University of Alberta

Anaerobic Degradation of Oil Sands Tailings

by

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Dedication

Dedicated to my family.

A mi mamá y papá que siempre me han dado su apoyo a lo largo de mis estudios.

A mi hermano Raúl por sus conversaciones invaluables.

Muchas gracias.

Abstract

The objective of this study was to determine if there was any difference in the bioremediation of oil sands process-affected water (OSPW) and to quantify and identify bacteria present in different scenarios. Two reactors were compared in this study: an acetic acid amended OSPW bioreactor, (AAAO bioreactor) and a HiPOx treated OSPW bioreactor (HTO bioreactor). The AAAO bioreactor contained 1750 mL OSPW and 250 mL of mature fine tailings (MFT). The second bioreactor contained the exact amount of OSPW and MFT with the only difference that OSPW was treated with an advanced oxidation process (HiPOx). The AAAO bioreactor was able to remove 70% of COD and 15% of naphthenic acids (NA). The HTO bioreactor removed 48% of COD and 19% of NA removal under nitrate reducing conditions. Bacterial quantification showed that sulfate reducing bacteria (SRB) was the dominant specie at the end of the AAAO bioreactor operation with a final $4.2x10^6$ copy number per gram. In contrast, the HTO bioreactor showed that total bacteria was the dominant specie with $7.0x10^7$ copy number per gram. A community analysis was performed on both bioreactors. In the AAAO bioreactor bacteria identified were *Acidovorax sp., Acidovorax ebreus, Acidovorax defluvii, Cryobacterium psychrotoleans, Brachymonas petroleovorans*, and uncultured members of the Desulfocapsa and Syntrophacea genus. In the HTO bioreactor, identified bacteria were *Acidovorax sp., Hydrogenophaga defluvii, Rhodoferax sp., Desulfotomaculum sp., Pseudomonas stutzeri* and uncultured members of the Desulfocapsa genus.

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List of Symbols, Nomenclature or Abbreviations

°C Degrees celcius

MPa Mega Pascals

BTEX Benzene, toluene, ethylbenzene and xylenes

CH4 Methane

CT Composite tailings

COD Chemical oxygen demand

CHWE Clark hot water extraction method

DGGE Denaturing gradient gel electrophoresis

DNA Deoxyribonucleic acid

DO Dissolved oxygen

DNTP Deoxyribonucleotide triphosphates

EDTA Ethylenediaminetetraacetic acid

FTIR Fourier transform infrared spectroscopy

g Gram

HiPOx Advanced oxidation process

HPLC High-performance liquid chromatography

IC Ion chromatography

L Liter

m Meters

MFT Mature fine tailings

MLBS Mildred Lake Settling Basin

MNP Most probable number

NA Naphthenic acids

NCBI National Center for Biotechnology Information

NaOH Sodium hydroxide

NRB Nitrate reducing bacteria

OSPW Oil sands process-affected water

OSA Oil sands area

PAH Polycyclic aromatic hydrocarbons

PCR Polymerase chain reaction

RDP Ribosomal database project

qPCR Quantitative polymerase chain reaction

SAGD Steam assisted gravity drainage

SARA Fractionation of saturated, aromatic, resin and asphaltenes

SCO Synthetic crude oil

SRB Sulfate-reducing bacteria

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

TiO_2 Titanium dioxide

UV Ultra violet light

Chapter 1: Introduction

1.1 Introduction

1.1.1 Oil sands industry

The oil reserves in northern Alberta, Canada, named as the oil sands area (OSA), span 140,000 square kilometers; one of the largest reserves in the world with established reserves of 168.7 billion of barrels remaining (Teare *et al.* 2012). The extraction of bitumen from oil sands requires 12 barrels of water per one barrel of bitumen produced (Mikula *et al.* 2008). Large quantities of water that needs to be stored on site due to zero discharge policies enforced by the government (Alberta environment 2010). This has led to the construction of more than 70 km^2 of tailing ponds containing slurry waste (composed of sand, clays bitumen and tailings). Moreover, the province of Alberta has a zero discharge policy for all process-affected water, meaning this water must be contained on site, thus creating the huge logistical problem of accommodating the tailings produced. Therefore, there is a progressive decrease in the quality of stored water in the tailing ponds, and there is a need to use more fresh water in the process to continue with the recovery of the bitumen (Allen 2008).

The in situ extraction process requires 12 barrels of water for one barrel of produced bitumen, but about 70% is reused, which then decreases the use to 4 barrels of water for each barrel of bitumen produced. Recycled water poses additional challenges because it has undergone different treatments than other tailings wastewater such as the addition of calcium sulfate, which causes increased alkalinity and hardness in water (Allen 2008).

1.1.2 Reclamation issues

Oil sands tailings water and mature fine tailings pose an environmental problem mainly because of the huge volumes that are stored on site and the toxic nature of the waters. The MFT inventory is estimated at 650 million m^3 (Beiber

& Sego 2008), and the increase in the heavy metals, organics and salts in oil sands tailings water is an important problem. Companies operating on OSA areas are required by law to perform reclamation at the end of their lease. Reclamation is the return of the land or water to a useful state; although not necessarily the same state as before it was mined. Water and sediments need to be treated for reclamation purposes. Remediation technologies available to remove pollutants from water or soil can be chemical, physical and biological or any combination of the three. In addition, the lease provided to oil sands companies requires them to develop new methods and strategies in order to reclaim tailings ponds. In addition to chemical and physical remediation processes for reclamation, biological remediation options have been explored to tackle this problem.

1.2 Bioremediation

Bioremediation is defined as a process where living organisms (i.e. bacteria) degrade or transform toxic or hazardous contaminants into less toxic compounds. Biological decontamination methods can degrade a broad range of environmental pollutants with no toxic intermediates (Debarati *et al.* 2005). Another formal definition of bioremediation states that bioremediation is a "managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination" (Madsen 1991).

One of the most sound and successful biodegradation applications was in Prince William Sound, Alaska, where large-scale bioremediation was performed to clean up the Exxon-Valdez oil spill. Bioremediation consisted of applying fertilizers to the surface of oil-contaminated beaches (Pritchard *et al.* 1992). Bioremediation has been applied on a smaller scale in other cases (Boopathy 2000).

Bioremediation technologies can be classified into two categories: in situ and ex situ. In situ technologies deal with remediating the contamination on site

while ex situ technologies treat the contamination off site. Some examples of ex-situ technology are land farming, composting, bioventing, biofilters, bioaugmentation or bioreactors. These technologies have been used alone or coupled with chemical or physical treatments to achieve remediation goals. For instance, land farming has been used before as a way to biodegrade oil sludge over a number of years (Genouw *et al.* 1994).

Ex-situ bioremediation is the scope of this work. Bioremediation by the use of anaerobic bioreactors is an option that has to be investigated to treat oil sands tailings. Anaerobic bioreactors use the bacterial communities present in mature fine tailings (MFT) as an initial inoculum to degrade target pollutants in oil sands tailings. Indigenous microbial communities from MFT have been proven to thrive in oil sands tailings.

1.3 Microbial communities

The biodegradation of acyclic aromatic compounds by bacterial consortia have been reported in anoxic environments including oil-contaminated sediments (Massias *et al.* 2003), enhanced anaerobic bioremediation in ground water (Cunningham *et al.* 2000), and aquifers (Gieg *et al.* 1999). Biodegradation of alkanes and aromatic compounds found in fuels have been reported in laboratory tests using microbes from contaminated environments under iron- (Botton & Parsons 2006), sulfate- (Edwards *et al.* 1992), and nitrate reducing conditions (An *et al.* 2004). These studies demonstrate that bacteria can degrade a broad variety of organic compounds under different conditions and using different electron acceptors. In addition, short chain n-alkanes' degradation from oil sands under methanogenic conditions and long n-alkanes' degradation under anaerobic conditions using bacteria from MFT has been reported previously (Siddique *et al.* 2006; Siddique *et al.* 2011). Therefore, the use of bioreactors in anaerobic conditions may provide a tool to degrade toxic compounds in oil sands tailings water.

An example of previously successful bioremediation project with oil sands tailings water was the transfer of tailings water to shallow pits were its toxicity decreased over a 1- 2-year time frame attributed to microbial activity (Boerger & Aleksiuk 1987). Using the native bacteria present in mature fine tailings to biodegrade the organic material is a potential method to remediate these water bodies (Herman *et al.* 1994).

Chemical technologies have also been used to degrade toxic compounds and pollutants in water bodies, including oil sands tailings. Such technologies include ozonation (Hwang *et al.* 2013) and advanced oxidation processes (Afzal *et al.* 2012) which have been reported to decrease the concentration of both organic matter and naphthenic acids (NA).

Both biological and chemical technologies have advantages and disadvantages. The disadvantage to bioremediation is that it takes considerable time for the bacteria to adapt to the new environment, and it is possible that organic material may not be degraded at all. The main advantage to bioremediation is that it is inexpensive. On the other hand, oxidation processes can degrade complex organic compounds in a short period of time, but oxidation alone may not be able to degrade all of the hydrocarbons or NA present. Using these technologies together may enhance water treatment and provide helpful insight into what can be achieved by bioremediation alone, or with the use of coupled technologies as previous studies have suggested (Martin *et al.* 2010; Gamal El-Din *et al.* 2011).

1.4 Research objectives

This thesis is part of a larger project, which focused on the design of a bioreactor to treat oil sands tailings water. The selection of microorganisms to from biofilms from mature fine tailings and their possible use in a bioreactor is part of a large project to engineer a bioreactor to treat oil sands tailings. Additionally, these reactors are mainly designed to achieve organic matter and NA removal.

In this regard, this thesis focuses on the degradation of compounds found in oil sands tailings waters. Two scenarios were set up; one is the use of raw oil sands tailing water (untreated) and pretreated oil sands tailings water. Treated tailings water has undergone an oxidation process (HiPOx), which is meant to degrade complex organic compounds to more readily degradable simple organic compounds. Then these process waters were subjected to biodegradation using native microorganisms found in MFT by anaerobic bioreactors. The study of these bioreactors through chemical and biological analysis provides further understanding of the processes occurring. Using molecular biology tools, dominant bacteria will be identified in the reactors. This is with the objective to understand the best treatment and the bacteria involved in the biodegradation process.

The overarching goal of this project was to investigate if bacterial communities present in mature fine tailings were capable of degrading organic matter and NA present in affected mine tailings water under anaerobic conditions.

1. Determine anaerobic process performance using indigenous bacteria from mature fine tailings. The objective is to determine until what extent contaminants are removed from oil sands tailings using this process.

2. Determine if coupled technologies are more effective at treating oil sands tailings. The use of advanced oxidation and anaerobic process together is expected to remove more contaminants than a single process used alone. The comparison between anaerobic process and anaerobic-advanced oxidation may provide information of what is the best option to treat oil sands tailings.

3. Quantify bacteria present in each bioreactor. The quantification of nitrate reducing bacteria, sulfate reducing bacteria and total bacteria may provide addition information of the ongoing biodegradation process. Additionally, the quantification may provide information of what kind of bacteria becomes dominant at the end of the process.

4. Relate chemistry to the quantification of different bacteria in the bioreactors. This will give information of the community structure in different time points on the bioreactors. Additionally, it will provide information of what kind of bacteria thrive within the bioreactors.

5. Identify dominant bacteria present in untreated and pretreated oil sands tailings by the use of denaturing gel gradient electrophoresis. This will provide a profile of the predominant bacteria present under different scenarios across time. Additionally, the collected chemistry data can be correlated with the bacteria identified in each bioreactor. In other words, identify what organisms are responsible for the degradation of organic matter, NA and sulfate reduction. This information may provide a more robust picture of the chemical degradation with identified bacteria and may provide insightful information of the ongoing biodegradation process.

This research is significant because it will provide insight into the bacterial interactions in two different scenarios. The first scenario is a bioreactor, which contains fresh oil sands tailings water and bacterial community native to MFT.

The second scenario is an identical reactor with one difference: oil sands tailings water that has been treated using HiPOx technology. Moreover, this research will provide further information about how these reactors work and how they can be improved. Additionally, it will demonstrate what can be achieved by using anaerobic boreactors to treat oil sands tailings.

In order to achieve the objectives of this study, a variety of methods were used. Bioreactors experiments were performed using 2-Liter vessels, under nitrogen atmosphere to assure anaerobic conditions. Sampling was performed on the reactors and different chemical parameters were measured such as pH, chemical oxygen demand (COD), and naphthenic acid concentrations. Ion Chromatography (IC) was used to monitor the concentrations of anions such as sulfate and nitrate. DNA was extracted from the MFT and sampled water from each of the bioreactors, followed by polymerase chain reaction (PCR). Subsequently DGGE was performed to create a profile of the bacterial population present in both reactors. Finally, cloning and sequencing were used to identify the bacteria present in both reactors.

1.5 Thesis Outline

This thesis comprises four chapters. A detailed literature review of research in this field is presented in Chapter 2. Chapter 3 gives an overview of materials and methods used in this study. Additionally a detailed description of the results and discussion are provided. At the end of Chapter 3 a brief significance of these findings is presented to the oil sands community. Conclusions, recommendation and the relevance of this research to engineering will be provided in Chapter 4.

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Chapter 2: Theoretical Background and Literature Review

2 Background and literature review

2.1 Oil sands

Canada has one of the world's largest oil reserves, right behind those of Saudi Arabia and Venezuela (Teare *et al.* 2012). Reserves of oil in northern Alberta have been calculated to contain more than 174 billion barrels of bitumen, spanning an area of 142,200 km^2 (Alberta government 2009). The oil sands region in northern Alberta has been divided into three deposits named the Athabasca, Cold Lake and Peace River deposits. The Athabasca deposit is the largest. Despite the costly extraction process required to obtain bitumen, increasing global energy demands and advances in bitumen extraction technologies have caused the oil sands industry to grow rapidly.

Alberta's oil sands industry began with the Great Canadians Ltd. Company in 1967. In 2013, more than 40 companies operate in the oil sands region. As a result of mining, 715 km^2 of land has been disturbed (Teare *et al.* 2012). In 2011, the government reported an estimated bitumen production of 1.7 million barrels per day. Production has been predicted to double to 3.7 million barrels per day within the next decade (Teare *et al.* 2012). The potential revenue from the extraction and upgrading processes render the oil sands an attractive resource for industry and government.

The oil sands are composed of 80% solids (quartz sand, silt, clay), 10% water and 10% bitumen as well as other minor amounts of minerals (titanium, zirconium, tourmaline and pyrite) (National Energy Board 2004). When these components are intermixed, a 10 nm-thin water film exists between the bitumen and sand particles. The structure of oil sands grains, water and bitumen can be seen in Figure 2.1. The recovery of bitumen is performed by liberating the sand grain and water from the mixture (Masliyah *et al.* 2004). Using fresh water in the hot water extraction process results in an alkaline wastewater product that is brackish and toxic due to the organic acids leached from bitumen (Allen 2008a).

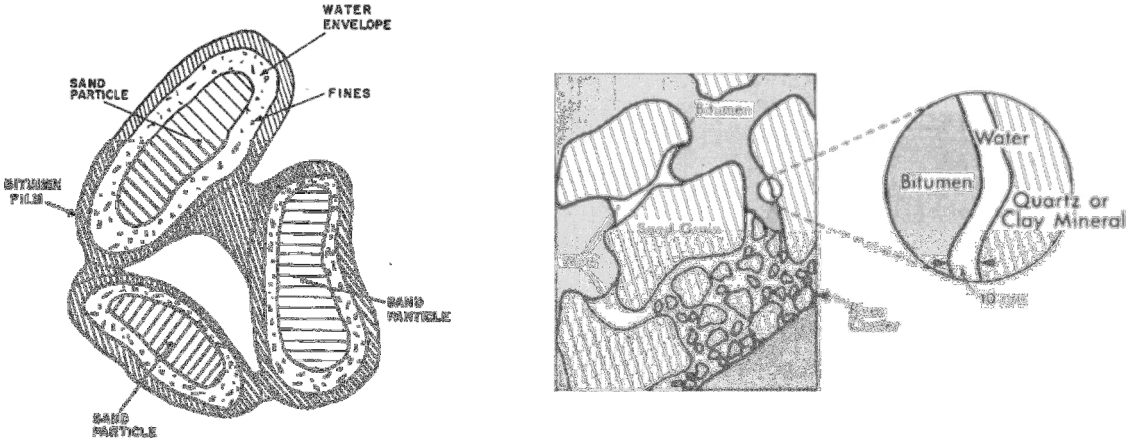

Figure 2. 1 Structure of oil sands in northern Alberta, Canada. Adapted from (Berkowitz & Speight 1975; Masliyah *et al.* 2004).

Oil sands are classified as low-grade (6-8% bitumen), average grade (8 to 10% bitumen) or high-grade (>10% bitumen) ore. While the bitumen quantity varies depending on the location, mineral solids and water remain fairly constant within the range of 83 to 86%. Fine particle content affects the recovery of ore; higher fines content in sand grains produces a lower bitumen recovery (Zhu 2013).

2.2 Bitumen

Oil sands are unconsolidated sand deposits soaked in a viscous petroleum liquid referred to as bitumen. At room temperature, bitumen resembles cold molasses, with a high viscosity, so it doesn't flow easily. Bitumen is black and composed of saturated hydrocarbons, high aromatic compounds, resins and asphaltenes. Although bitumen is one of the most complex compounds found in nature, petroleum fractions can be estimated. For instance bitumen from the Athabasca and Cold Lake deposits was subject to fractioning, resulting in a composition of 17 to 20% saturated hydrocarbons, approximately 39% aromatic, 24% resins and 15 to 17% asphaltenes. Peramanu *et al.* provide a complete list of the elemental analysis and physical properties (Peramanu *et al.* 1999).

Heavy metal content in bitumen has also been quantified in the µg per gram range. Elements found include Ti, V, Al, Ca, Dy, Sm, Ba, Eu, Na, Mn, K, Cl. A complete particle distribution of the mentioned metals, mined from the Athabasca deposit, has been published by Syncrude (Schutte *et al.* 1999).

2.3 Extraction processes

Before it is pumped through pipelines to refineries where it is further refined to produce gasoline, jet fuel and other oil-based products, bitumen undergoes an extraction and upgrading process to produce synthetic crude oil (SCO) (Masliyah *et al.* 2004). The most widely used technique for bitumen extraction is the Clark hot water extraction process. This process includes mining, extraction and upgrading. It is an interrelated process where the mining operation along with the material recovered ultimately affects the upgrading operation.

2.3.1 Mining

2.3.1.1 Surface mining recovery

Surface mining, or open pit mining, is a technique where oil sands are mined using power shovels and massive mine trucks. It is used on deposits less than 50 to 75 m below the surface.. Approximately two tonnes of oil sands must be dug up, moved and processed to produce one barrel of oil. Surface mining uses the Clark hot water extraction process to separate bitumen from the mined oil sands, with recovery efficiency from 88 to 95%. Only 20% of the oil sands in the Fort McMurray area are shallow enough to be recoverable by surface mining (Allen 2008a; Alberta government 2009).

2.3.1.2 In situ recovery

The remaining 80% of the oil sands are in deep deposits, typically located 400 m below the surface. These deposits are recoverable by in situ technology. Steam-assisted gravity drainage (SAGD) is the most common in situ technology used in the oil sands region. SAGD uses two parallel horizontal wells, one above the other. Steam is injected into the upper well, lowering the viscosity of the bitumen and allowing it to flow to the lower well, where it is pumped to the surface. SAGD recycles approximately

90% of the used water and utilizes a net 0.5 barrels of water per barrel of oil produced (Alberta government 2009).

