4343-4350.

Siddique, T., Penner, T., Klassen, J., Nesbo, C. and Foght, J. M. 2012. Microbial communities involved in methane production from hydrocarbons in oil sands tailings. Environmental Science & Technology. **46**(17): 9802-9810.

Siddique, T., Penner, T., Semple, K. and Foght, J. M. 2010. Anaerobic biodegradation of longer-chain n-Alkanes coupled to methane production in oil sands tailings. Environmental Science & Technology. **45**(13): 5892-5899.

Smith, R. L., Buckwalter, S. P., Repert, D. A. and Miller, D. N. 2005. Small-scale, hydrogen-oxidizing-denitrifying bioredctor for treatment of nitrate-pontaminated drinking water. Water Research. **39**(10): 2014-2023.

Syron, E. and Casey, E. 2008. Membrane-aerated biofilms for high rate biotreatment: Performance appraisal, engineering principles, scale-up, and development requirements. Environmental Science & Technology. **42**(6): 1833-1844.

Tan, S. Y., Yu, T. and Shi, H. C. 2014. Microsensor determination of multiple microbial processes in an oxygen-based membrane aerated biofilm. Water Science and Technology. **69**(5): 909-914.

Tan, S. Y. 2012. Multiple microbial processes in membrane aerated biofilms studied using microsensors. PhD Thesis, University of Alberta. Edmonton, Canada.

Tan, S. Y. and Yu, T. 2007. Fabrication of an amperometric $H_2S$ microsensor and its application in wastewater biofilms. Proceedings of International Workshop on Monitoring and Sensors for Water Pollution Control, Beijing, China.

Terada, A., Hibiya, K., Nagai, J., Tsuneda, S. and Hirata, A. 2003. Nitrogen removal characteristics and biofilm analysis of a membrane-aerated biofilm reactor applicable to high-strength nitrogenous wastewater treatment. Journal of Bioscience and Bioengineering. **95**(2): 170-178.

Throback, I. N., Enwall, K., Jarvis, A. and Hallin, S. 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology. **49**(3): 401-417.

Timberlake, D. L., Strand, S. E. and Williamson, K. J. 1988. Combined aerobic heterotrophic oxidation, nitrification and denitrification in a permeable support biofilm. Water Research. **22**(12): 1513-1517.

Tolker-Nielsen, T. and Molin, S. 2000. Spatial organization of microbial biofilm communities. Microbial Ecology. **40**(2): 75-84.

Tsushima, I., Kindaichi, T. and Okabe, S. 2007. Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. Water Research. **41**(4): 785-794.

Vanloosdrecht, M. C. M. and Heijnen, S. J. 1993. Biofilm bioreactors for wastewater treatment. Trends in Biotechnology. **11**(4): 117-121.

von Wintzingerode, F., Gobel, U. B. and Stackebrandt, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews. **21**(3): 213-229.

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y. and Gevertz, D. 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Applied and Environmental Microbiology. **62**(5): 1623-1629.

Wagner, M. and Loy, A. 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology. **13**(3): 218-227.

Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A. and Stahl, D. A. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. Journal of Bacteriology. **180**(11): 2975-2982.

Wang, X. H., Wen, X. H., Criddle, C., Wells, G., Zhang, J. and Zhao, Y. 2010. Community analysis of ammonia-oxidizing bacteria in activated sludge of eight wastewater treatment systems. Journal of Environmental Sciences-China. **22**(4): 627-634.

Waters, D. L. E. and Shapter, F. M. The Polymerase Chain Reaction (PCR): General Methods. Cereal Genomics: Methods and Protocols. R. J. Henry and A. Furtado. Totowa, Humana Press Inc: 65-75.

Xia, X., Zhang, X., Feng, H. and Zhao, L. 2010. Diversity and quantification analysis of functional genes in a lab scale denitrifying quinoline-degrading bioreactor. Weishengwu Xuebao. **50**(12): 1613-1618.

Yamagiwa, K., Ohkawa, A. and Hirasa, O. 1994. Simultaneous organic-carbon removal and nitrification by biofilm formed on oxygen enrichment membrane. Journal of Chemical Engineering of Japan. **27**(5): 638-643.