2.3.2 Hot-water extraction process

Lumps of mined oil sands are crushed and mixed with hot water. This mixture is hydrotransported in a pipeline to stirred tanks. When the mixture is hydrotransported, oil sands are stripped from oil and bitumen is liberated from sand grains. Next, a caustic chemical solution such as sodium hydroxide (NaOH) is added, along with air, to produce conditioned slurry. Aerated bitumen floats to the surface of a separation vessel where it is removed, leaving behind water or tailings. Bitumen as froth consists of 60% bitumen, 30% water and 10% solids. Bitumen is then recovered and stored in tanks for further processing. Secondary separation vessels are used to recover as much as bitumen possible. Water used to extract bitumen is called tailings, process affected water or oil sands tailing water (OSTW). Tailings go to a thickener where rejects (large particles and fines) are stored in tailing ponds. Remaining water can be recycled back into the process or stored in tailings ponds. A coarse fraction of solids from tailings quickly settles from the fine fraction. Fine particles such as clay and silt settle very slowly. Over a period of 2-3 years, fines content reaches 30 to 40% weight and the suspension is denominated mature fine tailings (Farkish & Fall 2013).

Process affected water is of great importance for current research since this alkaline water contains an organic fraction (NA) that is the most toxic component present. Other ions such as calcium, magnesium, bicarbonate, sodium and heavy metals (cadmium, chromium, copper, nickel, lead and zinc) also contribute contribute to the water's toxicity (Clemente & Fedorak 2005; Allen 2008b).

2.3.3 Upgrading bitumen

The final stage is the upgrading process where bitumen is transformed into synthetic crude oil. The main function of this process is to reduce the bitumen viscosity by breaking down high molecular hydrocarbons (through C-C breakage) into distillable fractions with boiling points less than 525°C. Primary upgrading processes are based on thermal cracking, coking, or hydro-conversion. Thermal cracking consists of heating the

bitumen mixture to 475°C to 500°C. This removes the side chains from the high molecular weight compounds and reduces the viscosity.

Several methods are used to upgrade residues from vacuum distillation. One of these methods is delayed coking, where thermal cracking takes place in a furnace. Temperatures reach from 480°C to 515°C and gas is generated on coking drums. Coke is the main by-product of this process (Sawarkar *et al.* 2007). Fluid coking consists of a hot bed of coke particles (500°C to 540°C) that thermally crack bitumen and create different light products such as gases and gas oils (Gray 2002; Li *et al.* 2012). Coke is also a by-product of fluid coking. Some research has focused on how to manage and use coke in useful ways (Small *et al.* 2012). Finally, hydrocracking aims at getting rid of undesired elements (sulfure, nitroged or oxygen) by heating feeds at 400 °C and applying 8 to 15 Mpa of pressure. High quality products such gasoline and kerosene are produced from this method (Alfke *et al.* 2000).

Extracting the value-added product, bitumen, from oil sands formations is costly from beginning to end. The least expensive method for extracting bitumen is mining, which requires approximately $9 to $12 dollars per barrel. Other methods such as SAGD elevate the operating cost to $9 to $14 dollars per barrel (National Energy Board 2006). Mining and upgrading to synthetic oil is more costly and on the order of $18 to $22 dollars per barrel Synthetic oil production is the most expensive because it requires an additional process to refine the bitumen. This can be energy consuming but it creates various end products. Mining extraction is usually the least expensive of the methods to extract bitumen mainly because lumps of oil sands can be transported and treated; the yield bitumen recovery is high, up to 95%. Additionally, mining extraction accounts for 65% of the total production of oil sands and may stabilize around this figure by 2010(Allen 2008a). SAGD is a fairly efficient process that recycles 90% of the steam and recovers up to 70% of the bitumen in the deposit (Alberta government 2009).

2.4 Process affected water

Process affected water used in the hot water extraction process has had contact with bitumen and caustic solutions. This disturbs the water quality, as it gets contaminated with naphtha, bitumen, clay, sands and heavy metals. Water is essential for separating bitumen from oil sands and the amount required for the extraction is

large. Water that has been used in the process is stored in tailing ponds where it sediments, allowing the surface water to be reused in the process. The more water is recycled, the more contaminants are dissolved. Eventually the water quality decreases and fresh water is withdrawn from the environment. The need to treat the stored water from these ponds is a challenge that oil sands companies face today.

2.4.1 Contaminants present in process affected water

Oil sands companies base their operations and profits in bitumen extraction. One of the greatest environmental challenges today is dealing with the enormous amount of stored tailings and process-affected water.

Tailings consist of process-affected water, coarse and fine sediments, inorganic and organic products and residual bitumen left behind after extraction. Process affected water contains dissolved metals, ions, and organic compounds at different concentrations (Allen 2008a). The nature and concentration of both of these is largely dependent on the tailing pond itself. Concentrations of contaminants and water characteristics ultimately vary from pond to pond (Golby *et al.* 2012).

Typically, process-affected water has a hardness of between 15 to 25 mg/L Ca^{+} and 5-10 mg/L Mg^{+2} and an alkalinity between 800-1000mg/L HCO_3^{-}. Total dissolved solids range from 200 to 2500 mg/L and are mainly comprised of sodium, bicarbonate, chloride and sulfate ranging from 500-700, 75-550, and 200-300 mg/L, respectively (Allen 2008a). Several tailing ponds water samples were subject to different chemistry parameters to assess their quality as reported by Allen. Dissolved organic carbon (DOC) ranges from 26 up to 67 mg/L, biochemical oxygen demand (BOD) ranges from 10 up to 70 mg/L, chemical oxygen demand (COD) ranges from 350 to 525 mg/L. Oil and grease ranges from 9 to 31 mg/L. Naphthenic acids range from as low as 3 to 68 mg/L. Phenol detected as little as 0.8µg/L to 1.8 mg/L. Cyanide is detected as well; it is from 0.004 to 0.5 mg/L Polycyclic aromatic hydrocarbons (PAH) are detected in Syncrude MLSB as low as 0.01mg/L; BTEX detected are below 0.01mg/L from the same source. Allen provides further information on the ponds characterized in the following reference (Allen 2008b).

Other components found in process-affected water include bitumen, naphthenic acids (NA), asphaltenes, benzene, creosols, humic acids, fulvic acids, phenols,

phthalates, polycyclic aromatic compounds (PAHs) and toluene as shown in Table 3.4 (Allen 2008a). NA are complex mixtures of cycloaliphatic acids which have been studied extensively in petroleum reservoirs and in oil sands tailings (Scott *et al.* 2008). NA are found in bitumen and are released during the water extraction process; these compounds are important to mention since they are attributed to the toxicity found in process affected water. Extensive toxicity studies have been completed to elucidate this problem (Rogers *et al.* 2002).

Process affected water (previously used in the hot extraction process) is stored on site in tailings ponds, which are containment structures that hold the water until reuse. These ponds are engineered to allow the settlement of the sand and fines from the extraction process, allowing expression of the water, which can then be recycled. This process allows up to 80 to 85% of the process-affected water to be recycled (Syncrude 2005); since not all water can be recycled, water must also be withdrawn from fresh water sources (for instance, the Athabasca River) to maintain operation (Allen 2008a). As oil production increases, so does water withdrawal. Due to a zero-discharge policy for process affected water, it must be contained on site for the purpose of protecting the environment. This creates the enormous volumes of tailings, which remains a challenge for the industry to treat. Discharging the affected water into the environment would only be allowed once a technology is available to decontaminate particular stored water. Consequently, strategies to treat process-affected water are necessary to reduce environmental impacts and to increase the amount of water that can be recycled back into the process. Several technologies have been explored to investigate possible methods to treat process affected water.

2.4.2 Naphthenic acids

Oils sands tailings water contains high concentrations of inorganic ions and organic compounds which accumulate in the tailing ponds as a result of the extraction process. Of these, NA are of greatest concern and comprise 80% of the dissolved organic matter in the tailings ponds (Allen 2008a). NA are alkyl- substituted cyclic and aliphatic carboxylic acids, which enter the water phase during the extraction process. NA concentrations in tailings pond water range on average from 40 to 70 mg/L, although values as high as 130 mg/L in fresh tailings waters have been reported (Holowenko *et al.* 2002). NA can be described by the general formula $C_nH_{2n+Z}O_2$ where

n indicates the carbon number and *Z* determines the number of rings in a specific homologous series. Predominant structures found in process-affected water contain 5- or 6-carbons in different combinations, creating a complex mixture of compounds (Holowenko *et al.* 2002). Since the naphthenic acid composition found in oil sands tailings water is rather complex, some authors have suggested using more descriptive terminology to talk about the mixture. Some possibilities include the use of "oil sands tailings water acid-extractable organics" (OSTWAEO) or "acid extractable organics" (AEO) to differentiate from simple naphthenic acids (Grewer *et al.* 2010). Despite the extensive research done on the subject, there is still debate regarding how to measure and quantify NA.

Several techniques have been used to measure NA, such as synchronous fluorescence spectroscopy. The principle behind fluorescence spectroscopy is that light directed to the sample is absorbed by the sample, and some other molecules react to the light and fluoresce. The fluorescent light is emitted in all directions. Usually the detector is placed 90° from the emitted light to avoid transmitted or reflected light, and the signal is recorded. Fluorescence spectroscopy is a simple and fast method to monitor NA and it was used to quantify the organic fraction present in this study. This method is relatively fast and relatively cheap but it doesn't give detailed information on the characterization of the sample. Other methods to characterize NA are high-performance liquid chromatography (HPLC), mass spectrometry (MS), or gas chromatography–electron impact mass spectrometry (GC-MS) which, despite being time consuming, can provide qualitative data useful for comparing NAs from different sources (Scott *et al.* 2005). More equipment and methods are available to characterize mixtures of NA according to their retention time (Wang & Kasperski 2010). For further information on this topic refer to the following review (Headley *et al.* 2013).

It is important to accurately measure and quantify NA, to determine which NA are present and their internal structures, because larger structures represent a source of toxicity and may be recalcitrant or hard to biodegrade in the environment. Oil sands tailing waters and their components can leak or migrate from water to the sediment and possibly to the groundwater through seepage and into the underlying soils or water systems. The seepage process is intricate and could include physical, chemical or biological transformation before the waters migrate to underlying layers (Holden *et al.* 2011).

NA are known to be toxic to a wide range of organisms including bacteria, plants and rats (Clemente & Fedorak 2005). Acute toxicity test conducted in previous studies with Wistar rats have shown that from 3 to 300 mg/kg of NA had the liver as the target organ (Rogers *et al.* 2002). Another independent study demonstrated that tadpoles also showed reduced growth and liver dysfunction due to NA in the environment (Melvin *et al.* 2013). The evidence shows that NA have detrimental effects in the development and in organs of model organisms, which means they are likely to have the same effect on humans. In other words the presence of NA in the environment is toxic and there is a need to find a way to either prevent the leakage or a way to clean the acids once they are present in the environment.

2.5 Treatment of OSPW

2.5.1 Physical methods

A physical process to treat water means using filters, centrifuges or absorbents. The physical process itself does not change the water's chemical composition. Physical processes have been applied to oil sands tailing waters. Settling has been used primarily on the oil sands tailing ponds to produce a slow clarification, but it does not provide detoxification. Other methods that have been used include filtration, ultrafiltration and reverse osmosis which all three have poor suspended solid removal (MacKinnon & Boerger 1986). The dewatering of tailings is possible because dewatering removes water using a cyclone (Chalaturnyk *et al.* 2002). Centrifuges have also been used and they have certain advantages such as the recovery of large amounts of process water in a continuous process. Some disadvantages include high capital and operational costs. Tailings can be consolidated by changing pH, adding flocculating agents, agglomeration, bacterial treatment, and freeze-thaw dewatering. The advantages are the release of water that improves the densification rates. However, the effects of consolidation are modest. The use of fly ash as an absorbent added to tailings followed by filtration has also been studied (Bakhashi *et al.* 1975) as has high-intensity sound waves, but neither process has been implemented because both are too costly (Chalaturnyk *et al.* 2002).

2.5.2 Chemical methods

Chemical processes utilize a diverse set of liquid chemicals or even gases to treat oil sands tailings waters. Commercially, calcium sulfate ($CaSO_4$) is used to produce composite tailings (CT) by blending MFT and cyclone underflow. Another way to consolidate tailings is with the use of lime, $Ca(OH)_2$ (Chalaturnyk *et al.* 2002). The advantages of using a chemical reagent are that the reagent can reverse the effects of caustics used in the extraction process; a disadvantage is that there is limited knowledge of coagulants and flocculants (BGC Engineering Inc 2010). Other chemical treatments include adding coagulants and flocculants that help to clarify suspended solids but have, in certain cases, exhibited poor detoxification effects (MacKinnon & Boerger 1986). Coke has been studied as a potential absorbent for contaminants (Small *et al.* 2012). Using activated carbon to remove organic compounds is inexpensive but has high operation and maintenance costs (BGC Engineering Inc 2010).

2.5.2.1 Ozonation technologies

Ozonation and the use of hydrogen peroxide have been proven to reduce pollutants in waste water (Andreozzi *et al.* 2000). Other studies have used UV light and H_2O to determine the relative reactivity of the organic fraction in oil sands tailings (Afzal *et al.* 2012). The HiPOx process, which uses ozone and hydrogen peroxide, has been used in the past to treat oxygenates (MTBE and TBA) with relative success (Bowman 2005). Similar observations using ozone and hydrogen peroxide to degrade certain NA have also been noted (Perez-Estrada *et al.* 2011).

Oil sands tailings water underwent an advanced oxidation process (HiPOx) before placing it in the HiPOx treated OSPW bioreactor, (HTO bioreactor). This advanced oxidation process uses ozone (O_3) and hydrogen peroxide (H_2O_2). When the hydrogen peroxide is in a solution part of the hydroperoxide anion (HO_2^-) reacts with ozone arising a series of chain reactions that include hydroxyl radicals that interacts with ozone. Two reactions can be written to follow the process:

$$H_2O_2+2O_3\text{-}\rightarrow 2OH^{\bullet}+3O_2$$

$HO_2^- + O_3 \rightarrow HO_2^\bullet + O_3^\bullet$

Radical hydroxyl ($OH^\bullet$) ions are very reactive and unselective. They attack and oxidize most of the organic molecules leaving behind mineralized materials (Poyatos *et al.* 2010).

This process can easily be automated and can be used for the degradation of practically all compounds, which may include NA. This technology has been proven with other contaminants such as trihaloamnines, dimethyl sulfoxide (DMSO), sodium dodecylbenzenesulfonate (SDBS) and tested on oil sands tailings water (Poyatos *et al.* 2010; Perez-Estrada *et al.* 2011).

2.5.2.2 HiPOx™ process

The HiPOx™ process destroys contaminants in an efficient and quick way by exposing them to oxidants (ozone and hydrogen peroxide) under pressure. The injection of oxidizing agents promotes uniform mixing and improves the reaction between oxidants, radicals formed (OH•) and contaminants present in water. Oxidants are injected "in-line" trough different sparging tubes facing downstream from each oxidant. A typical molar ratio of H_2O_2/O_3 ranges between 0.5 to 4.0 but precise molar ratios depend on the varieties of COD present. After the addition of oxidants high intensity mixing is required. Intense mixing is achieved under high pressure which facilitates high intensity mixing/reactions stages. The use of inline static mixers or mixer with moving parts is recommended. The patent states that a pressure drop from 0.1 to 10 psig is a criterion for intense mixing. Residence time of the ozone/hydrogen peroxide and water mixture in the mixing zone is the time necessary to consume all of the ozone present in the mixture for proper use of the technology. The treatment of contaminated water in a series of mixing and reactions stages allows the use of less ozone, more contaminants are destroyed and are more efficient and economical to operate. The amount of mixing and reaction stages depend on the nature of the COD present and the removal required for the water (Bowman 1998).

The HiPOx™ unit process is depicted below. The influent enters the unit where oxidants are added. Afterwards, the effluent is subject to mixing and allowed a determined residence time according to the nature of the water. Finally, the water can undergo post-treatment usually by the use of several HiPOx™ units in series. The image below shows a single HiPOx™ unit.

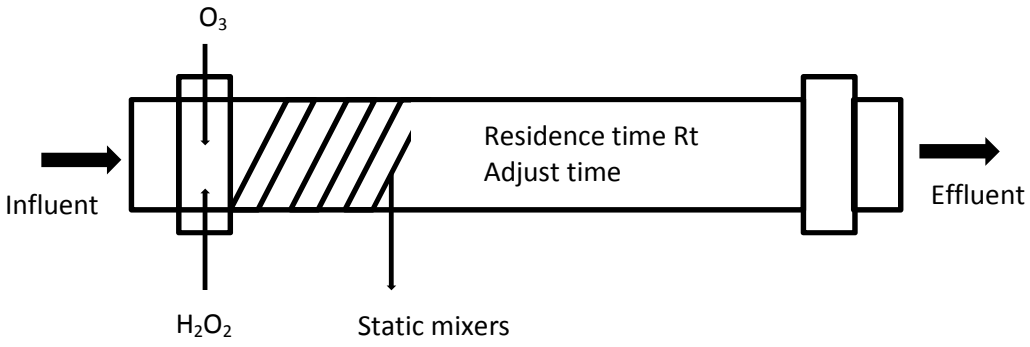

Figure 2. 2 Schematic of HiPOx™ advanced oxidation system. Modified from Bowman 2005.

2.5.3 Biological methods

Biological process can include natural attenuation, the use of biomass or the use of bacterial communities to treat the oil sands tailings. Natural processes involving the storage of tailings under aerated conditions provide detoxification but the clarification process is a rather slow two-year period One other accelerated natural process involves using fertilizer or biomass to provide detoxification (MacKinnon & Boerger 1986). Biological processes include bioremediation by indigenous microorganisms in the sediments of oil sands tailings water. Bioremediation using native organisms has the advantage to be relatively inexpensive and can degrade a variety of hydrocarbons. A patented biological process known as the Thiopac system uses sulfate reduction coupled with anaerobic steps (Van Lier *et al.* 1999). It can be used to remove sulfate from an effluent (Muyzer & Stams 2008). Other biodegradation options have looked at the aerobic biodegradation of NA with the use of bacteria, like *Pseudomonas putida* (Johnson *et al.* 2013). Bioremediation technologies include using indigenous micro alga to biodegrade NA and remove heavy metals (Quesnel *et al.* 2011; Mahdavi *et al.* 2012). One of the advantages of anaerobic degradation is that it is relatively cheap and requires less space than aerobic treatments. However, it is a relatively slow process.

2.5.3.1 NA biodegradation

The degradation of the organic fraction of oil sands has been proposed by either model NA and commercially NA compounds. Proposed mechanisms to degrade aliphatic and alicyclic carboxylic acids include aromatization pathways and α and/or β pathways. Generally all mechanisms involve producing hydroxylated intermediates. The majority of microorganisms then degrade aliphatic and alicyclic carboxylic acids by a β-oxidation pathway which involves forming a new carboxylic acid that has two fewer carbons than its predecessor. Another pathway in which NA are metabolized include aromatization on a cyclohexylcarboxilic acid performed by arthrobacter. The end product forms a ketone. To degrade aromatic carboxylic acids may involve forming dihydroxy intermediates split from an aliphatic carboxylic acid from mono and aromatic compounds from polycyclic aromatic hydrocarbons (Whitby 2010).

The biodegradation of NA have been studied extensively over the past decade. Microbiological studies have been made to analyze what bacteria are responsible for the biodegradation of NA in oil sands tailings. One study identified a *Pseudomonas stutzeri* and *Alcaligenis denitrificans, Acinetobacter calcoaceticus, Pseudomonas fluorescens* as naphthenic acids degrading organisms (Herman *et al.* 1994). Other authors point out that commercial NA reached a 95% degradation and partially degraded NA from OSPW by members of *Pseudomonas* (Del Rio *et al.* 2006). Researchers have point out that *Mycobacterium spp., Brevibacterium erythrogenes, Achromobacter sp., Corynebacterium sp., Rhodococcus sp., Acinetobacter sp., Alcaligenes spp., Flavobacterium spp. Moraxella spp., Micrococcus, and Bacillus sp* biodegrade recalcitrant NA (Whitby 2010).