Yamagiwa, K., Ohkawa, A. and Hirasa, O. 1994. Simultaneous organic-carbon removal and nitrification by biofilm formed on oxygen enrichment memrane. . Journal of Chemical Engineering of Japan. **27**(5): 638-643.

Yu, T. 2000. Stratification of microbial process and redox potential changes in biofilm. PhD Thesis, University of Cincinnati. Cincinnati, USA.

Yu, T. and Bishop, P. L. 2001. Stratification and oxidation-reduction potential change in an aerobic and sulfate-reducing biofilm studied using microelectrodes. Water Environment Research. **73**(3): 368-373.

Zhang, T. C. and Bishop, P. L. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. Water Environment Research. **68**(7): 1107-1115.

# Appendix A

**Data forFigure 4-3: Profile of $H_2S$ and $O_2$ in Membrane Aerated Biofilm (Profile of $O_2$)**

| Depth (μm) | $O_2$ (mg/L) | Depth (μm) | $O_2$ (mg/L) |
|---|---|---|---|
| -100 | 0 | 1335 | 5.45 |
| -50 | 0 | 1360 | 5.75 |
| 0 | 0 | 1385 | 5.90 |
| 50 | 0 | 1410 | 6.09 |
| 100 | 0 | 1435 | 6.46 |
| 200 | 0 | 1460 | 6.59 |
| 300 | 0 | 1485 | 6.82 |
| 310 | 0 | 1510 | 7.22 |
| 360 | 0 | 1535 | 7.43 |
| 385 | 0 | 1585 | 8.50 |
| 410 | 0 | 1635 | 8.80 |
| 435 | 0 | 1685 | 9.68 |
| 460 | 0 | 1735 | 10.65 |
| 485 | 0 | 1800 | 13.65 |
| 585 | 0 | 1850 | 15.87 |
| 685 | 0.09 | 1900 | 16.89 |
| 735 | 0.24 | 1950 | 16.87 |
| 785 | 0.57 | 2000 | 16.85 |
| 835 | 0.78 | | |
| 885 | 1.14 | | |
| 935 | 1.59 | | |
| 985 | 1.93 | | |
| 1035 | 2.25 | | |
| 1085 | 2.71 | | |
| 1135 | 3.13 | | |
| 1185 | 3.64 | | |
| 1235 | 4.06 | | |
| 1260 | 4.47 | | |
| 1285 | 4.97 | | |
| 1310 | 5.27 | | |

**Data for Figure 4-3: Profile of $H_2S$ and $O_2$ in Membrane Aerated Biofilm**

**(Profile of $H_2S$)**

| Depth ($\mu m$) | $H_2S$ ($mg.L^{-1}$) |
|---|---|
| 0 | 10.41 |
| 50 | 10.35 |
| 100 | 10.22 |
| 110 | 8.47 |
| 160 | 6.09 |
| 185 | 5.03 |
| 210 | 3.56 |
| 235 | 1.04 |
| 260 | 0.08 |
| 285 | 0.08 |
| 300 | 0 |
| 350 | 0 |
| 400 | 0 |
| 450 | 0 |

**Data for Figure 4-7: The Vertical Distributions of SRB Biovolume in the Membrane Aerated Biofilm in an $O_2$-based MABR**

| Depth (Distance from Surface to Substratum) (μm) | Volume ($\mu m^3$) |
|---|---|
| 0-225 | 107.18 |
| 225-450 | 351.39 |
| 450-675 | 574.95 |
| 675-900 | 623.73 |
| 900-1125 | 276.93 |
| 1125-1350 | 280.60 |
| 1350-1575 | 92.19 |
| 1575-1800 | 0 |
| 1800-2025 | 15.60 |
| 2025-2250 | 130.60 |
| 2250-2475 | 86.19 |
| 2475-2700 | 5.69 |

**Data for Figure 5-3: Calibration of $H_2S$ Microsensor**

| Total Sulfide Concentration | | Respond Current |
|---|---|---|
| μM | mg/L | pA |
| 10 | 0.34 | 8 |
| 100 | 3.4 | 35 |
| 400 | 13.6 | 236 |
| 2500 | 85 | 1058 |
| 10000 | 340 | 2900 |

**Data for Figure 5-4: Potential Response of ORP Microsensors in Reference Solutions**

| Reference Solutions | Nominal Potential* (mV Vs. Ag/AgCl) | Measured Potential (mV Vs. Ag/AgCl) | |
|---|---|---|---|
| | | #1 ORP Microsensor | #2 ORP Microsensor |
| pH 4 Quinhydrone Solution | 265 | 256 | 255 |
| pH 7 Quinhydrone Solution | 88.4 | 75 | 85 |
| Ferrous-Ferric Standard Solution | 463 | 439 | 433 |

* The values of nominal potentials were for 23°c

**Data for Figure 5-6: Profile of $O_2$ in MFT and in Gypsum Amended MFT (Profile of $O_2$ in MFT)**

| Depth (Distance from Interface of Bulk Water and MFT Sediment) (μm) | $O_2$ Concentration (mg/L) |
|---|---|
| 0 | 4.57 |
| 200 | 4.43 |
| 400 | 4.36 |
| 500 | 4.36 |
| 600 | 4.36 |
| 800 | 4.36 |
| 1000 | 4.29 |
| 1200 | 4.22 |
| 1400 | 4.00 |
| 1600 | 3.64 |
| 1700 | 3.29 |
| 1800 | 2.93 |
| 1900 | 2.64 |
| 2000 | 2.00 |
| 2100 | 1.50 |
| 2200 | 0.93 |
| 2300 | 0.43 |
| 2400 | 0.21 |
| 2500 | 0.00 |
| 2600 | 0.00 |
| 2700 | 0.00 |
| 2800 | 0.00 |
| 2900 | 0.00 |
| 3000 | 0.00 |
| 3100 | 0.00 |
| 3200 | 0.00 |

**Data for Figure 5-6: Profile of $O_2$ in MFT and in Gypsum Amended MFT (Profile of $O_2$ in Gypsum Amended MFT)**

| Depth (Distance from Interface of Bulk Water and MFT Sediment) (μm) | $O_2$ Concentration (mg/L) |
|---|---|
| 0 | 4.72 |
| 200 | 4.57 |
| 400 | 4.22 |
| 500 | 3.93 |
| 600 | 3.57 |
| 700 | 3.07 |
| 800 | 2.43 |
| 900 | 1.86 |
| 1000 | 1.21 |
| 1100 | 0.71 |
| 1200 | 0.29 |
| 1300 | 0.00 |
| 1400 | 0.00 |
| 1500 | 0.00 |
| 1600 | 0.00 |
| 1700 | 0.00 |