Since literature has shown that some NA are more reactive than others it is important to know what type of NA are present to apply the adequate technology to break them down. Numerous studies have been conducted to determine ways to clean up these compounds. One of the technologies available today involves using an advanced oxidation process (AOP) to remediate NA. One paper describes using UV light along with H_2O_2. This paper reported that NAs with more atoms in their rings, increased alkyl branching and a single saturated ring on their structure, or a high number of carbons, could be degraded more quickly (Afzal *et al.* 2012). Biodegradation can degrade small structures at which AOP is less efficient. Hence, using these two technologies together may increase the efficiency removal of NA, as this research proposes (Afzal *et al.* 2012). Other investigations have shown that the structure of NA is involved in their degradation (tertiary carbons relative to a carboxylic group); ozone may react directly in the oxidation process but secondary reactions with radicals affect NA reactivity. Hydrogen peroxide (H_2O_2) may react with metals on the tailings, forming other oxidizing reagents (Afzal *et al.* 2012). Ozone and hydrogen peroxide in a solution form hydroxyl radicals and hydroxyl peroxide anions in a series of chain reactions. These anions, which are highly reactive, which allow to decompose the pollutant producing carbon dioxide, water and inorganic ions or at least transform compounds into more innocuous compounds (Poyatos *et al.* 2010). Partial decomposition of non-biodegradable contaminants can lead to biodegradable intermediates. For these reasons combined pre-treatments of AOP combined with the biological process are both cost efficient and viable from an economic perspective (Cañizares *et al.* 2009).

It has been reported that generally recalcitrant NA have high molecular weight, that have alkyl chains or methyl substituted cycloalkane rings (Smith *et al.* 2008; Paslawski *et al.* 2009). The methyl groups can create difficulties to NA biodegradation (Herman *et al.* 1993; Smith *et al.* 2008), mixed bacterial population can degrade NA by methyl substitution on cycloalkane rings (Herman *et al.* 1993; Headley *et al.* 2002; Smith *et al.* 2008) which shows the importance of microbial consortia for complete biodegradation (Johnson *et al.* 2011). Larger species of NA tend to be more recalcitrant. A study found that the recalcitrant species in NA is due to their cyclization periods. This means that NA acid with more rings on their structure, are harder to degrade. This trend was observed in cyclization periods (Z) from -2,-4,-6 and -8 (Han *et al.* 2008).

It has also been reported that after ozonation, microbial bioremediation accelerates, but still the mechanism behind this bioremediation is not yet understood (Scott *et al.* 2008; Martin *et al.* 2010b; Perez-Estrada *et al.* 2011). It's not only the quantification of NA that is important, but also their internal structure in a given sample.

The compounds that remain after AOPs process are small linear hydrocarbons or innocuous compounds. These hydrocarbons are not very reactive, so it is necessary to use oxidation processes and another method to break them down. Since the use of biodegradation can degrade small hydrocarbons, the use of the native microbial community has been studied to investigate, evaluate and assess its potential role in biodegradation.

There is little knowledge of biodegradation itself but this is a cheap technology that aims at removing the remaining fraction of hydrocarbons. NA can be treated by AOP and the biodegradation process which would be an economic way to reduce toxicity and undesirable characteristic. However, there is limited information about this method (Kannel & Gan 2012).

2.6 Anaerobic biodegradation of hydrocarbons

Anaerobic degradation of petroleum hydrocarbons by microorganisms was long believed to occur at negligible rates and was considered of minor importance (Widdel & Rabus 2001). Nonetheless, microbial degradation hydrocarbon compounds have been reported under anaerobic conditions (Mbadinga *et al.* 2011). These days anaerobic degradation of aliphatic and aromatic hydrocarbons is an alternative to the bioremediation process. Anaerobic bacteria are capable of using substrates in pure and complex mixtures such as crude oil (Holliger & Zehnder 1996).

Several organisms can degrade alkanes and also aromatic hydrocarbons. Such organisms are classified according to what electron acceptor the use, or what products they produce. These organisms are capable of breaking down compounds by oxidizing them; several organisms are more effective than others in doing so. The activation energy is involved in this process. Certain reactions are more favorable thermodynamically than others and certain reactions will enable the bacteria to take advantage of this energy more effectively than others. For instance, the biodegradation of benzene is thermodynamically more favorable if nitrate is present and this process is carried by nitrate reducing bacteria. This degradation has a free energy of 496.2kj per mol. In comparison, if benzene is subject to other electron acceptors such as iron or sulfate, they are less favorable with only -39.6 kJ/mol or -49.6 kJ/mol respectively (Spormann & Widdel 2000).

The degradation of hydrocarbon is subject to its substrate and the oxidizing agent used to degrade it. For instance, the aerobic process that uses oxygen rapidly degrades hydrocarbons. The oxidant or, in other words, the electron donor, oxygen, is thermodynamically more favorable than other electron donors. Other electron acceptors are available when the oxygen is absent. The most favorable electron donor in the absence of oxygen is nitrate, followed by iron, sulfate and carbon dioxide (CO_2). These electron acceptors can be reduced by certain organisms which provide energy for their functions. Microorganisms are classified according to the electron acceptors used; nitrate reducers use nitrate (NO_3^-), iron reducers survive with iron (Fe^{+3}), sulfate reducers utilize sulfate (SO_4^{-2}) and methanogens use carbon dioxide (CO_2). Anoxic or anaerobic environments include soil, groundwater, wetlands or soil reservoirs as well as

oil sands tailing water bodies where most of the mechanisms taking place are considered anaerobic.

2.6.1 Sulfate reducing bacteria (SRB)

Sulfate reducing bacteria are anaerobic microorganisms that are widely spread in environments that lack oxygen (anoxic). They use sulfate ($SO4^{-2}$) as a terminal electron acceptor to degrade organic compounds, resulting in the production of sulphide (S^{2-}). Sulphide can be further degraded by chemolithotrophic sulphur bacteria or by phototrophic sulphur bacteria to elemental Sulphure (S^0) (Muyzer & Stams 2008).

To date, SRB can be divided in two groups according to their metabolic function: incomplete oxidizers (iSRB), which mainly degrade organic matter to acetate (CH_3COO^-), and complete oxidizers (cSRB) which complete the degradation to carbon dioxide (CO_2)(McDonald 2007). Those bacteria that can degrade compounds to carbon dioxide also use acetate as a growth substrate and usually use the citric acid cycle (*Desulfobacter postgatei*) and the acetyI-CoA pathway (used by *Desulfobacterium, Desulfotomaculum* and *Desulfococcous* species and *Desulfobacca acetoxidans*) (Muyzer & Stams 2008). Incomplete SRB appear to grow faster than cSRB, for instance in sulfate fed reactor ISRB will dominate (Hilton & Oleszkiewicz 1988) while cSRB take longer period of time to become well-established (McDonald 2007).

2.6.2 Nitrate reducing bacteria

Denitrification is the process where nitrate reduction takes place and ultimately further reduces to molecular nitrogen (N_2). This process is achieved by nitrate-reducing microorganisms. Compounds found in water such as ammonium, nitrate, or nitrites are common contaminants and there is a need to treat these compounds because they can become a problem (toxic) if they exist in large quantities. The denitrification process is performed by anaerobic facultative bacteria which use nitrate as an electron acceptor (Fernández et al. 2008).

The majority of denitrifying bacteria are heterotrophic and use organic compounds as their electron acceptor; nonetheless, a limited number of bacteria can

perform a chemolithotrophic denitrification and use inorganic compounds such as reduced sulphur compounds, hydrogen, ammonium nitrite, ferrous iron (Straub *et al*. 1996) or even uranium (IV) (Beller 2005) as their electron donor for nitrate reduction (Fernández et al. 2008).

Nitrate-reducing prokaryotes are a diverse group of microorganisms categorized as alpha, beta, and gammaproteobacteria, as well as Gram positive bacteria. All of them share a similar biochemical mechanism to degrade nitrate (Philippot 2005). The process of denitrification carried by microorganisms and can be represented by the following equation:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2\,(g)$$

The enzyme responsible for nitrate reduction is called nitrate reductase. Nitrate (NO_3^-) and nitrite (NO_2^-) are reduced by the nitrate reductase to form nitric oxide (NO). Further reduction of nitric oxide along with nitrous oxide (N_2O) is performed by nitrous oxide reductase to form nitrogen (N_2). Some organisms can only carry out the first steps.

Nitrate (NO_3^-) can be used as an electron donor by heterotrophic bacteria or denitrifiers. Denitrifying bacteria can use both O_2 or NO_3^- as electron acceptors but if the oxygen concentration is sufficiently high, denitrifying bacteria prefer the use of oxygen (Seifi & Fazaelipoor 2012). Denitrification can be improved by external carbon sources such as acetate, methanol or glycol (Morgan-Sagastume et al. 2008).

Technological applications of biological denitrification can be used to treat a variety of water that contains a high concentration of nitrate. A study conducted by Nair *et al.,* developed a process to degrade nitrate in high concentrations by pre-treating and acclimating the nitrate-reducing bacteria using a effluent water from a fertilizer company; this bacteria was capable of degrading nitrate waste as high as 9032 ppm NO3-N, the highest reported, according to the author (Nair et al. 2007).

In order to evaluate the nitrate-reduction process taking place in the environment, genetic techniques can be used. Certain genes code for the enzyme, which performs a certain reaction. For instance, sulfate reduction is carried out by an enzyme called dissimilatory sulfite reductase (*dsrAB*). The denitrification process can be regulated by several genes such as *nirK,* which produces the enzyme nitrite reductase. Other genes such as *narG, nirk, nirS* or *nosZ* help to track down the denitrification

process (Madigan *et al.* 2009). Methanogens by methane production are usually followed by *mcrA* that encodes methyl coenzyme m reducatse. These genes are commonly used for monitoring the activity, or determining the presence of these bacteria in an environment.

2.6.3 Iron-reducers

Iron (Fe^{+3}) is abundant in nature and can be used by diverse bacteria as an electron acceptor for metabolisms. Iron reduction supports anaerobic respiration. It is thought that Fe (III) reducers are capable of oxidizing important fermentation by-products (Lovley 1991). The reduction potential is slightly electropositive (Fe^{+3}/Fe^{+2}, E=+0.2V at pH 7) and the iron reduction can be coupled to organic or inorganic electron donors (Madigan *et al.* 2009).

The iron-reducing bacteria couple the oxidation of hydrogen (H_2) or organic substrates to the reduction of ferric iron. This reaction provides energy by reducing Fe^{+3} to Fe^{+2} in anoxic and under non-sulfidogenic environments. Iron-reducing bacteria compete and interact with other microorganisms in anaerobic environments (Fredrickson & Gorby 1996).

Research has studied the energetics on Fe^{+3} in a gram-negative bacterium *Shewanella putrefaciens* by using various organic electron donors. Other important iron reducers to mention are *Geobacter, Geospirilllum* and *Geovibrio* (Madigan *et al.* 2009). Geobacter oxidizes acetate with Fe^{+3}, yielding a highly exergonic reaction (Madigan *et al.* 2009). Given the wide range of metabolic capabilities, iron-reducing bacteria may be important in the bioremediation of contaminated soils and aquifers.

2.6.4 Methanogens

The production of methane by specialized bacteria is referred to as methanogenesis. This group of bacteria, named methanogens, performs methanogenesis in anaerobic environments. The process reduces (CO_2) by hydrogen (H_2) to form methane (CH_4). The electrons to reduce CO_2 can come from formate, carbon monoxide or even certain alcohols (Madigan *et al.* 2009).

Two main processes occur when hydrocarbons are degraded. Once the organic matter has been decomposed into simpler compounds, such as acetate and hydrogen, acetoclastic methanogens uptake this substrate and transform it to methane and carbon dioxide. Other methanogens (hydrogenotrophic methanogens) take up dissolved carbon dioxide and hydrogen to produce methane and carbon dioxide (Siddique *et al.* 2011). Organic matter can be degraded to methane and CO2 in a variety of anoxic environments, from fresh water sediments, swaps, water-logged soils, rice paddies or sewage treatment plants, and methanogenesis is one of the last terminal electron-accepting processes to occur (Schink 1997).

Methanogens are classified as archea in the kingdom of euryarchaeota; they are further classified in five orders: Methanobacteriales, Methanococcales, Methanomicrobiales Methanosarcinales and Methanopyrales (Ferry 2010). Methanococcales is a marine species order; all the families are slightly halophylic and chemolithotrophic and use hydrogen gas to reduce CO_2 to CH_4. Methanomicrobiales reduce CO_2 to CH_4 using H_2, formate or alcohols; almost every species of this order requires acetate as a carbon source for growth. Methanosarcinaceae is the most metabolically versatile microorganism in the Methanosarcinales order, which can use as much as seven substrates to grow, including acetate; the order of Methanosarcinales grows and produces methane (CH_4) from acetate; it also produces energy for the metabolism by methyl groups from methanol and methylamines (Barber 2001; Ferry 2010).

2.7 Microbial communities in tailing ponds

The oil sands tailings contain a wide variety of microorganisms as found by several authors (Holowenko *et al.* 2000; Fedorak *et al.* 2003). Even though initially believed to be originated from the Athabasca river, bacterial and archeal population studies (using 16S rRNA and next generation sequencing technology) proved that these communities were "strikingly distinct from Athabasca rivers or tributary sediments" (Yergeau *et al.* 2012). Microbial populations in sediments close to the tailing ponds were similar in fine tailing ponds. For comparison, bacterial diversity is significantly lower in tailing ponds sediments (Yergeau *et al.* 2012). The results provided imply that the unique characteristics of tailing ponds harbor a specialized microbial community which may be originated from the river, oil sands bitumen or the extraction process itself. Once in the pond, organisms interact between each other (for instance, gene transfer) and adapt to their environment (gene expression). Organisms that are the most successful at adapting will survive and prevail (Ramos-Padrón 2013), creating a microbial community uniquely adapted to this environment.

Tailing ponds are mostly anaerobic. Most of the activity is done by anaerobic bacteria and archea. Since there is no oxygen present, other elements and/or compounds can be used as terminal electron acceptors. Nitrate (NO_3^-), ferric iron (Fe^{3+}), sulfate (SO_4^{2-}) and carbon dioxide (CO_2) are among the possible electron acceptors (Madigan *et al.* 2009). The energy obtained from the oxidation of an electron donor (for instance organic matter, hydrocarbons and sugars) varies depending on which electron acceptor is used. The most energy-producing electron acceptor reaction is nitrate reduction followed by iron, sulfate, and, finally, carbon dioxide (Madigan *et al.* 2009).

In tailing ponds the enumeration of sulfate reducing bacteria is reported before this study and it was reported an average MPN of 10^9 SRB cells/g of MFT, to 10^4-10^5 SRB per gram (Holowenko *et al.* 2000; Salloum *et al.* 2002; Fedorak *et al.* 2002). Methanogens encountered in tailings range from 10^5 to 10^6 methanogens per gram (Holowenko *et al.* 2000b).

One of the first attempts to identify bacteria within the tailing ponds was studied by Fought. Aerobic, anaerobic and sulfate-reducing bacteria counts were 106 cells/mL,103 cells/mL and 104 cells/mL. The predominant aerobic bacteria found were

Alcaligenis and *Acinetobacter*. Aerobic mineralization of compounds was performed using glycolic acids and glutamic acid within four days at 15°C by microbial populations from all depths (Foght *et al.* 1985).

Other authors have reported different compositions of microorganisms on sediments, specifically from MFT from Mildred Lake Settling Basin (MLSB) and West In Pit (WIP) (Penner & Foght 2010). It was found that most of the bacteria in the sediments correspond to Beta-proteobacteria, which is a very metabolically diverse group which contains chemoheterotrophs, photolithotrops, methylotrophs and this diversity is associated with sulphur-, nitrate- and iron-reducing mechanisms. One of the species encountered was a sulfur-oxidizing *Thiobacillus*. Also found was *Rhodoferax ferrireducens,* which is capable to reduce of Fe (III) or nitrate. *Thayera* was also found on sediments and it is believed to be responsible for degrading toluene under nitrate-reducing conditions. Sulfate reducing organisms were also found on these sediments which belong to Delta-proteobacteria and were *Desulfocapsa, Desulfatibacillum* and *Desulfobubaceae.* Also Syntrophus was considered to be related to one of the clones fond in the study. Fermicutes, Peptotrotococcaceae families were also found. The archeal organisms found were related to *Methanosaeta spp*. which is an acetoclastic microorganism (Penner & Foght 2010).

Bordenave showed that the organism methanogen *M.Barkeri* and the nitrate-reducing bacteria *Thauera sp*. strain N2, found in MFT sediments, have been shown to help in the sedimentation process, which aids in the conglomeration of clay particles (Bordenave et al. 2010).

More studies have been done in bacterial population from MFT from oil sands talings. One of the Sidique's studies pointed out the diversity of bacterial and archeal communities in MFTs; his findings were that sulfate-reducing genera in the genus of Desulfotomaculum and Desulfosporosis and genus *Cryptoanaerobacteres*, *Desulfosporosinus* all capable of anaerobic hydrocarbon degradation (Siddique et al. 2012).

Syntrophus and *Smithella* groups were abundant in hydrocarbon amended cultures and among these organisms at least the first genus is involved in methanogenic n-hexadecane degradation. Smithella sequences were also found independently in uncultivated methanogenic oil sands (Siddique et al. 2012). Betaproteobacterial

sequences, which are closely related to iron-reducing *Rhodoferax fermentans*, were also found in sediments. The betaproteobacterial sequences were found in the control enrichment culture, and it is suggested they play a secondary function and are out-competed by other species that readily degrade hydrocarbons. Other studies have reported the presence of clones, closely related to *Rhdoferax ferrireducens* (98.8%similarity), which can oxidize propylbenzene with ferrous iron production under iron-reducing and anaerobic conditions (Eriksson et al. 2005).Acidovorax has been found in other studies (Eriksson *et al.* 2003; Lin *et al.* 2007). Iron is believed to play an important role in biodegradation; for instance iron (Fe II) can be an electron source for iron-oxidizing microorganisms under anoxic and oxic environments and Fe (III) can function as a terminal electron acceptors in an anoxic environment for iron-reducing microorganisms (Weber *et al*. 2006)

Archea organisms such as acetoclastic methanogens (Methanosaetaceae) were found in n-alkanes-enriched cultures. Hydrogenotrophic methanogens (Methanomicrobiales) were found in enriched BTEX cultures. In contrast, these two methanogens were found in naphta-amendend cultures (Siddique et al. 2012) This information shows that certain bacteria can degrade different substrates under different environments.

Microcosms with oil sands tailings and MFT have been subject to restriction fragment length polymorphism (RFLP). Bacterial DNA has shown the existence of species related to Acidovorax and Rhodoferax species. Those species has been linked to degrade organic compounds (Eriksson *et al.* 2003; Lin *et al.* 2007). Acidovorax has been reported as part of a microbial community which degrade PAH coupled with nitrate reduction(Eriksson *et al.* 2003). Other species found were delta-proteobacteria and other clones grouped with Desullforomonadales, Desulfuromonas, Acetivibrio and Acidobacteriaceae (Li 2010).

Mesocosms were analyzed as well (on a 7-Liter MFT and and 233 mL of citrate, polyacrylamide or Albiuan diluent). PCR-DGGE analysis found Rhodoferax species among *Acidaminobacter hydrogenoforms*, which are capable of degrading citrate as a carbon source. This study showed that those organisms are the dominant groups in the mesocosms (Li 2010).

Megacosmos analysis (containing 2000 L of MFT and 800 L of water) were subject to PCR-DGGE analysis as well. The samples were taken at time zero, 5 months and 10 months. Columns were sampled for analysis. Samples revealed the presence of sequences belonging to Rhodoferax. Chloroflexi (found on Syncrude MFT) was found along with Duslfotomaculum which is a sulfate reducer bacteria. Spirochaeta appeared on MFT. Other sequences found were Polaromonas and Tepidomonas (Li 2010).

Li concluded that "Albian MFT contains some Bacterial and Archeal species similar to those detected in Syncrude MLSB MFT" study conducted by Penner in 2006. Although tailing ponds are similar in their chemical and bacterial composition, each one of them should be considered separately for management and reclamation purposes (Li 2010).

Furthermore, DNA analysis and pyrosequencing has been performed in water from a tailings pond at Syncrude (Pond 6) which it is considered to remain "somehow active" since in 2010 it stopped to receive fresh tailings (Ramos-Padrón 2013). Pyrosequencing enables rapid characterization of microbial communities. It is faster and has greater sequence depth than cloning and Sanger sequencing. Pyrosequencing makes it possible to assess hundreds of microbial communities.

2.7.1 Activity in ponds

The activities on tailing ponds are an example of anaerobic degradation of hydrocarbons and the different bacteria present in this environment that make the degradation possible. Tailings ponds from Suncor show the presence of abundant euryarcheota and proteobacteria. The latter is among one of the largest and most metabolically diverse of all Bacteria (Madigan *et al.* 2009). Acinobacter, syntropus, desulfocapsa, pseudomonas and methanogens were detected, along with other genera. See figure 2.7.

A study conducted by Ramos-Padrón on Suncor tailing ponds number 5 and 6 and their bacterial community that used a 16s rRNA and pyrosequencing approach is presented in the following section.

More in-depth studies performed in tailing ponds measured the sulfate-reducing and methanogenesis rates. Sulfate-reducing bacteria was most prominent at depths of 11 to 15 meters where anaerobic conditions were prevalent. Sulfate-reducing organisms were *Desulfocapsa, Desulfurivibrio,* and *Desulfobacterium.* For instance, the members of Desulfocapsa can use hydrocarbons. These genera are capable of disproportionation (oxidation-reduction) of elemental sulfur, sulfite and thiosulfate to sulfide and sulfate occurs; these genus are capable to grow on a hydrogen-sulfide scavenging agent like ferric iron (Finster *et al.* 1998; Winderl *et al.* 2008). Other syntrophs were also found and belong to *Pelotomaculum, Syntrophus*, and *Smithela.* Total methanogens include the genera *Methanosaeta, Methanoregula, Methanolinea,* among others (Ramos-Padrón *et al.* 2011)**.**

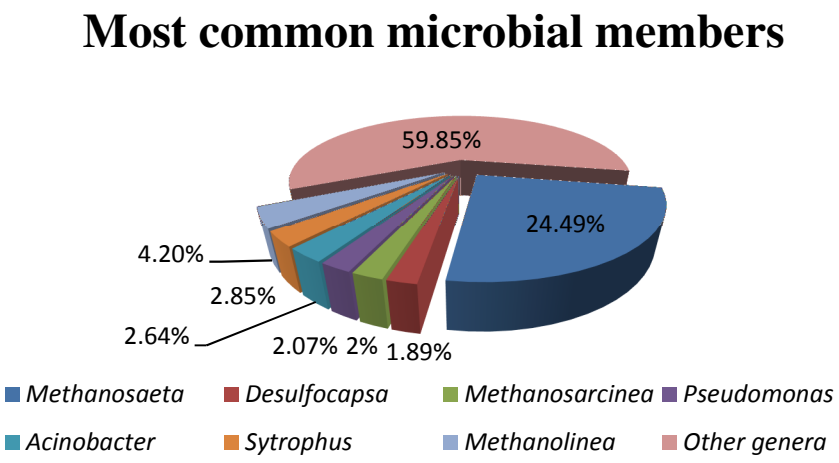

Figure 2. 3 Average of three-year samples of most common genera found in Suncor tailings pond #6; modified from (Ramos-Padrón 2013).

Moreover, a bacterial community profile was performed at different depths of the tailings pond, which provides information about the different bacteria present in the pond. Sulfate reduction and the methanogenesis rate were studied, along with the presence of sulfate-reducing bacteria, total syntrophs and methanogens. It is important to note that sulfate-reducing bacteria had larger numbers at depths of 11 to 15 meters below the pond's surface; this also corresponds with the sulfate reduction rate in the pond. On the other hand, syntrops and methanogens were distributed throughout the whole depth of the tailings pond. Among the sulfate reducers found were genera

Desulfocapsa, Desulfurivibrio, Desulfobacterium, Desulfuromonas, Desulfotomaculum, Desulfobulbus, Desulfomicrobium, Desulfobacca, Desulfofustis, Desulfosarcina, Desulfobacter, and *Desulfovibrio*. The total syntrophs reported belonged to *Pelotomaculum, Syntrophus*, and *Smithella*.

2.7.2 Bacterial community in a closed pond

A pond was closed in 2010 where bacterial community had already achieved methanogenesis. Members of *Methanosaeta* were identified in this pond. Once the pond was closed, no more tailings water was discharged. The lack of additional water made the bacterial community change from methanogens to putative hydrocarbon degraders (*Pseudomonas sp*.). This shift most likely occurred because when the pond was dewatered, it put stress on methanogens, along with the presence of oxygen and the availability of less biodegradable compounds. Pseudomonas appears to be degrading hydrocarbons in the tailing ponds along with *Acidovorax sp.* Stopping the discharge of tailing waters to the ponds when the bacterial community has become methanogenic, is a good approach to stop methane release and allow other alternative microbial activities (i.e., hydrocarbon degradation) (Ramos-Padrón 2013).

Golby and associates studied an oil sands tailings pond sludge samples from 0.45 m below the surface. They do not provide information about the tailings pond itself. The samples were used to produce two different kinds of biofilms: one that used a growth medium and one that did not. Those biofilms were subjected to pyrosequencing 454. Different bacteria were identified in biofilms with and without growth medium. This provides an example of what bacteria can grow and form biofilms in oil sands tailings and provides background information on what bacteria can be found in tailings and in a bioreactor.

According to Golby's results, the sludge contained 59% of proteobacteria of the total community. This was also the case for all biofilm community percentages between 65% to 73% of proteobacteria. Deltaproteobacteria accounted for were less than 1% of the total community compared to 7% in the sludge. Alphaproteobacteria were 5% of the total community compared to the initial sludge of 2.35% of the total community. On the

other hand Betaproteobaceria and Gammaproteobacteria remained comparable between biofilms and sludge with a 45%, 10% respectively of the total community. Chloroflexi was detected at 1.10% and 2.10% in sludges samples. Chloroflexi remained in less than 1% of the total community for other cultured samples. Fermicutes remained at 2% on sludge but had 8.20% on aerobic inoculum with growth medium and 5.7% on anaerobic medium with growth medium in the bacterial community. Euryarchea populations disappeared in all biofilms. The sludge and other biofilm cultured remained in the same proportions as those found by other researchers (Golby *et al.* 2012; Ramos-Padrón 2013).

The most abundant genus on the sludge was Brachymonas at 17.2%, followed by Acidovorax (6.2%) Variovorax (5.7%), Rhodofexas, and Thioalkalispira (3.7%). It is worthwhile mentioning that under different conditions of growth and with the addition of medium, some genus were more abundant than others. In anaerobic biofilms, which contained no growth medium, Hydrogenophaga was the predominant (19.5%), then Rhodoferax (9.9%), Methyloversatilis (9.9%), Magnetospirillus (6.5%) and Acidovorax (4.0%). The addition of the anaerobic medium (for freshwater or the GA medium) showed Methyloversatilis was predominant (17.6%), then Pseudomonas (8.4%), Thauera (8.0%), Azocarcus (6.0%), and *Acholeplama* (5.1%)

I would also acknowledge the presence of the Hydrogenophaga species. Even though its abundance on sludge is less than 1%, this microorganism has been identified in other wastewater treatment communities (Anders *et al.* 1995; Magic-Knezev *et al.* 2009) and it has also been isolated with other microorganisms as well such as Rhodoferax, Acidovoras, and Pseudomonas which were also found by authors from Calgary (Golby *et al.* 2012). The importance of this study was that it identified the abundance of this microorganism in isolated biofilms.

Other studies have relied on enriched culture from oil sands tailings ponds. The enriched microbial community culture was reported to be anaerobic and to degrade small hydrocarbon structures, alkanes ranging from C6- to C10, which is known as short-chain alkane degrade culture, SCADC and occurs under methanogentic conditions. The DNA was subject to 454 pyrosequencing, Illumina and Pyrotag sequencing. The Phylum found was very similar to that of other bacterial communities found in tailing waters. The main phylum corresponds to Proteobacteria, Fermicutes,

Bacterioodete, Spirochates, and Chloroflexi, which corresponds to 90% of the sequence reads. Additionally, Euryarcheota is predominant over Crenarcheota.. The study suggests the potential primary degradation of hydrocarbons with the presence of *Methanosaeta concilii*, *Syntrophus aciditrophicus*, *Desulfobulbus propionicus* and a sulfate reducer, *Desulfatibacillum alkenivorans* AK-01 (Tan *et al.* 2013).

The disimilatory sulphite reductase α- and β-subunits were detected in members of Delta-proteobacteria and Clostridia. The author states that this means that the anaerobic culture is capable of shifting from sulfate or sulphite reduction (Tan *et al.* 2013). Sequences found were related to Methanomicrobia, which have evidence of dsR-like protein in methanogens (Susanti & Mukhopadhyay 2012).

2.7.3 Activities and identification in sediments

Among the bacteria identified by pyrosequencing 454 performed on sediments from Syncrude and Suncor tailings ponds, bacteria found belonged to *Betaproteobacteria, Bacteriodetes, Deltaproteobacteria, Alphaproteobacteria, Chloroflexi, Firmicutes, Gammaproteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Cyanobacteria,* and *Epsilonproteobacteria* among others (Yergeau *et al.* 2012). The most abundant taxa found in the tailings ponds sediments are strict or facultative anaerobes (*Rhodoferax, Smithella, Thiobacillus*) (Yergeau *et al.* 2012) including *Pseudomonas, Thauera, Brachymonas* and *Acidovorax,* which were described in other research (Siddique *et al.* 2006; Penner & Foght 2010; Ramos-Padrón *et al.* 2011). I would point out the importance of the identification of *Geobacter, Rhodoferax, Smithella, Flavobacterium, Thiobacillos, Acidobacteria, Verrucomicrobi, Leptolinea, Ottowia, Salinimicrobium, Methylibium, Idionella, Leteobacter, Wollinea, Curvibacter, Desulforomonas, Hydrogenophaga, Polaromonas* and *Pseudomonas*. These organisms had over 100 sequences identified from each sample (if each taxa is combined with the identified sequences from all the sediment samples and added). Sediments from pong 6 from Suncor were used in our bioreactors for inoculum and It is expected that bacteria identified on this pyrosequencing study made by Yergeau, may be present in the bioreactors as well.

For archea species, *Methanospirilum Methanosarcina, Methanosaeta, Methanobrevibacter Methanolinea, Methanoculleus* and *Methanocorpusculum* were

found. *Methanobacterium*, although absent from the three tailings ponds, was present in Athabasca sediment and other studied samples. Almost all archea found were from either the *Methanocorpusculacea* or *Methanomicrobaceae* families (Yergeau et al. 2012).

2.8 Bioreactors using tailings ponds water

Studies comparing the bacterial communities in tailings ponds and bioreactors have demonstrated that the bacteria present in the bioreactors are comparable to those found in tailings ponds. In this regard bioreactors are a good approximation, in a laboratory setting, of what is happening on the tailing ponds.

Bioreactors studies had investigated the bacterial population shifts using oil sands tailings waters from Syncrude's West In Pit (WIP). Terminal restriction fragment length polymorphism (TRFLP) was done in a series of bioreactors. This study identified genus and species that include Proteobacteria (55%) and putative nitrate-, iron- and sulfate-reducing and hydrocarbon degraders, related to Thauera, Rhodoferax, and Desulfatibacillum. The study also suggests that there was no significant dissimilarity between populations in the WIP and those present in bioreactors (Chi Fru *et al.* 2013). Even though the pond studied is different from the Suncor pond, the bacteria present is similar in composition to that found in Syncrude tailings ponds, which reinforces the idea the bioreactors simulate the same environment present on tailings ponds.

This research found species very similar to those found in previous studies of oils sands tailings ponds: genus comprising *Acidovoras, Desulfocapsa, Hydrogenofaga, Desultomaculum, Pseudmonas, Anaerlonea, Rhodoferax, Syntrophacea, Cryobacterium and Brachymonas. Hydrogenophaga defluvii* was present in one of our reactors. This microorganism,as well as *Rhodoferax* and *Acidoborax* (Golby *et al.* 2012) has been found in anaerobic cultivated biofilms. *Brachymonas petroleovorans* was also found at the end of one of the AAAO bioreactor and it was present in water samples in the present research. *Cryobacterium psychrotolerans* was found only in the present research. Other uncultured bacteria pertaining to Chloroflexi genus, Syntrophea, Acidovorax and Desulfocapse were present on the reactors.

Since the reactors were sealed this allowed anaerobic conditions. Bacteria found on the reactors are mainly anaerobic and were also found in the tailing ponds. Moreover, bacteria present in the reactors are consistent with previous studies, which suggest that they are a useful medium through which to study tailings ponds.

2.9 Methods for studying microbial community structure in tailing ponds

A major part of the bacterial communities in nature have not been culturable; only less than 1% are culturable in the lab and the only way to obtain information about the uncultured microorganisms is by studying their nucleic acids, lipids and proteins. Nucleic acids can be used to analyse genomes or genes from the 16S or 18S rRNA for prokariotes and eukariotes. In recent years the field of microbial ecology has increased and many techniques have been developed to describe and characterize the functions and the phylogenies of microorganisms. These molecular techniques can be classified as partial community analysis approaches and whole analysis approaches. Partial community analysis includes denaturant gel gradient electrophoresis (DGGE), clone libraries, qPCR, fluorescence in-situ hybriditation, and DNA microarrays, to mention a few. Whole communities include genome sequencing, G+C fractionation, metagenomics, metaproteomics, preteogenomics and metatrascriptomics. These techniques characterize the structural, functional and metabolic diversity in the environment (Rastogi & Sani 2011). I will only describe a few methods to analyze environmental samples that were used in this work.

2.9.1 Culture-based methods

The majority of microorganisms, 99%, have never been cultured in laboratory conditions. This has stimulated development of new ways to separate a particular microbial specie in a pure culture. Culture-based techniques are useful to isolate bacteria from a sample. The culture is usually grown in an enriched media that is suitable for growth , providing resources (nutrients) under specific conditions (temperature, pH) that allow an organism to flourish. Enrichment cultures can affirm the presence of an organism but can never be certain of the absence of an organism (enrichment may provide insufficient nutrients). Isolation of an organism from the environment or enrichment culture can be grown on agar plates, agar shake or liquid media. Even though techniques for isolating microorganisms are widely established, a large part of the bacteria cannot be isolated in laboratory conditions. Other methods are needed to

identify bacteria (Madigan *et al.* 2009) because laboratory methods do not precisely mimic the environment in which certain organisms can grow.

2.9.2 Community analysis by 16S RNA

The enormous amount of bacteria that cannot be cultured can be identified by uncultured-based methods. Molecular biology techniques allow microbiologists to detect uncultivated organisms.

The use of 16S rRNA gene has been used to monitor community shifts and compare different communities (Muyzer *et al.* 1993) and has become a standard tool of molecular biology. This gene is an ideal marker mainly because it is universal to cells and is highly conserved structurally and functionally. This gene can be amplified using specific universal primers. Usually 16S RNA is used for bacterial identification which is capable of classifying bacteria. The use of special primers is crucial for the amplification procedure and this can target several genes or functions from a bacteria. These techniques require the extraction of high-quality nucleic acids prior to amplification and final analysis (Deng 2008 Today the use of the 16S rRNA gene is widespread and online resources such as BLAST (Sayers *et al.* 2009) and the Ribosomal Database Project (RDP) are available and helpful for identifying specific or novel species (Cole *et al.* 2009).

The use of polymerase chain reaction (PCR) was used to amplify 16S RNA gene, dissimilatory sulfate reductase (*dsrB*) and nitrate reductase (*nirK*). Only 16S RNA gene was used to monitor community shifts using denaturant gel gradient electrophoresis (DGGE).

2.9.3 qPCR for functional genes

Quantitative polymerase chain reaction (qPCR) produces quantitative information from a DNA sample and is usually done in a single process (Higuchi et al 1993). QPCR can investigate microbial diversity by targeting specific genes or functions within the sample. This will give an idea of the numbers of target organisms (i.e., sulfate-reducers vs. nitrate-reducers) present in a sample. By using different functional genes, qPCR allows the detection and quantification of microbial gene copy

numbers from samples that have their origin in the environment in real time (Smith *et al.* 2006). For instance genes for denitrification can be targeted by primers to identify genes such as *nirk* (nitrite reductase) or *dsrB* (sulfate reduction) (Throb□ck *et al.* 2004; Geets *et al.* 2006). Other genes that are conserved regions of DNA such as 16S (prokaryotes) or 18S (eukaryotes) can provide information about the numbers of the bacteria population.

QPCR uses a fluorescent dye to measure the replication process in a PCR cycle, and dyes can be SYBR green or TaqMan. These dyes attach to double strand DNA and emit a flourescent signal when are bind to DNA; later these dyes are degraded by the polymerase. This allows the amplification cycle to be recorded by measuring the emitted light. The cycle threshold or Ct is the cycle where the florescence is detectable over the background in the exponential phase of replication and the values measured are used for quantification (Smith *et al.* 2006). Probes have been designed and used to provide a rough estimate of the total gene copies of the 16S rRNA, from all the microbial population present in a given sample. QPCR was used in this study to assess the numbers of total bacteria, sulfate-reducer bacteria and nitrate-reducer bacteria.

2.9.4 Denaturant gel gradient electrophoresis (DGGE)

In 1993, Gerard Muyzer presented this technique to assess complex microbial communities in the environment. This method is based on the amplification of 16S nucleic acids fragments to be analyzed into polyacrylamide gels which contain a linearly increasing gradient of denaturants, in this case urea or formamide. DNA fragments then move along the gel and melt or denature until they stop, at different points in the gel, making a banding pattern that can be compared to/with different environmental samples (Muyzer *et al.* 1993). DGGE is a robust and rapid technique that allows to identify individual populations; it is a valuable, popular and well-established method which can be used to compare different samples as well as different microbial populations such as bacteria, archea and eukarya in a variety of environments (Muyzer *et al.* 1993; Ferris *et al.* 1996; Casamayor *et al.* 2002; Cherobaeva *et al.* 2011). This method has been applied to study activated sludge with success (Nielsen *et al.* 1999). In theory, each band represents a single kind bacteria but from time to time there are problems resolving the DGGE banding pattern, resulting in mixed populations of bacteria from a single band; moreover, this method can be used only with DNA

fragments equal to or less than 500 base pairs and is subject to biases from the PCR reaction as well. Therefore, these results should be analyzed with great care.