**Data for Figure 5-7: Profile of $H_2S$ in MFT and in Gypsum Amended MFT (Profile of $H_2S$ in MFT)**

| Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.00 | 34 | 5.33 | 61 | 7.69 | 88 | 8.87 | 115 | 8.51 |
| 2 | 0.00 | 35 | 5.80 | 62 | 7.69 | 89 | 8.51 | 116 | 8.63 |
| 3 | 0.00 | 36 | 5.92 | 63 | 7.69 | 90 | 8.87 | 117 | 8.51 |
| 5 | 0.00 | 37 | 6.27 | 64 | 7.69 | 91 | 8.75 | 118 | 8.51 |
| 7 | 0.00 | 38 | 6.27 | 65 | 7.57 | 92 | 8.51 | 119 | 8.63 |
| 9 | 0.00 | 39 | 5.56 | 66 | 7.22 | 93 | 8.63 | 120 | 8.51 |
| 11 | 0.00 | 40 | 6.39 | 67 | 7.33 | 94 | 8.40 | 121 | 8.51 |
| 13 | 0.00 | 41 | 6.39 | 68 | 7.69 | 95 | 8.51 | 122 | 8.75 |
| 15 | 0.00 | 42 | 6.63 | 69 | 7.81 | 96 | 8.87 | 123 | 8.63 |
| 16 | 0.00 | 43 | 6.86 | 70 | 7.81 | 97 | 8.75 | 124 | 8.40 |
| 17 | 0.00 | 44 | 6.63 | 71 | 7.81 | 98 | 8.51 | 125 | 8.28 |
| 18 | 0.00 | 45 | 6.98 | 72 | 7.92 | 99 | 8.63 | | |
| 19 | 0.00 | 46 | 7.10 | 73 | 8.04 | 100 | 8.40 | | |
| 20 | 0.00 | 47 | 6.86 | 74 | 8.16 | 101 | 8.51 | | |
| 21 | 0.00 | 48 | 5.68 | 75 | 8.16 | 102 | 8.51 | | |
| 22 | 0.00 | 49 | 6.86 | 76 | 8.51 | 103 | 8.75 | | |
| 23 | 0.00 | 50 | 6.98 | 77 | 8.40 | 104 | 8.51 | | |
| 24 | 0.00 | 51 | 7.22 | 78 | 8.16 | 105 | 8.63 | | |
| 25 | 0.00 | 52 | 7.33 | 79 | 8.04 | 106 | 8.75 | | |
| 26 | 0.14 | 53 | 7.33 | 80 | 8.75 | 107 | 8.75 | | |
| 27 | 0.73 | 54 | 7.45 | 81 | 8.75 | 108 | 8.63 | | |
| 28 | 1.55 | 55 | 7.57 | 82 | 8.51 | 109 | 8.51 | | |
| 29 | 2.14 | 56 | 7.57 | 83 | 8.51 | 110 | 8.51 | | |
| 30 | 3.09 | 57 | 7.57 | 84 | 8.63 | 111 | 8.28 | | |
| 31 | 3.79 | 58 | 7.69 | 85 | 8.51 | 112 | 8.40 | | |
| 32 | 4.15 | 59 | 7.69 | 86 | 8.51 | 113 | 8.40 | | |
| 33 | 5.09 | 60 | 7.69 | 87 | 8.63 | 114 | 8.51 | | |

**Data for Figure 5-7: Profile of $H_2S$ in MFT and in Gypsum Amended MFT (Profile of $H_2S$ in Gypsum Amended MFT)**

| Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) | Depth (mm) | $H_2S$ (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.00 | 31 | 15.12 | 59 | 50.41 | 86 | 85.58 | 113 | 113.08 |
| 2 | 0.00 | 32 | 16.30 | 60 | 52.30 | 87 | 86.88 | 114 | 115.44 |
| 4 | 0.00 | 33 | 17.48 | 61 | 54.19 | 88 | 87.11 | 115 | 115.91 |
| 6 | 0.00 | 34 | 18.55 | 62 | 56.90 | 89 | 88.65 | 116 | 116.62 |
| 8 | 0.00 | 35 | 19.84 | 63 | 59.02 | 90 | 90.42 | 117 | 115.67 |
| 9 | 0.00 | 36 | 21.14 | 64 | 60.91 | 91 | 93.37 | 118 | 119.80 |
| 10 | 0.00 | 37 | 22.32 | 65 | 62.21 | 92 | 94.19 | 119 | 116.62 |
| 11 | 0.00 | 38 | 23.50 | 66 | 65.16 | 93 | 95.37 | 120 | 117.80 |
| 12 | 0.00 | 39 | 24.68 | 67 | 65.40 | 94 | 96.20 | 121 | 117.32 |
| 13 | 0.00 | 40 | 20.55 | 68 | 65.87 | 95 | 96.79 | | |
| 14 | 0.00 | 41 | 22.32 | 69 | 67.88 | 96 | 95.96 | | |
| 15 | 0.00 | 42 | 23.27 | 70 | 69.41 | 97 | 97.73 | | |
| 16 | 0.00 | 43 | 25.27 | 71 | 71.42 | 98 | 101.51 | | |
| 17 | 0.00 | 44 | 26.57 | 72 | 72.71 | 99 | 100.33 | | |
| 18 | 0.00 | 45 | 28.10 | 73 | 74.13 | 100 | 101.04 | | |
| 19 | 0.61 | 47 | 29.64 | 74 | 76.02 | 101 | 103.63 | | |
| 20 | 1.67 | 48 | 30.94 | 75 | 77.79 | 102 | 104.58 | | |
| 21 | 3.09 | 49 | 32.59 | 76 | 77.43 | 103 | 103.63 | | |
| 22 | 4.27 | 50 | 34.12 | 77 | 80.03 | 104 | 104.58 | | |
| 23 | 5.45 | 51 | 36.01 | 78 | 81.68 | 105 | 103.63 | | |
| 24 | 6.63 | 52 | 37.90 | 79 | 83.10 | 106 | 104.81 | | |
| 25 | 7.81 | 53 | 38.73 | 80 | 77.43 | 107 | 106.47 | | |
| 26 | 8.99 | 54 | 40.73 | 81 | 74.96 | 108 | 106.94 | | |
| 27 | 9.69 | 55 | 42.38 | 82 | 78.61 | 109 | 107.41 | | |
| 28 | 11.58 | 56 | 44.27 | 83 | 79.21 | 110 | 108.35 | | |
| 29 | 12.53 | 57 | 45.92 | 84 | 81.57 | 111 | 109.77 | | |
| 30 | 13.94 | 58 | 47.46 | 85 | 84.04 | 112 | 111.66 | | |