The fragments amplified from PCR using the 16S rRNA gene were used in DGGE. DGGE was made with the objective to monitor and identify the bacteria present. Each fragment is separated on the DGGE according to its GC content. Theoretically each band represents a single organism. Each band contains a DNA sequence that was compared to databases (NCBI or RDP) that allows identify an organism. Since DGGE only assess what species are in the sample, the use of this technique is merely qualitative. A quantitative technique (qPCR) makes it possible to determine the numbers in a certain specie (i.e., nitrate reducers) but cannot provide information about the actual identity of the bacteria present. DGGE and qPCR techniques were used together to provide a better picture of the process ongoing in the reactors.

2.9.5 Pyrosequencing

Pyrosequencing is a powerful technique that generates information faster than any previous method. For instance, in pyrosequencing a DNA sample molecule is broken in segments of 100 base pairs. Then each fragment is attached to a bead and further amplified by PCR. Each bead now has several copies of the fragment and are placed in a plate with millions of wells, which each one contains a single bead. The nucleotides are then sequentially added in a fixed order; sequencing reactions occur at the same time in what is described as "massive parallel" sequencing. Massive parallel sequencing provides information about several identities of samples in a relatively short time. This technique can now be performed in laboratory with DNA sequencing technologies and creates a huge amount of information for a given sample (Madigan *et al.* 2009).

Massive parallel sequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis. A reaction mixture carries four enzymes to detect nucleic acid sequences during DNA synthesis. In pyrosequencing, the sequencing primer is coupled with a single stranded DNA biotin-labeled template and mixed with enzymes: DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phophosulfate (APS) and luciferin. Deoxyribonucleotide triphosphates, (dNTPS) are added separately and constantly to the reaction mixture. Nucleic acids are replicated and PPi is released

by the activity of the polymerase. Each nucleotide incorporated in the DNA strand releases inorganic pyrophosphate (PPi). PPi is converted to ATP, which drives a luciferase reaction, producing a visible light which is detected by a photon detection device. The light and the intensity of the light are proportional to the added nucleotide, and a sequence is recorded (Petrosino *et al.* 2009; Fakruddin *et al.* 2012).

This technology has been evolving and now it provides a high-throughput analysis. Sequence reads are up to 350 base pairs and can run parallel analysis of up to 300,000 samples, generating significant information in very short time frame, usually as short as four hours. Pyrosequencing analysis has been used to identify bacteria, fungi, viruses, mutations, clone checking, environmental bacterial communities or even genome sequencing, to mention a few applications (Petrosino *et al.* 2009; Fakruddin *et al.* 2012).

2.10 Conclusions

The use of indigenous bacterial communities and their possible anaerobic degradation of oil sands tailings were investigated in this work. Several parameters were studied such as the degradation of organic matter (COD), tracking or naphthenic acid, and anion levels (SO_4^{-2} and NO_3^-). Quantification of target genes for total bacteria (16S), sulfate-reducing organisms (*dsrB*) or nitrate-reducing organisms (*nirK*) was performed and compared against the chemistry parameters measured. Finally, the bacterial community was assessed by identifying the dominant bacteria present using DGGE, cloning, sequencing and online databases such as National Centre for Biotechnology Information (NCBI).

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Chapter 3: Anaerobic degradation of oil sands tailings

3 Introduction

The Athabasca oil sands located in northeastern Alberta, Canada contain deposits of 174 billion bitumen barrels (Teare *et al.* 2012). Bitumen is extracted through either surface mining or in situ processes. Surface mining requires transporting the ore to an extraction plant where it undergoes a Clark hot water extraction process. The resulting mixture of bitumen, sand, clay and organic matter is aerated and the bitumen rises to the surface, forming froth, which is separated and further processed at an extraction plant. The residual bitumen, sand, clay, and water used in the extraction process are called tailings and are stored on site in tailings ponds due to a zero discharge policy enforced by the Alberta government. It is estimated that there are more than 70 km^2 of tailings ponds in the Athabasca oil sands region (Allen 2008a). Tailings ponds also allow the sands and fine clays to settle out of the water fraction (known as oil sands process water, or OSPW), allowing water to be recycled back into the extraction process. Periodically, fresh water is withdrawn from the Athabasca river to supplement the recycled OSPW, creating a larger inventory of water stored on site. Therefore it is essential to treat oil sands tailings water for future, responsible discharge to the environment. OSPW has numerous contaminants and fine particles. Several contaminants are known to be harmful. The organic fraction, NA, is particularly important since it has been associated with toxicity in animals (Rogers *et al.* 2002). Hydrocarbons associated with bitumen extraction, such as benzene, toluene, phenol and PAH hydrocarbon, are toxic (Brownlee *et al.* 1999; Allen 2008a). Heavy metals content has also been indicated as toxic (Clemente & Fedorak 2005).

Over the years several treatment solutions have been proposed to treat oil sands tailings. Chemical and biological treatments have been used to treat tailings. Chemical treatments include metallic coagulants and ozone, which remove organic fractions from oil sands tailings (Scott *et al.* 2005; Pourrezaei *et al.* 2011). Biological treatments include indigenous microbial populations from oil sands (Herman *et al.* 1994). Aerobic packed-bed bioreactors (Huang *et al.* 2012) have been used to degrade the organic fraction in tailing ponds. These technologies can have an impact in the treatment of oil sands tailing waters. Nevertheless, the chemical process does not remove all of the organic fraction; it leaves behind small structures. Biodegradation can remediate short and long hydrocarbons present in indigenous community of oil sands. The use of

coupled technologies, chemical and biological technologies may enhance water treatment and may give more information of what can be achieved with these technologies as previous literature have suggested (Afzal *et al.* 2012). Additionally there is little information about anaerobic biodegradation of oil sands tailing; this is a knowledge gap that needs to be filled.

This research investigates the capacity of the bacterial community from mature fine tailings (MFT) to treat oil sands tailings water under anaerobic conditions. More specifically, this study will compare the remediation performance and bacteria present in treated and untreated oil sands tailings. Chemical analysis, quantification and identification of dominant bacteria will enable further understanding of the bioreactors′ performance. The use of anaerobic bioreactors may provide an economical and effective technology to treat oil sands tailings water.

3.1 Materials and Methods

3.1.1 Sample Source

Mature fine tailings and oil sands tailings water were provided by Suncor Energy Inc. Oil sands tailings water was collected from Suncor Pond 1A and shipped to University of Alberta and stored at 4°C until used. MFT were collected from the same pond and shipped and stored at 4°C until used. One part of the oil sands tailings water was subject to a chemical oxidation treatment by an oxidation process (HiPOx) prior to use in this study.

3.1.2 Experimental Design

Bioreactors were prepared in 2-Liter crystal glassware bottles (h x d: 250mm x 138mm) with screw caps. Reactors were then placed in an anaerobic bag with an inert atmosphere (N_2 99.9%) to ensure anaerobic conditions. Acetic acid amended bioreactor, AAAO bioreactor, consisted of fresh oil sands tailings and 250 mL of MFT. Fresh oil sands tailing and MFT were pumped separately into the bottle using an electric pump attached to a hose. Once in the bioreactor, 3 mg/L mL of acetic acid was added, or as a 300 mg/L as COD to provide a primary substrate to microorganisms. It was added to

adjust the pH to 7. HiPOx treated OSPW bioreactor, HTO bioreactor, consisted of pretreated oil sands tailings which have undergone a HiPOx process. Pretreated water volume of 1750 mL and 250 mL MFT were pumped separately into the bottle using an electric pump attached to a hose. No amendments were added in this bioreactor

Sampling was done by filling the anaerobic bag, shaking the entire reactor for complete homogenization. Afterwards the screw cap was taken off and 20 mL of sample from each bioreactor was removed. Chemical and biological analyses were then performed on each sample as outlined below.

3.1.3 Analytical Methods

Chemistry parameters were monitored such as pH, oxidation reduction potential and dissolved oxygen. Samples were measured on samples as is, with no centrifugation step. These parameters were measured using a pH probe (Accumet AR15), an Accumet metallic ORP electrode (Fisher Scientific) and an optical dissolved oxygen probe (model HQ30d, Hach) respectively. Instruments were calibrated periodically against known standard solutions.

Samples were then centrifuged using a Multifuge® 3 L-R Heraeus at 3750 rpm for 30 minutes to remove the fine tailings. Water was filtered using a vacuum pump through a 0.45µm cellulose media (Milipore) filter. Water was then ready for chemical oxygen demand (COD) analysis and was subjected to a COD protocol according to Standard Methods of Water and Wastewater, method number 5220 D (American Public Health Association 1999). Digestion was performed using a Digital Reactor Block 200 (Hatch, Loveland, USA) at 150°C for two hours. Spectrometer readings were done at 420 nm wavelength using a spectrophotometer Ultrospec 1100 Pro (Biochrom, Cambridge, UK).

Remaining water samples were filtered again using a 20mL plastic syringe and a 30-mm Teflon syringe filter with a filtering media of 0.45µm (Thermo scientific) for Ion Chromatography. Anions were measured using a Thermo Scientific Dionex ICS-5000 Ion Chromatography (IC) apparatus using an AS14A anion exchange column (4x250 mm ion pack) and an eluent stream (8.0 mM Na_2CO_3, 1.0 mM $NaHCO_3$).

Measurement and analyte quantification were performed by a Dionex CD25 Conductivity Detector and Chromeleon Client v6.50 software respectively (Holden *et al.* 2011). The major anions measured were sulfate and nitrate. The mean detection limit was less than 0.2 mg/L for the mentioned anions.

NA quantification was measured using a Varian eclipse fluorescence spectrophotometer with a scan rate of 600 nm/min (Lu *et al.* 2013). Fluorescence emissions were recorded from 260 to 600 nm with 1 nm increments at specific wavelengths 250 to 450 nm with 10 nm increments. The excitation and emission slit was set to 10 nm and 5 nm. Absorbance was recorded on a Shimadzu UV2401-PC. UV-Vis spectrophotometry and wavelengths obtained were from 250 to 600 nm with 1 nm increments. Fluorescence intensity data was corrected according to Tucker's method. Detection limit was1.0 picoMolar fluorescein using a standard cell.

3.1.4 DNA extraction

Biological samples were taken as follows: reactors were agitated to homogenize the sample, then nitrogen was filled into a plastic bag where reactors were placed, then reactors were opened and two-10 mL were withdrawn for replicate reactors for a final 20 mL sample. Samples were taken at different intervals from the HTO bioreactor and AAAO bioreactor and stored at -20°C until DNA was extracted. The DNA was extracted using a PowerSoil® DNA Isolation Kit (MO BIO laboratories) according to the manufacturer's protocol, except that 60 µL 2X EDTA and a solution of lysis buffer (0.5 M EDTA and 5% sodium dodecyl sulfate) were added to aid in the cell lysis and remove fatty acids from samples. Extracted DNA samples were stored in a freezer (-20°C) for further use.

3.1.5 PCR amplification of the 16S rRNA gene

Amplification of extracted DNA was performed using a 25 µL reaction mixture, in triplicate. The final concentrations of each component were as follows: approximately 20 ng of template DNA, 1X buffer mixture (Invitrogen), 1.5 mM of $MgCl_2$, 200 µM of each deoxynuclesoide triphosphate (dNTP), 0.5 µM of each primer, 0.1U Taq DNA Polymerase (Invitrogen) and nuclease-free water (Thermo scientific). PCR amplification of the 16S rRNA genes for total bacterial analysis was performed

using 341F-GC (5'-CCT ACG GGA GGC AGC AG-3') with a GC rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') as suggested before and 907 R (5'-CCG TCA ATT CAT TTG AGT TT-3') primers according to reported literature (Muyzer *et al.* 1993; Lima & Sleep 2007). The PCR reaction used the following cycle: 95°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 3 minutes and, lastly, a final extension step at 72°C for 5 minutes. The reaction mixture was amplified using "My Cycler" (Bio-Rad laboratories, CA, USA). The resulting PCR products were inspected on a 2% agarose gel stained with 1X SYBR® Safe DNA Gel Stain (Invitrogen) and photographed under UV light. Fragments of the correct size were pooled together and used for DGGE analysis.

3.1.6 DGGE

A DGGE profile was performed using a Dcode™ Universal Mutation Detection System (Biorad, Hercules, CA). Electrophoresis was carried out in 0.75-mm polyacrylamide gels (6% [wt/vol] acrylamide-bisacrylamide 37.5:1) containing 20 to 70% urea-formamide denaturing gradient for the first gel and 30% to 70% for the second gel (where 100% indicates a 7 M urea and 40% [vol/vol] formamide). The gel was run at a constant voltage of 100 V for 480 minutes at 60°C in the 1X TAE buffer (40 mM tris base, 20 mM acetic acid glacial, 1mM EDTA, pH 8 and dH_2O) for the first gel. For the second reactor a second gel was run at a constant voltage of 100V for 460 minutes at 60°C in the 1X TAE buffer. Gels were stained using SYBR Safe DNA Gel Stain (Invitrogen) 1X in Micropore water for 30 minutes. The gel was photographed using a UV transluminator apparatus. Excised bands were cut using sterile razor blades and washed with 150 µL of water twice, after which 15 µL of water were added to the excised band. The water and the excised band were stored overnight at 4°C in the refrigerator to allow passive diffusion of DNA to water. For a complete methodology, the reader is encouraged to consult the following reference (Green *et al.* 2009).

3.1.7 Cloning

DNA in water solution from the excised bands was amplified again using the primers and protocols described above in Section 3.1.5. Two microliters from the eluate were used as a template for PCR amplification. Amplification was checked on a 2% agarose gel to confirm correct product size. PCR products were purified using Exosap-it (Affymetrix, CA, USA) prior to cloning. The resulting products were cloned in a vector pGEM® 5Zf(+)-T Vector system (Promega, Madison, USA) by TA cloning. Ligations reactions were transformed into competent *Escherichia coli* JM109, plated, and incubated overnight at 37°C. Three colonies were picked from each transformation and plasmid was recovered using a QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen, Valencia, USA). Further amplification using M13 forward (5'-CAG GAA ACA GCT ATG AC-3') and M13 reverse primers (5'-CGC CAG GGT TTT CCC AGT CAC-3') plasmid were used. Each reaction consisted of 1 X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µL template (extracted plasmid), 1 µM of each of the primers and 1.25 U Taq polymerase. The reaction mixture was filled with sterile water to reach a final volume of 25 µL.

Amplification was performed using the following PCR conditions: 94°C for 10 minutes, then 25 cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and 30 seconds, and a final extension step at 72°C for 5 minutes. Product sizes were inspected on a 2% agarose gel. PCR products of the appropriate size were purified using Exosap-it according to the manufacturer's instructions. The resulting DNA was then diluted accordingly for sequencing at The Applied Genomics Centre (TAGC), Department of Medicine, University of Alberta.

3.1.8 Sequence analysis

Sequences obtained were inspected using Sequence Scanner Software v1.0 (Applied Biosystems). Sequences were screened for vector contamination of nucleic acid sequences using an online vector screener (Vecscreen) available online through NCBI (National Center for Biotechnology Information) (Sayers *et al.* 2009). Sequences were further curated to delete primers sequences. The best sequences were then chosen for the downstream analysis. The sequences were compared to existing databases such as NCBI or Ribosomal database project, realease 10 (RDPII). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al.* 2011). Sequences were aligned using an embedded version of ClustalW (Pairwise aligment and multiple alignment 3 and 1.8 gap opening penalty and gap extention penalty, respectively). A phylogenetic tree was constructed using a Neigborg-Joining method, with a pair-wise deletion and 2000 bootstrap values (Higgins *et al.* 1994; Sanders *et al.* 2010).

3.1.9 Quantitative PCR (qPCR)

Amplification of gene 16S, *dsrB* and gene *nosZ* was performed to quantify the total bacteria, sulfate reducers and nitrate reducers respectively (Henry *et al.* 2006; Foti *et al.* 2007; Lima & Sleep 2007). The total bacteria reaction mixture contained 1X of Eva Green buffer, 625 nM of each primer and a DNA template of approximately 20 ng. For sulfate reducers the reaction mixture contained 1X Eva green buffer, 200 nM of each primer (0.2 µM), approximately 15 ng of DNA and water for a 20 uL reaction mixture. For nitrate reducers targeting the *nosZ* gene the reaction contained 1X Eva green buffer, 100 nM of each primer (0.1 µM), approximately 15 ng of DNA and water for a 20 uL reaction mixture. The protocol for total bacteria was as follows: 94°C for 3 minutes, followed by 40 cycles of 94°C for 45 seconds and 59°C for 45 seconds. The amplification protocol for sulfate reducers was 95°C for 3 minutes, 39 cycles of 95°C for 40 seconds, and 55°C for 40 seconds. The amplification protocol for nitrate reducers was 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, and 62°C for 30 seconds. Fluorescence readings were done at the end of each cycle. A melting curve was programmed from 65°C to 95°C measuring the fluorescence every 0.5°C on all protocols. Standards were prepared using a pure culture of *Pseudomona putida* (ATTC 25922), *Desulfovibrio vulgaris* (ATTC 29579) and *Nitrosospira multiformis* (ATTC 25197). The number of gene copies was calculated using the copy numbers for each standard curve. The gene copies were calculated based on the following equation: copy number = $(N \times A \times 10^{-9})/(660 \times n)$, where N is the Avogadro number (6.02×10^{23} molecules per mol), A is the molecular weight of the molecule in the standard, and n is the length of the amplicon in base pairs (Li *et al.* 2009). This known copy number was serially diluted in triplicate to create a standard curve. Samples from the bioreactors were also analyzed in triplicate. Data was analyzed using CFX Manager™ Software (Biorad laboratories).

Table 3. 1Gene target and primer sequences

Gene	Primers	Sequence	Reference
16 S	341f[a,b]	5'-CCT ACG GGA GGC AGC AG-3'	(Muyzer *et al.*
(Universal)	534r	5'-ATT ACCGCGGCTGCTGG-3'	1993; Lima
	907r	5'-CCG TCA ATT CAT TTG AGT TT-3'	& Sleep 2007)
dsrB	dsr2060f	5'-CAA CAT CGT YCA YAC CCA GGG-3′	(Foti *et al.* 2007)
	dsr4r	5′-GTG TAG CAG TTA CCG CA- 3'	
nosZ	nosZ2f	5'- CGCRACGGCAASAAGGTSMSSGT-3′	(Henry *et al.* 2006)
	nosZ2r	5′-CAKRTGCAKSGCRTGGCAGAA- 3'	

[a]F, forward primer; R, reverse primer.[b]Primer with a 40-bp GC clamp rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3').

3.2 Results

In this section the results are presented starting with the AAAO bioreactor and then with the HTO bioreactor. Bioreactors' performance was based on their ability to degrade organic matter in form of COD and the organic fraction NA. Then the electron acceptors (SO_4^{-2}, NO_3^-) were monitored and correlated with the degradation of COD or NA. Next quantified nitrate reducers, sulfate reducers and total bacteria are discussed and related to the chemistry collected. Finally, a more detailed look was presented about the microbial communities on each bioreactor.

3.2.1 AAAO Bioreactor

The AAAO bioreactor containing 1750 mL of OSPW and 250 mL of MFT was able to reduce the COD in the first 16 days from 564 to 289 mg/L. This degradation may be due to the rapid biodegradation of the substrate added and the COD existing in the OSPW indicating a potential co-metabolism. These results indicate a reduction of 49% of organic matter. After this time, the degradation of organic matter slowed down substantially and a final concentration of 170 mg/L was observed on day 72. Overall, organic matter in the reactor was reduced by 70%. For the first 18 days, COD reduction correlated with sulfate reduction, yielding a 94% reduction at day 18, from 132 to 8 mg/L. It can be seen that sulfate (electron acceptor), along with living microorganisms, is oxidizing the organic matter (electron donor) and biodegradation is taking place. The fact that biodegradation was taking place led us to use a different electron acceptor, nitrate, once sulfate was depleted later on the experiment (day 16). For naphthenic acids the concentration reduced from 30 to 29 mg/L for the first 5 days. When nitrate was added on day 16, nitrate began to decrease slowly until day 31. To incorporate nitrate, which is a more thermodynamically favorable electron acceptor than sulfate, may allowed to degrade the remaining electron donors. Sulfate, on the other hand, began to increase after nitrate was added on day 18, reaching its maximum value at 107 mg/L on day 34. In this case the sulfide was oxidized by the nitrate, increasing the sulfate concentration again. This process continued until nitrate was depleted, on day 30, and sulfate reduction took place again using the remaining available electron donors. COD continued to decrease slowly until the last measurement on day 72. Finally, naphthenic acids decreased from 30 to 26 mg/L yielding an overall 14% reduction.

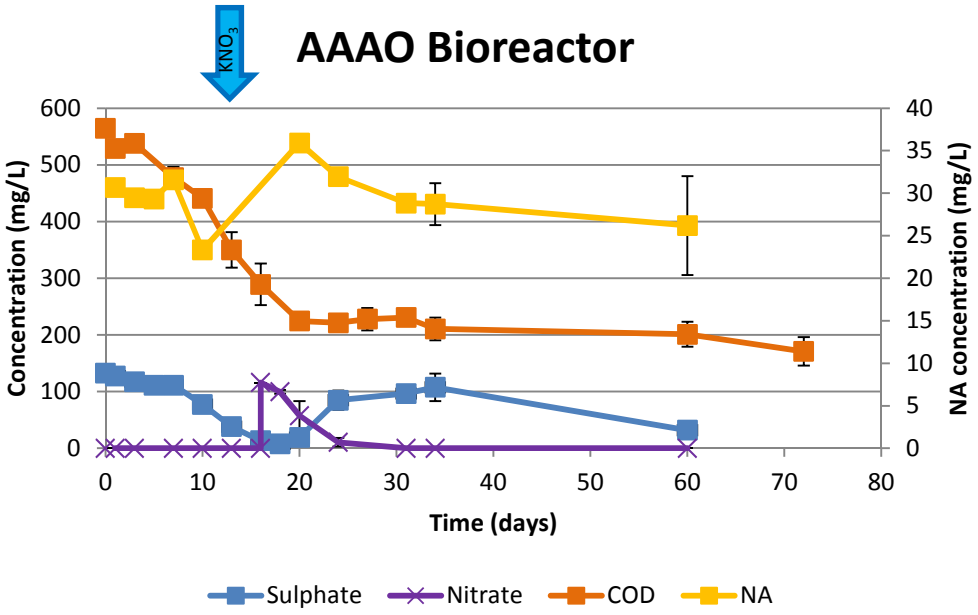

Figure 3. 1 AAAO bioreactor (oil sands tailing water 1750mL, 250 mL MFT) electron acceptor and electron donor concentrations over time. Chemical oxygen demand is abbreviated as COD, naphthenic acids as NA. The arrow indicates the addition of nitrate in the form of KNO_3 to the reactor. Error bars indicate one standard deviation.

Quantification of nitrate-reducing, sulfate-reducing and total bacteria was performed for this reactor. Initial copy numbers of nitrate-reducing bacteria were determined to be $1.92x10^3$ copy numbers per gram and remained at this concentration for the first 10 days. By day 72 the numbers increased one order of magnitude to $1.6x10^4$ copy numbers per gram for a total increase of 733%. The numbers for sulfate-reducing bacteria remained between $1.69x10^6$ to $4.29x10^6$ copy numbers per gram of sample for a final increase of 154% during the first 72 days. Finally, for total bacteria, the initial quantification was $4.68x10^6$ copy numbers per gram, but dropped by day five to a value of $4.44x10^5$ copy numbers per gram. At the end of the experiment the total bacterial count was $9.59x10^4$ copy numbers per gram for a final reduction of 97% of total bacteria. The final data on day 72 shows sulfate-reducing organisms at $4.29x10^6$ copy numbers per gram which is higher than the total bacteria, at $9.59x10^4$ copy numbers per gram which shows an increase of 154% in numbers of this type of bacteria. This may be due to the fact that sulfate reducing bacteria are specialized bacteria that can thrive in this special environment, out competing total bacteria in numbers. Before reaching the former statement, it

was thought that total bacteria needed to be in higher numbers than sulfate reducing bacteria and it was attributed to experimental biases as a result of poor or incomplete DNA extraction, damaged DNA, which resulted in a reduced amplification of PCR, yielding a lower copy number than expected. After several attempts to correct this value using different DNA extraction methods and making sure we had the best amplification protocol, it was clear that this value was correct. It is clear then, that sulfate reducing bacteria were in higher numbers in this bioreactor using the methods used. Other reasons may be primer design based on only known species or inhibitory substances present in samples (i.e. humic acids), which may explain the difference between total bacteria method and sulfate reducing method. The method of total bacteria not always comprises the entire bacterial community, and there have been special cases reported where sulfate reducing bacteria are found to be in higher numbers (Ramos-Padrón, *et al.* 2013). This appears to be one of them.

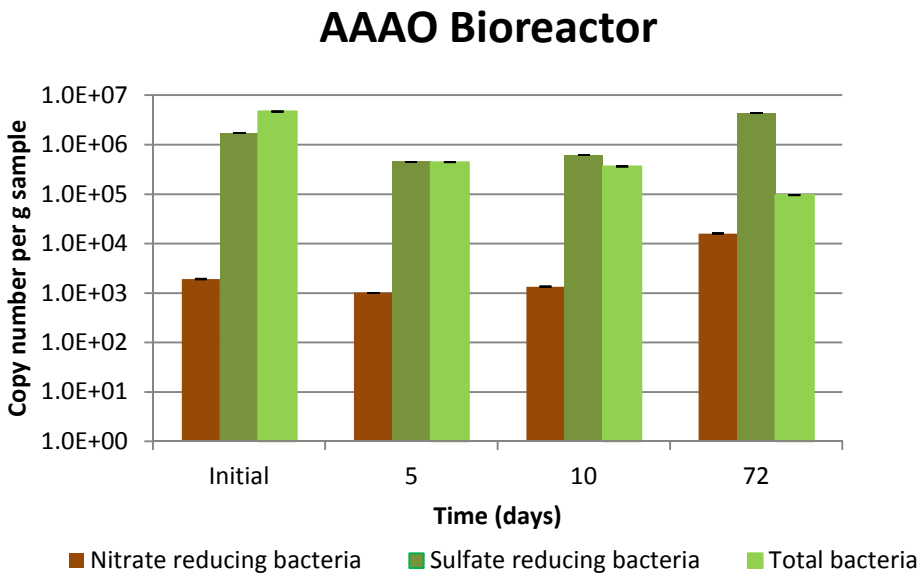

Figure 3. 2 qPCR quantification by copy number of nitrate reducers using *nosZ* gene, sulfate reducers using *dsrB* gene and total bacteria using *16S* gene, per gram of sample. Copy number per gram reported according to the formula gene copies/g sample=(gene copy number)x (vol DNA extracted (µL)/(µL DNA per reaction x 0.5 g sample) used by (Golby *et al.* 2012) . Error bars indicate one standard deviation.

3.2.2 HTO Bioreactor

The HTO bioreactor contained 1750mL of OSPW treated through a $HiPO_x$ process and an inoculum 250mL of MFT. HTO bioreactor was able to reduce the organic matter in the first 60 days from 266 mg/L to 163 mg/L. These results indicate a reduction of 38% of organic matter. This information correlated with the sulfate reduction during the first 60 days of operation, which yielded a 82% reduction from the initial sulfate concentration. Nitrate remained constant, below detection limits < 0.2 mg/L during the day 0 to day 195. Naphthenic acids increased from 3 mg/L up to a maximum of 15 mg/L, likely due to desorption from the MFT over a period of 200 days. When nitrate was added NA decreased to 16 mg/L. It is important to mention that the dominant electron acceptor was sulfate. Sulfate concentration continued to decrease as COD degradation was taking place until it reached a stable point of 70 mg/L. COD values reached an approximate value of 170 mg/L on day 52. Here the organic matter degradation is inhibited by sulfide. It has been reported that sulfide concentrations of 200 mg/L decrease COD removal from anaerobic digesters; sulfide interferes with the hydrocarbon uptake, affecting bacteria growth (Hilton & Oleszkiewicz 1988). In the second phase of the experiment, another electron acceptor, NO_3, was added, to allow for denitrification conditions. During this phase nitrate levels spiked to 116 mg/L, but decreased to zero within the next 14 days. The sulfate concentration in this reactor increased from 70 mg/L to a final concentration of 200 mg/L during the same time period. The final COD values reached 138 mg/L for an overall reduction of 48%. Finally, the naphthenic acids decreased from 15.0 mg/L to almost 12.1 mg/L, under nitrate reducing conditions to achieve a 19% biodegradation; probably because the bacteria used nitrate as an electron acceptor, which led to their degradation.

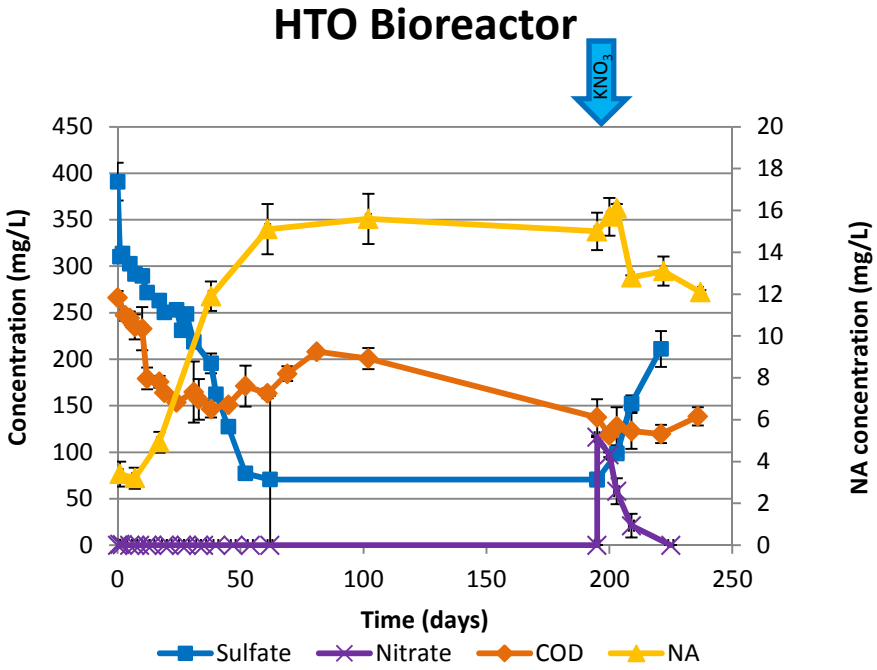

Figure 3. 3 HTO bioreactor (oil sands process water treated with HiPOx 1750mL, 250 mL MFT) electron acceptor and electron donor concentrations over time. Chemical oxygen demand is abbreviated as COD, naphthenic acids as NA. The arrow indicates the addition of nitrate in the form of KNO_3 to the reactor. Error bars indicate one standard deviation.

A survey of the bacterial population from the reactor, which included OSPW and MFT sediment, was performed for total bacteria, sulfate-reducing bacteria and nitrate-reducing bacteria. The MFT contained nitrate-reducing bacteria in low numbers ($1.61x10^3$ copy number per gram). The sulfate-reducing bacteria were found in quantities $5.44x10^5$ copies per gram of sample. Total bacteria from the MFT were in the order of $1.42x10^7$ copies per g. Follow-up samples were taken over the course of the experiment on days 0, 90, 195 and 210. An increasing trend was observed for total bacteria for a final $7.00x10^7$ copy numbers per gram or a 394% increase. In contrast, a decreasing trend was observed for nitrate-reducing bacteria from $1.6x10^3$ to $6.34x10^1$ with an overall reduction of 96%. Additionally a final increase of 480% was observed in sulfate-reducing bacteria with a final count of $3.15x10^6$ copy numbers per gram on day 210. This increasing trend correlates with the sulfate chemistry data collected from the bioreactor. It is important to underscore that total bacteria is the dominant specie type in this bioreactor.

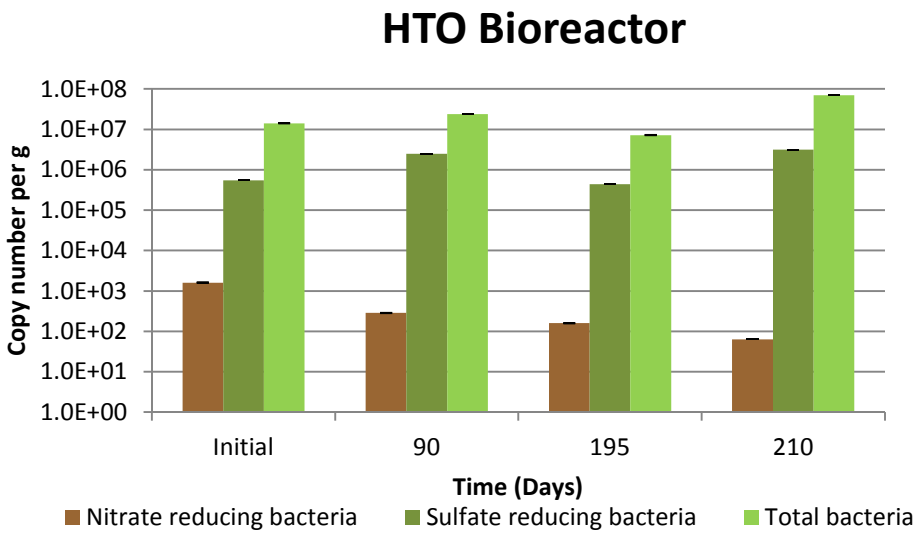

Figure 3. 4 qPCR quantification by copy number of nitrate reducers using *nosZ* gene, sulfate reducers using *dsrB* gene and total bacteria using *16S* gene, per gram of sample. Copy number per gram reported according to the formula gene copies/g sample=(gene copy number)x (vol DNA extracted (µL)/(µL DNA per reaction x 0.5 g sample) used by (Golby *et al.* 2012) . Error bars indicate one standard deviation.

3.2.3 Summary and treatment comparison

The chemistry alone is important to discuss between the AAAO and HTO bioreactor. For the AAAO bioreactor alone the overall COD removal was 70%. Naphthenic acids removal achieved 14% by the end of the experiment. Overall, 76% of the sulfate was reduced. Complete nitrate reduction is observed.

The use of advanced oxidation process is interesting to discuss. Initial and final values to assess the HiPOx^{TM} process were measured from fresh oil sands tailings. Their values are 341 mg/L, 71 mg/L, 140 mg/L for COD, NA and SO_4^{-2} respectively. Nitrate in fresh oil sands tailings were below detections limits, (b.d.l). Naphthenic acids after HiPOx treatment were below detection limits. HiPOx process could reduce COD from 341 to 266 mg/L to achieve a 22% removal of COD. A complete removal of naphthenic acids is observed with the use of the HiPOx^{TM} process. On the other hand an increase of sulfate is observed from 140 to 390 mg/L to achieve an increase of 177%. The sulfate increase was likely from the bitumen and hydrocarbons that contain sulfure in their structure. When the advanced oxidation process took place the hydrocarbon structure may have been disrupted and sulfur was liberated. Since oxidants were present when the sulfur was released, it could be oxidized again to form sulfate. This is likely the reason why it was observed as an increase of sulfate by the use of an advanced oxidation process.

In the HTO bioreactor, the anaerobic phase yielded an overall COD removal of 48%. Naphthenic acids were removed 19% under nitrate reducing conditions. Nonetheless, a desorption of NA from MFT is observed under sulphate reduction and an important increase is observed. Overall 46% of sulfate was reduced. Complete nitrate reduction was observed.

The use of coupled technologies such as advanced oxidation–anaerobic processes achieved 67% COD removal from 341 to 111 mg/L. Additionally, from 71.6 to 12.1 mg/L of NA, for 83% naphthenic acid removal is observed. On the other hand a sulfate increase was observed from 140.9 to 211 mg/L for a final sulfate increase of 49%.

A comparison of this data can be seen in the figure below. One of the best treatments found was a HiPOx™ coupled with anaerobic process (HiPOx™-HTO bioreactor) to degrade target pollutants present in oil sands tailings.

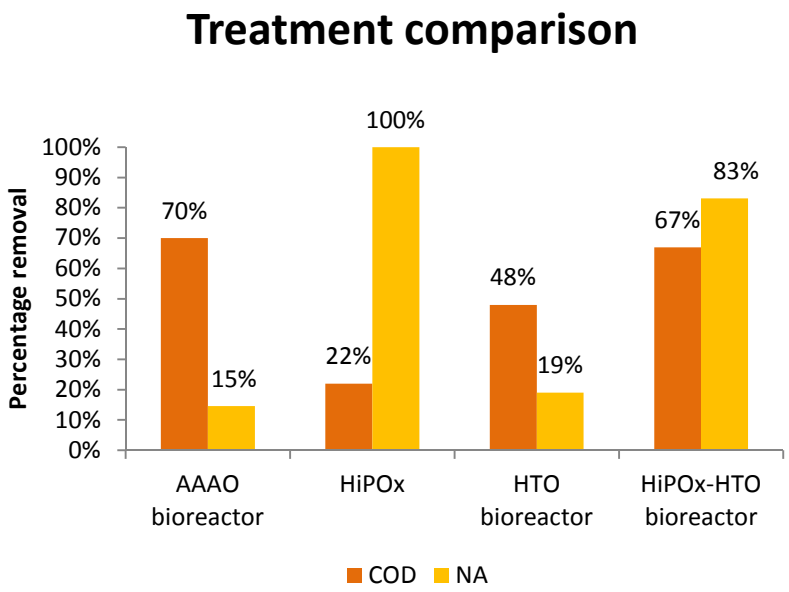

Figure 3. 5 Comparison between different treatments for oil sands tailings. The best treatment is an HiPOx-anaerobic treatment.

3.2.4 Bacterial Community Changes in the Reactors

Molecular biology tools were used to better understand which microorganisms are involved in biodegrade the organic matter, naphthenic acids, sulfate reduction and nitrate reduction in the two reactors. To monitor the shifts in the bacteria populations, a bacterial community analysis DGGE was performed (see Figure 3.5). For the two bioreactors, MFT was used as a reference line in both gels. Subsequent days were sampled and monitored. These lines were named according to the day sampled and are shown at the top of each line. For the HTO bioreactor, bands were named as T (Treated) and for the AAAO bioreactor, U (Untreated). They were numbered accordingly. Bands with an asterisk (*) were cloned and sequenced, but no readable sequence could be obtained from them and they were discarded.