**Data for Figure 6-2: Calibration of $H_2S$ Microsensor**

| Total sulfide concentration | | Respond current |
|---|---|---|
| μM | mg/L | pA |
| 0 | 0 | 2 |
| 10 | 0.34 | 8 |
| 100 | 3.4 | 42 |
| 200 | 6.8 | 90 |

**Data for Figure 6-3: Potential Response of ORP Microsensors in Reference Solutions**

| Reference Solutions | Nominal Potential* (mV Vs. Ag/AgCl) | Measured Potential (mV Vs. Ag/AgCl) | |
|---|---|---|---|
| | | #1 ORP Microsensor | #2 ORP Microsensor |
| pH 4 Quinhydrone Solution | 265 | 256 | 255 |
| pH 7 Quinhydrone Solution | 88.4 | 75 | 85 |
| Ferrous-Ferric Standard Solution | 463 | 439 | 433 |

* The values of nominal potentials were for 23°C

**Data for Figure 6-4: Calibration of pH Microsensor**

| pH | The Potential Readings | |
|---|---|---|
| | Before Measurement (mV) | After Measurement (mV) |
| 6 | 393 | 411 |
| 7 | 330 | 350 |
| 8 | 276 | 290 |
| 9 | 228 | 244 |

**Data for Figure 6-6: Profiles of $O_2$, ORP, pH, and $H_2S$ in a Piece of OSPW Biofilm**

| Depth μM | DO $mg.L^{-1}$ | Depth μM | ORP mV | Depth μM | $H_2S$ $mg.L^{-1}$ | Depth μM | pH |
|---|---|---|---|---|---|---|---|
| -150 | 8.25 | -100 | 411 | -300 | 0 | -50 | 7.44 |
| -100 | 8.13 | -50 | 410 | -200 | 0 | 0 | 7.44 |
| -50 | 8.13 | 0 | 410 | -100 | 0 | 50 | 7.51 |
| 0 | 8.25 | 50 | 415 | 0 | 0 | 100 | 7.59 |
| 50 | 7.91 | 100 | 378 | 100 | 0 | 150 | 7.62 |
| 100 | 7.34 | 150 | 342 | 200 | 0 | 200 | 7.64 |
| 150 | 6.44 | 200 | 311 | 300 | 0 | 250 | 7.64 |
| 200 | 5.99 | 250 | 275 | 400 | 0 | 300 | 7.66 |
| 250 | 5.65 | 300 | 169 | 500 | 0 | 350 | 7.70 |
| 300 | 5.31 | 350 | 113 | 600 | 0.16 | 400 | 7.64 |
| 350 | 5.20 | 400 | 85 | 650 | 0.16 | 450 | 7.60 |
| 400 | 4.97 | 450 | 57 | 700 | 0.16 | 600 | 7.59 |
| 450 | 4.74 | 500 | 33 | 750 | 0.42 | 700 | 7.59 |
| 500 | 4.41 | 550 | 21 | 800 | 0.92 | 800 | 7.60 |
| 550 | 4.07 | 600 | -4 | 850 | 1.43 | 900 | 7.66 |
| 600 | 3.84 | 650 | -18 | 900 | 1.68 | 1000 | 7.70 |
| 650 | 2.94 | 700 | -78 | 950 | 1.68 | | |
| 700 | 2.37 | 750 | -81 | 1000 | 2.19 | | |
| 750 | 1.69 | 800 | -71 | | | | |
| 800 | 0.68 | 900 | -91 | | | | |
| 850 | 0.00 | 1000 | -110 | | | | |
| 900 | 0.00 | | | | | | |
| 950 | 0.00 | | | | | | |