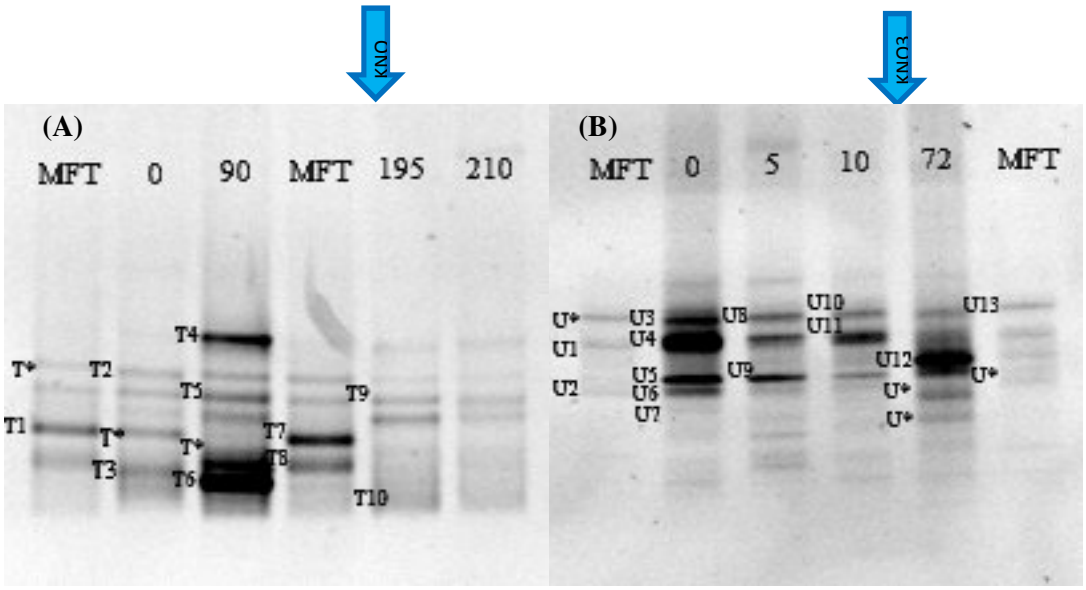

Figure 3.5 Denaturing gel gradient electrophoresis (DGGE). Image (A) corresponds to HTO bioreactor and subsequent days. Image (B) corresponds to AAAO bioreactor. Lanes represent the day a sample was withdrawn from the reactor. Bands are labeled as T (treated) or as U (untreated) and numbered in order. Unreadable sequences were assigned an asterisk (*) and discarded.

A BLAST search determined the closest matches and percentages of similarity for the sequences retrieved (Table 3.2 and Table 3.3). These tables include similarity value for each sequence and their taxonomic group.

Table 3. 1 Sequences from HTO bioreactor and their closest match using NCBI. Maximum identity, taxonomic group and accession number are presented.

Band name	Top hit matches from NCBI using Blastn Eubacteria 16S (550 base pairs)- HTO bioreactor	Max identity	Taxonomic group	Accession number
T1	Acidovorax sp., isolate G8B1	99%	*Acidovorax*	AJ012071
T2	Uncultured bacterium clone TSAX19	99%	*Desulfocapsa*	AB186853
T3	Hydrogenophaga defluvii, strain hyd1	100%	*Hydrogenophaga*	AM942546
T4	Uncultured bacterium clone DNA-R3-G9	99%	*Desulfocapsa*	JN885798
T5	Uncultured bacterium clone Fort Lupton 494 16S	99%	*Desulfocapsa*	GU453459
T6	Rhodoferax sp. clone MLSB10m6D	99%	*Rhodoferax*	EU517536
T7	Desulfotomaculum sp. ECP-C5	98%	*Desultomaculum*	AF529223
T8	Pseudomonas stutzeri RCH2	100%	*Pseudomonas*	CP003071
T9	Uncultured Chloroflexi bacterium clone MLSB 20m 12A	99%	*Anaerolinea*	EF420218
T10	Acidovorax defluvii strain b268	99%	*Acidovorax*	EU434475

Table 3. 2 Sequences from AAAO bioreactor and their closest match using NCBI. Maximum identity, taxonomic group and accession number are presented.

Band name	Top hit matches from NCBI using Blastn Eubacteria 16S (550 base pairs)-AAAO bioreactor	Max identity	Taxonomic group	Accession number
U1	Uncultured Comamonadaceae bacterium clone D25_14 or its synonym Acidovorax sp. JS42	99%	*Acidovorax*	EU266893
U2	Acidovorax ebreus TPSY Uncultured bacterium clone CB13	100% 100%	*Acidovorax* *Diaphorobacter*	NR_074591 KC211862.1
U3	Uncultured bacterium clone N-207	99%	*Desulfocapsa*	HQ218648
U4	Acidovorax defluvii strain b332	99%	*Acidovorax*	EU434521
U5	Acidovorax ebreus TPSY Uncultured bacterium clone CB13	100% 100%	*Acidovorax* *Diaphorobacter*	NR_074591 KC211862.1
U6	Rhodoferax sp. clone MLSB10m6D 1	99%	*Rhodoferax*	EU517536
U7	Beta proteobacterium PB7	100%	*Rhodoferax*	AY686732
U8	Uncultured eubacterium WCHB1	100%	*Syntrophacea*	AF050534
U9	Cryobacterium psychrotolerans strain 0549	98%	*Cryobacterium*	NR_043892
U10	Uncultured bacterium cloneTSAX19	99%	*Desulfocapsa*	AB186853
U11	Acidovorax sp. JW26.2a	99%	*Acidovorax*	FN556569
U12	Brachymonas petroleovorans strain CHX	99%	*Brachymonas*	AY275432
U13	Uncultured Acidovorax sp. clone CP3.3.33	99%	*Acidovorax*	JN697518.

On the AAAO bioreactor DGGE, a comparable amount of lines can be observed on the reference line and day 0. The image shows a close banding pattern between each line. The reference line represents MFT and the banding pattern is the original inoculum on the reactors. This means that the reactors have the same dominant bacteria as the starting inoculum. Bands T1, T2 and T3 were identified as an *Acidovorax* species, which is an unculturable species under the *Desulfocapsa* genus and *Hydrogenophaga defluvii* respectively. Acidovorax species belonged to the initial community baseline found on MFT inoculum. As time progresses (day 90), more bands were defined. One of the most important were T4, T5 and T6. They are important because they provide evidence that sulfate reduction is taking place and these bacteria have been found and cultured in tailing pods before (Golby *et al.* 2012). The sequences T4 and T5 are identified as organisms of the family *Desulfobulbaceae* under genus *Desulfocapsa.* Sequence T6 was identified as an organism in the *Rhodoferax* genus. On day 195, the banding pattern changes again. Only four defined bands can be seen, including a diffused line at the bottom. Bands T9 and T10 were identified as uncultured organisms from the genera *Anerolinea* and *Acidovorax defluivii.* Nitrate was added on day 195. Similar banding patterns can be seen on days 195 and 210. This is an indicator that the same bacteria was present at these stages and that a shift in electron acceptor did not change the microbial community dynamics in the last days of the experiment.

In the AAAO bioreactor DGGE, a profile and change of the bacterial community can be observed. The reference line represents MFT and the banding pattern is the original inoculum. Sequences U1, U2 and U13 represent the initial bacteria in the reactors. They were identified as unculturable organisms from the *Acidovorax* genus, uncultured bacterium from *Diahphorobacter* genus, and an uncultured *Acidovorax* clone respectively. *Acidovorax* species have been found on mesocosms studies and in other studies and have been reported to be sulfate reducers in anaerobic environments (Eriksson 2005; Li 2010; Byrne-Bailey *et al.* 2010). On day zero, four main bacteria populations can be seen as bands U3 to U7. The organisms were uncultured bacterium from the *Desulfocapsa* genus (U3), *Acidovorax defluvii* (U4), *Acidovorax ebreus* (or uncultured bacterium from *Diaphorobacter* genus) (U5), *Rhodoferax* clone, and uncultured *Rhodoferax* organisms (U6, U7). *Rhodoferax* species can grow in anaerobic environments and can use a wide variety of substrates as their electron donors; *Rhodoferax* species utilizes iron (Fe^{+3}) and nitrate as their electron donors, as it has

reported in other research (Penner & Foght 2010) The next line show three strong bands. Only bands U8 and U9 were selected for downstream analysis. Sequences were identified as an uncultured bacterium from the genus of *Syntrophacea* and *Cryobacterium psychrotolerans,* respectively. *Syntrophace* is reported to degrade hydrocarbon and other organic compounds (Gray *et al.* 2011). Line 10 has a banding pattern similar to that of the previous lanes but certain bands are becoming fainter. Finally, on day 72, the banding pattern is completely different from the original reference line. In this lane a strong band can be seen and it is designated as band U12 which was identified as *Brachymonas petroleovorans.* This last organism is reported to grow fairly slowly and can degrade linear alkanes and aromatics hydrocarbons (Rouviere & Chen 2003).

3.2.5 Dendograms: Analysis from DGGES

Banding patterns from both DGGEs were analyzed using the software *Gelcompare II* (Bionumerics). The patterns of both DGGEs were subject to numeric analysis calculated using binary and intensity matrices. Cluster gels analysis was performed using a clustering UPGMA method and a Persons correlation with an optimization of 0.5%.

The cluster analysis showed eight leaves. Four were from the HTO bioreactor and the remaining was from the AAAO bioreactor. The analysis, read from top to bottom, shows a clade with two leaves which are from Day 0 and Day 10 from the AAAO bio reactor. The similarity in the banding pattern is 79.3%. This means that the bacterial community is fairly similar to the reference community. The next clade has only one leaf and has a 76.8% similarity compared to the previous leaves. Also, the dissimilarity increases as more time goes by. The AAAO bioreactor leaves have a 50% similarity to each other, meaning that the community has changed and only half of the original bacteria are present.

The dendogram for the HTO bioreactor consists of a clade which bifurcates into two clades, each one consisting of two leaves each. The first two leaves are lanes from HTO bioreactor on days 0 and 72 respectively. The banding pattern similarity is 72%. This means that their compositions are very similar. The remaining leaves from the

HTO biorreactor on days 195 and 210 showed a similarity value of 74.4%. This means that over time the community composition changes. In comparison these two gels have a 44% similarity on their banding pattern. This means that different bacteria flourish in water compositions (fresh OSPW vs. treated OSPW), even though they had the same initial bacterial composition as seen on the reference line.

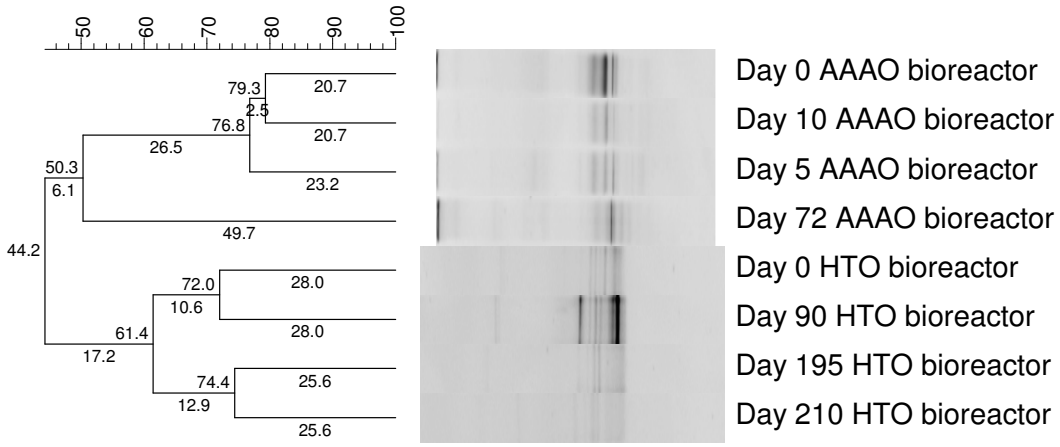

Figure 3. 6 The dendogram compares two DGGE gels from AAAO and HTO bioreactors and their banding pattern. The dendogram shows the similarity between each lane.

3.2.6 Phylogenetic Analysis

To analyse the obtained sequences required classification from an evolutionary standpoint. The objective was to understand the evolutionary relationships among sequences by comparing them with known organisms. The phylogenetic tree itself provides a hypothesis about the evolutionary history among their taxonomic groups. Therefore sequences were compared to their closest matches using known organisms. This method facilitates the classification of uncultured organisms with their family or genus.

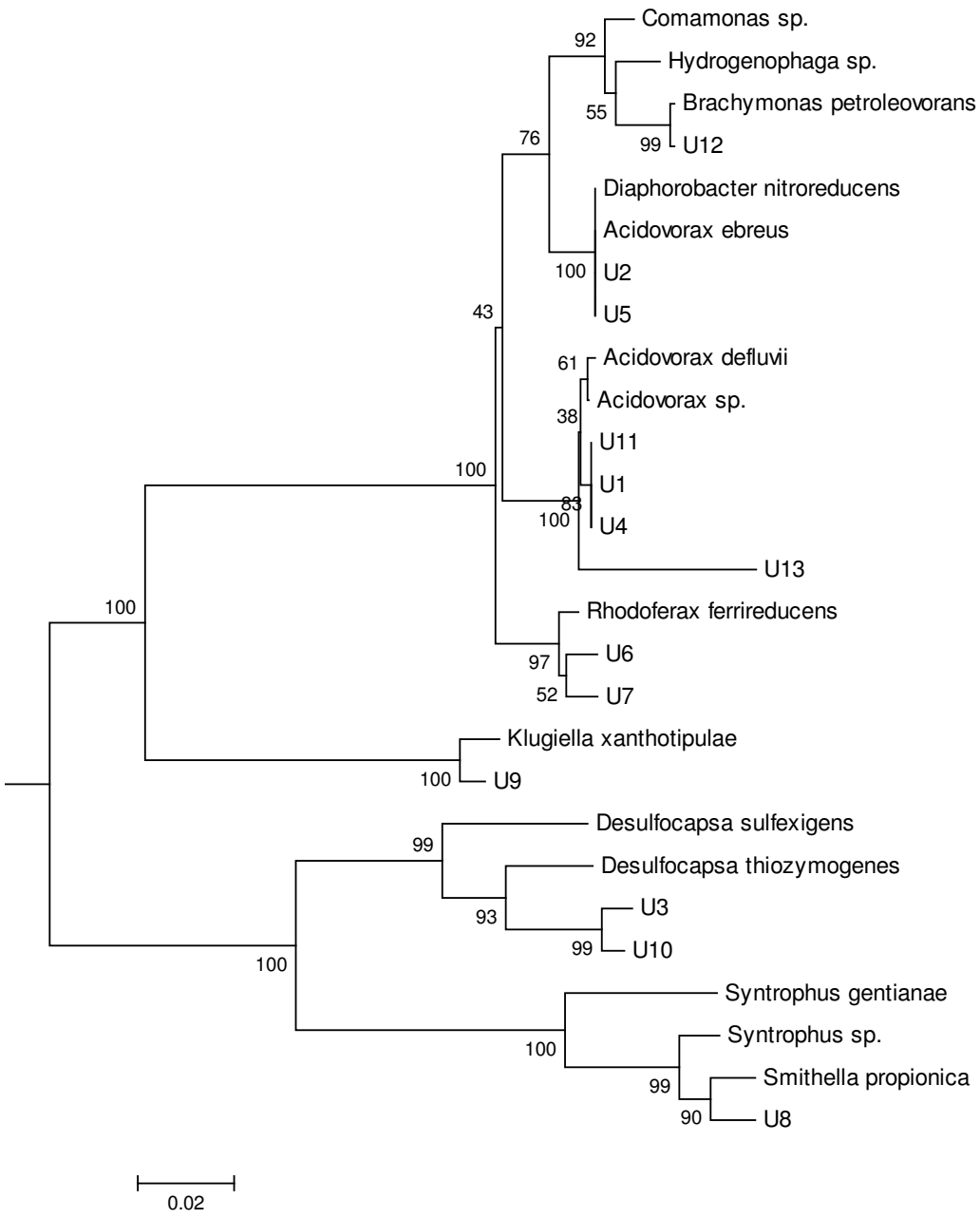

Figure 3. 7 Phylogenetic tree from AAAO bioreactor. Tree construction used was Neigborg-Joining method, with a pair-wise deletion and 2000 bootstrap values. Nucleotides substitution rates scale bar (0.02) is presented.

Sequence U1 is part of the Comamonadaceae family and is closely related to the genus *Acidovorax*. Band U1 shares the same characteristics as the U4 and U11 bands. Although band U13 is part of the same genus, it has no culturable related organisms. The sequence U2 is under the *Commamondaceae* family as well within the genus *Diaphorobacter*. It shares closely related characteristics with the U5 sequence; the sequences' share a bootstrap value of 100%. Sequences U2 and U5 were identified as *Acivodorax* organisms and unculturable organism respectively. Sequence U12 was identified as *Brachymonas petroleovorans* and clusters together with *Brachymonas petroleovornas* with a bootstrap value of 99% and with *Hydrogenophaga sp*. and *Comamonas sp.* with bootstrap values of 92 and 55 % respectively. Sequences U6 and U7 were identified as a *Rhodoferax* clones and *β-proteobacterium* respectively. The sequences mentioned are related to *Rhodoferax ferrireduces* and to the *Rhodoferax* genus. Sequence U9 clusters with a *Cryobacterium psychrotolerans* family *Microbacteriaceae* genus *Cryobacterium* with a 100% bootstrap value. This indicates that sequence U9 is identical to this organism. Sequences U3 and U10 cluster together in the family *Desulfobulbaceae* genus *Desulfocapsa*. U3 and U10 sequences have no similarity with cultivable species, but they have a large amount of unculturable bacteria from diverse sources. Finally, sequence U8 was identified as an unculturable eubacterium. Nevertheless, it clusters together with the family *Syntrophacea,* genus *Syntrophus* and with the *Smithella* genus.

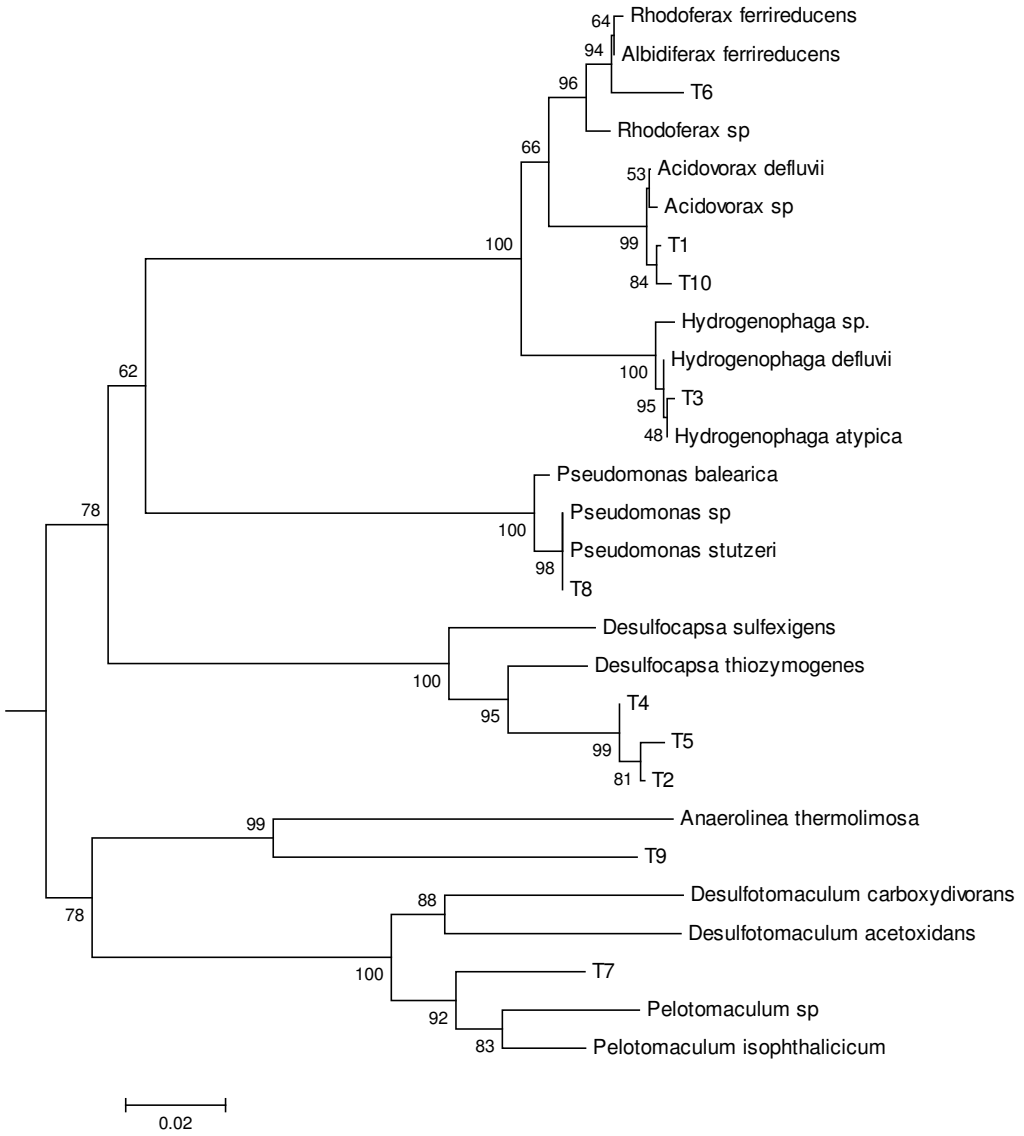

Figure 3. 8 Phylogenetic tree from HTO bioreactor. Tree construction used was the Neigborg-Joining method, with a pair-wise deletion and 2000 bootstrap values. The nucleotides substitution rates scale bar (0.02) is presented.