**Data for Figure 6-8: Real-time qPCR of dsrB Genes Amplified from Samples (#1, #2, #3, #4 Represent the Biofilm Samples Taken at 3 Months, 4 Months, 5 Months, and 6 Months)**

| Samples | Gene Copies | Standard Deviation |
|---|---|---|
| Sample #1 (3 Months) | 620 | 44 |
| Sample #2 (4 Months) | 1623 | 221 |
| Sample #3 (5 Months) | 9310 | 506 |
| Sample #4 (6 Months) | 8396 | 977 |

# Appendix B

**Chemical Oxidation Demand (COD) in Reactor 1**

| Water Retention Time (days) | Influent COD (mg/L) | Effluent COD (mg/L) | Removed COD (mg/L) |
|---|---|---|---|
| 7 | 202 | 198 | 4 |
| 7 | 202 | 200 | 2 |
| 8 | 200 | 194 | 6 |
| 9 | 204 | 192 | 12 |
| 7 | 201 | 192 | 9 |
| 6 | 192 | 188 | 4 |
| 14 | 198 | 190 | 8 |
| 12 | 200 | 192 | 8 |
| 15 | 198 | 189 | 9 |
| 21 | 192 | 182 | 10 |
| 20 | 191 | 182 | 9 |
| 28 | 195 | 182 | 13 |
| 30 | 198 | 184 | 14 |

**Chemical Oxidation Demand (COD) in Reactor 2**

| Water Retention Time (days) | Influent COD (mg/L) | Effluent COD (mg/L) | Removed COD (mg/L) |
|---|---|---|---|
| 7 | 860 | 190 | 670 |
| 7 | 872 | 204 | 668 |
| 8 | 865 | 152 | 713 |
| 9 | 853 | 186 | 667 |
| 7 | 847 | 138 | 709 |
| 6 | 878 | 134 | 744 |
| 14 | 882 | 108 | 774 |
| 12 | 872 | 90 | 782 |
| 15 | 869 | 90 | 779 |
| 20 | 864 | 87 | 777 |
| 21 | 873 | 85 | 788 |
| 28 | 879 | 72 | 807 |
| 30 | 889 | 68 | 821 |

**Nitrogen in Reactor 1 During Last 30 days**

| Samples | $NO_3^-$ (mg/L) | $NO_2^-$ (mg/L) | $NH_4^+$ (mg/L) | Total Nitrogen (mg/L) |
|---|---|---|---|---|
| 1 | 23.6 | 0 | 1.3 | 25.30 |
| 2 | 20.9 | 0 | 1.5 | 25.98 |
| 3 | 21.6 | 0 | 1.5 | 25.58 |
| 4 | 20.4 | 0 | 1.6 | 26.09 |
| 5 | 21.2 | 0 | 1.4 | 26.35 |

**Nitrogen in Reactor 2 During Last 30 days**

| Samples | $NO_3^-$ (mg/L) | $NO_2^-$ (mg/L) | $NH_4^+$ (mg/L) | Total Nitrogen (mg/L) |
|---|---|---|---|---|
| 1 | 9.6 | 0 | 8 | 91.98 |
| 2 | 0 | 0 | 17.7 | 48.16 |
| 3 | 0 | 0 | 20.6 | 45.96 |
| 4 | 31.6 | 0 | 2.7 | 41.99 |
| 5 | 38.8 | 0 | 2.7 | 44.60 |

**Sulfate in Reactor 1 and Reactor 2 During Last 30 days**

| Samples | R1 $SO_4^{2-}$ (mg/L) | R2 $SO_4^{2-}$ (mg/L) |
|---|---|---|
| 1 | 141 | 80 |
| 2 | 140 | 80 |
| 3 | 141 | 74 |
| 4 | 138 | 63 |
| 5 | 140 | 64 |