For the HTO bioreactor, sequences T1 and T10 cluster within the family of the *Comamonadaceae* genus *Acivodovorax*. The clone sequences cluster with bootstrap values of 99% for the known *Acidovorax* species and 84% in relation to each other. Clones T1 and T10 were identified as *Acidovorax sp* and *Acidovorax defluvii* species in a 99% maximum identity using NCBI and are placed with the *Acidovorax* genus. The

next branch, where the genus *Hydrogenophaga* resides, is also part of the *Comamonadaceae* family. It is in this branch that a clone was identified as *Hydrogenophaga defluvii*. This clone is related to other *Hydrogenopha* species (with a boostrap value of 95% to *Hydrogenofaga defluvii)*. The final genera within this family are *Albidiferax* and *Rhodoferax.* The clone T6 clusters with *Albidiferax ferrireducens* and *Rhodoferax ferrireducens*. Sequence T6 was identified as an unculturable *Rhodofera*x organism. There is a bootstrap value of 94% with these two organisms, indicating a close relationship. Sequences T2, T4 and T5 were identified as unculturable organisms. Nevertheless, these sequences are classified in the family *Desulfobulbaceae* under genus *Desulfocapsa*. These sequences have a bootstrap value of 95% to *Desulfocapse thiozymogenes* and cluster together with *Desulfocapse sulfoexigens*. Among these branches, bootstrap values are 81% for T2 and T5 sequences and 99 % for T4. Sequence T9 was identified as an unculturable species. It clusters together with *Anaerolinea thermolimosa* on the family *Anaerolineaceae* genus *Anaerolinea*. The last clone sequence, T7, rests with the *Peptococcaceae* family genus *Pelotomaculum.* It is also related to the *Desultomaculum* genus. Sequence T8 belongs to the *Pseudomonas* family and has bootstrap values of 98%.

3.3 Discussion

3.3.1 HTO bioreactor

For the HTO bioreactor, a quick reduction of organic matter along with a decrease in sulfate is observed in the first 60 days. This indicated that sulfate reduction was taking place, which was supported by the organisms found during this period of time, which are SRB and syntrophic bacteria. COD decreased until day 60 which shows that organic matter was being degraded. In this case the hydrocarbon of low molecular weight, NA, was oxidized from the HiPOx pre-treatment process. It is interesting to note here that the measurements of NA were increasing over time until day 200, when their values appeared to have stabilized. This may be due to the fact that the water treated with HiPOx has already eliminated NA acids and only a fraction of readably biodegradable organic matter is left behind in the water. The increase, then, in the NA concentration may be due to the desorption of NA from the MFT sediments until it reached an equilibrium and no further increase of NA was observed. It is interesting to note that after the addition of nitrate (1.6 mM), the naphthenic acids decreased, as degradation is taking place under nitrate-reducing conditions.

After another electron acceptor, nitrate, was added to the system; nitrate concentrations changed over a period of 25 days, and dropped below detection limits. This showed that the nitrate was rapidly consumed. Nitrate, which is an alternative electron acceptor, is thermodynamically more favorable than sulfate and it was rapidly consumed by present bacteria as an energy source. Sulphide, which was previously reduced form sulfate, was oxidized by bacteria using the introduced electron acceptor. This reversed the sulfate reduction, in other words sulfide (hydrogen sulfide or ferric sulfides) with nitrate yielded sulfate again. The sulphide oxidation is carried out by chemolithoautotrophic-denitrifying bacteria, which lead to the formation of sulfate (Beristain-Cardoso *et al.* 2006) which was what happened after the nitrate was added. Several sulfate-reducer bacteria are able to utilize a variety of electron acceptors such as oxygen, nitrite including nitrate (Dannenberg *et al.* 1992) and may be using the nitrate as a way to oxidize sulfide. Therefore sulfate increased slowly as the nitrate was consumed. If more measurements were taken after the nitrate was depleted, sulfate would again decrease due to the sulfate-reducing organisms present in the system.

Quantitatively speaking, nitrate reducers in the reactors went down almost two orders of magnitude. One explanation is that there was no nitrate available for those organisms to thrive in this environment during the experiment. On the other hand sulfate-reducing bacteria increased their numbers one order of magnitude from $5.44x10^5$ to $3.15x10^6$ copy number per gram. This increase is not surprising since sediments from MFT are known to contain specialized bacteria, especially sulfate reducers which are specially adapted to their environment. Sulfate reduction has been reported, as a first order degradation with a rate of about 192 mg/L·day (Salloum *et al.* 2002). In comparison the degradation rate in our experiments was slow because our system was anaerobic. Since the only additional substrate was nitrate, as electron acceptor, at the end of the experiment it was expected that the bacteria present may remain in approximately the same numbers, on the order of $1x10^7$ copy numbers per gram. By the end of the experiment more total bacteria was quantified ($7x1x10^7$ copy numbers per gram), probably because more specialized bacteria thrived and bacteria formed syntrophic relationships to survive.

Relationships among the chemistry data in the bioreactors and the bacteria found in the sequences from the DGGE study can be pointed out. For instance, on the first day of the reactor setup, the species Acidovorax, which is known to reduce a variety of hydrocarbons and denitrifies, was identified; this bacteria has also been found in activated sludge, and is probably one of the first bacteria to degrade organic matter in this reactor (Schulze *et al.* 1999).

Sulfate-reducing organisms can survive by symbiotic relationships or grow with a hydrogen-consuming partner, or are capable of utilizing H_2 and CO_2 (Siddique *et al.* 2012). The next microorganism identified, which is an uncultured bacterium clone from the Desulfocapsa genus, resides very close to *Desulfocapsa thiozymogenes*, which is a sulfate reducer found in freshwater. *Desulfocapsa thiozymogenes* can grow by incomplete oxidation or by a type of redox reaction (disproportionation) of either thiosulfate, sulfite or sulfur (Janssen *et al.* 1996), which may explain the sulfate reducing process in the first few days of the bioreactor operation. *Hydrogenophaga* species are known to degrade a variety of short molecular weight organic matter due to their diverse metabolic physiology. This species has also been isolated in activated sludge (Kämpfer *et al.* 2005). Additionally, we see in the first stage that these organisms have the ability to degrade short hydrocarbon species in wastewater.

After 90 days a clone closely related to *Desulfocapsa thizymogenes* was again present along with a *Rhodoferax* species. This means that these organisms have thrived in this environment. This family has been known to use H_2 and low molecular fatty acids, hydrocarbons such as toluene and potentially a wide range of linear hydrocarbons. *Rhodoferax sp.,* closely realated to *Rhodoferax ferrireducens,* was also found at this stage and it is worthwhile mentioning that this organism is a strict anaerobe which can oxidize propylbenzene with the reduction of Fe (III) or nitrate and that it grows at neutral pH (Finneran *et al.* 2003). Since almost all organic matter is depleted and *Rhodoferax sp*. has become dominant species and may be the principal organisms behind the hydrocarbon degradation process in the first 90 days.

Anaerolinea was found at the end of the experiment. Literature suggests that this microorganism is stimulated by the presence of hydrogenotrophic methanogens (Yamada *et al.* 2006). At the last stage of the experiment, methanogenesis was already occurring, as methane bubbles could be seen when mesocosms were agitated. It has also been established that *Anaeorliniea* are syntrophic organisms, which degrade carbohydrates with hydrogenotrophic methanogens, which supports the previous idea that methanogenesis was taking place (Yamada *et al.* 2006).

The presence of *Acidovorax defluvii* at this stage is important since this organism can reduce nitrate, nitrite, nitric oxide and nitrous oxide. This species also was reported to produce flocs in broth cultures and can help in the sedimentation process of organic matter (Schulze *et al.* 1999). *Acidovorax deflivii* was also first isolated from activated sludge. This microorganism was at the bottom of the other Acidovorax species on the DGGE profile and it has also been reported that this strain possess a higher G-C content (62%) (Schulze *et al.* 1999) .

During the last days of the experiment (195-210) the lane pattern remained the same. It is assumed that the bacterial composition remained basically unchanged even with the addition of nitrate as an alternative electron acceptor. It would be interesting to identify archeal communities at this stage. This could provide subject matter for future research.

The HTO bioreactor contained species found in other research. For instance, Acidovorax and Rhodoferax have been found in mesocosms and megacosmos in densification studies on oil sands tailings and in cloning studies from MFT samples (Li

2010). These genera are reported to degrade complex organic compounds (Eriksson *et al.* 2003). Acidovorax forms part of the bacterial consortium able to degrade polycyclic aromatic compounds in cultures from soils (Eriksson *et al.* 2003) (Lin *et al.* 2007). Hydrogenophaga and members of Pseudomonas were present in biofilm cultures from oil sands tailings (Golby *et al.* 2012). Pyrosequencing studies made from sediments of oil sands tailings demonstrated the presence of Desulfocapsa and Rhodoferax species (Yergeau *et al.* 2012). This mean that bacteria present in this study have been found previously in studies related to oil sands tailings, which support the idea that these bacteria are indeed part of the initial inoculum on the bioreactors. The synergetic interactions of these bacteria were able to degrade organic matter and NA.

3.3.2 AAAO bioreactor

For the AAAO bioreactor, organic matter measured as COD, it constantly and rapidly decreased over a period of 16 days. After this period, the organic matter decreased but very slowly, to reach a final value of 170 mg/L at the end of the experiment. This means that biodegradation of alkanes or hydrocarbons from hot water extraction were being degraded by indigenous bacteria from the sediments.

Naphthenic acids decreased during the experiment. The decrease in their concentration, ranges from 30 to 26 mg/L, may be due to biodegradation when the nitrate was introduced to the system. Additional evidence may be needed to determine what NA are being degraded by another method with more resolution and would make it possible to clarify to what extent the NA were biodegraded by the introduction of nitrate.

Sulfate reduction was completed in 16 days. Sulfate reducing bacteria may be responsible for this rapid sulfate reduction. A member of *Desulfocapsa* found at the beginning of the reactor set-up may have played an important role in this process. After the sulfate was depleted, nitrate was added to the system. An increase in nitrate is seen when spiked in the reactor. Since nitrate is a thermodynamically favorable electron acceptor, its introduction enables bacteria to consume nitrate. Nitrate can be consumed by certain species of sulfate reducers and denitrifying bacteria (Dannenberg *et al.* 1992; Beristain-Cardoso *et al.* 2006). Nitrate serves as an electron acceptor to oxidize sulfide

by these bacteria, which in turn produces sulfate. The increase of sulfate was visible, reaching a maximum value on day 34. Nitrate reached the minimum value at 31 mg/L on day 60. Nitrate levels decreased 15 days after the nitrate was introduced to the system.

The quantification of certain species in the bioreactor provided additional support for the ongoing process in the reactor. Quantification of nitrate reducers is one of the parameters measured. For instance, the initial value of quantified NRB remained unchanged, around $1x10^3$ copy numbers per gram for the first 10 days. On day 72, the levels of nitrate-reducing bacteria were $1.60x10^4$ copy numbers per gram which was probably because nitrate was added and more energy could be subtracted from the electron donor by NRB.

Sulfate-reducing bacteria numbers ranged from $4.55x10^5$ to $4.29x10^6$ copy numbers per gram throughout the experiment. The last increase in their numbers, on day 72, can be explained by the addition of the electron acceptor. Other possible explanation is that the sulfate reducing bacteria are specialized bacteria and can thrive in this environment, outnumbered total bacteria.

It is interesting to note the decrease in the numbers of total bacteria, up to one order of magnitude, from $4.68x10^6$ to $3.63x10^5$ copy numbers per gram during the first 10 days of operation. This means that the numbers of total bacteria decreased by an order of magnitude in copy numbers per gram. Then, at the end of the experiments, the total bacteria were below the copy numbers from SRB, which can be attributed to the reasons mentioned before. The last value in the quantification of total bacteria was $9.49x10^4$ copy number per gram.

Identified bacteria uncultured Camomanadacea or its synonym Acidovorax sp. JS42 was present in this bioreactor. This microorganism is reported to mineralize nitrotoluene by opening the ring in a series of consecutive steps (Rabinovitch-Deere & Parales 2012). *Acidovorax ebreus* is also identified here and it has a 99.8% similarity to *Acidovorax sp JS42*; *Acidovorax ebreus* is a facultative anaerobe and possesses an ability to denitrify and live in microaerobic environments. Also, it oxidizes simple alcohols and acids with oxygen and it also possesses the capability of nitrate respiration (Byrne-Bailey *et al.* 2010). Desulfocapsa genus is present in this reactor. Several strains within this genus are capable of sulphur disproportionation. Such is the case of

Desulfocapsa thiozymogenes catalogued as a sulfate reducer organisms*;* this microorganism can degrade alcohol (Finster *et al.* 1998). The same disproportionation of sulfure and thiosulfate is performed by *Desulfocapsa sulfoexigens* (Finster *et al.* 1998). As stated before, *Acidoborax defluvii* can reduce nitrate (Schulze *et al.* 1999). *Rhodoferax* was also present in this reactor. The microorganisms in this reactor can have specialized roles and because of this, organic matter can be decomposed. In this reactor removal of organic matter achieved up to 334 mg/L in 30 days along with sulfate reduction in 16 days. There was an overall decrease in NA of 15%.

Another uncultured microorganism was found. This microorganism is related to the syntrophus family. For instance *Syntrophus sp.* produces energy from the anaerobic oxidation of organic acids, with the end product of acetate and hydrogen and linear hydrocarbons (Dojka et al. 1998; Penner & Foght 2010).

Cyobrabacterium psychotolreans was also identified in this reactor as an aerobe organism. It may be present in the reactor here and it could respire using the added nitrate. This organisms is a nitrate reducer, it also grows at 20–22 °C with a pH of 6.0–7.0 (Zhang *et al.* 2007).

Desulfocapsa and Acidovorax strains continued to thrive by degrading organic matter as they did in the HTO bioreactor. By the end of the experiment a dominant species, *"Brachymonas petroleovorans,"* was present. This strain is capable of degrading alkanes in the range of C5 – C10, and is catalogued as a β- Proteobacteria of the *Comamonadacea* family (Rouviere & Chen 2003). Its presence suggests that hydrocarbon degradation is taking place in the mesocosms and probably at a very low rate. It can be hypothesised that other organisms function as a community, interacting with each other and forming syntrophic relationships. The genus syntropacea indicates that such bacterial interactions are taking place. This reactor started with five different dominant microorganisms. . It is important to point out that even at the end of the experiment, only one dominant species was present, which indicates a narrow, specialized consortium.

Further studies regarding the composition of archeal communities is needed to understand the chemical processes carried out by microbial communities

Acidovorax, Brachymonas and Rhodoferax species were found in tailings ponds from Syncrude as well as in previous work (Ramos-Padrón 2013). Brachymonas was reported previously as a denitrifier (Ramos-Padrón 2013). Syntrophus species were found on enrichment cultures on anaerobic cultures capable of hydrocarbon degradation (Siddique *et al.* 2011). Cultures found on these reactors correlate with previous studies on sediments or water from tailings ponds.

3.4 Dendograms

Using dendograms to analyze the banding patterns on DGGE provides information of how close the bacterial community was and how it diverged over time and treatment. For instance, in the AAAO bioreactor on day 0, the similarity between the first 10 days showed that the community was changing but still remains 76% related among those samples. Over time, the community evolved to the changing environment, due to the lack of nutrients, electron donors or acceptors until it diverged and reached only a 50% relatedness to the original inoculum.

For the second bioreactor, the similarities between day 0 and day 90 were apparent and they had a 72% similarity, which means they have a similar bacterial composition. Over time, and for different reasons (lack of nutrients, electron donors or acceptors), the community changed. At the end of the experiment with the HTO bioreactor, the bacterial community was only related 61% when compared to the initial and 90 days samples.

Finally, two treatments were compared (untreated and treated OSPW water). Even though they both had the same bacterial community inoculum, their similarity at the end of the experiment is only 44%. This means that the water quality plays an important role in developing bacterial communities in the bioreactors.

3.5 Summary and Conclusions

This research has studied different treatments for oil sands tailings. The use of anaerobic process was observed to decrease several target pollutants in oil sands tailings. Advanced oxidation is also useful in removing target contaminants. The use of these two technologies is promising for treat oil sands tailings. In addition, biological quantification and identification of bacteria in those reactors was investigated. The main conclusion from this work can be summarized in the following points:

1. Anaerobic process is a viable option to remove target contaminants in oil sands tailings. The acetic acids amended OSPW bioreactor (AAAO bioreactor) proved that a reduction from 564 to 170 mg/L of COD is possible. During the sampling period, 70% of COD was biodegraded. Finally, naphthenic acids concentration decreased, during the sampling period, from 30 to 26 mg/L for a biodegradation of 15%. This indicates that using anaerobic biodegradation could biodegrade target pollutants during this time frame.

2. The use of advanced oxidation coupled with anaerobic process can further remove target contaminants. On the anaerobic phase, in other words in the HiPOx treated OSPW bioreactor (HTO bioreactor), a biodegradation of COD from 266 to 138 mg/L is observed in the HTO bioreactor, for a 48% COD decrease. Under nitrate reducing conditions, naphthenic acids concentration decreased from 15 to 12.1 mg/L to achieved a 19% naphthenic acids biodegradation. Naphthenic acids were likely reduced by the use of nitrate, which is an alternative electron acceptor. The use of advanced oxidation and anaerobic process, improves the COD biodegradation to reach 67%, and biodegradation up to 83% respectively. This process needs to be optimized to further improve the COD and naphthenic acids biodegradation efficiency.

3. Quantification of bacteria present in AAAO bioreactor was assessed. The AAAO bioreactor showed that sulfate reducing bacteria became dominant with over a 4.29×10^6 copy number per gram of sample. Total bacteria was the second most abundant species with a 9.59×10^4 followed closely by nitrate reducing bacteria with 1.60×10^4 copy number per gram of sample (the total

bacteria method is biased since the primers are designed for only known sequences but provides a good estimation for environmental samples). This means that with only an anaerobic treatment, sulfate reducing bacteria are the best adapted species to its environment. This species can remove organic matter by the use of sulfate as their electron acceptor. Nitrate reducing bacteria and total bacteria followed sulfate reducing bacteria, indicating that they can survive but only in lower numbers.

4. Quantification of bacteria present in the HTO bioreactor was assed. The HTO bioreactor showed a significant amount of total bacteria throughout the experiment which dominated any other kind of bacteria, with a final $7.00x10^7$ copy number per gram. This indicates that the HiPOx™ process degrades complex organic compounds and naphthenic acids into smaller and simpler hydrocarbons, allowing an easy uptake for total bacteria for their microbial growth. In other words, the HiPOx™ process allowed to biodegrade organic matter and naphthenic acids by bacteria present in the bioreactor. Sulfate reducing bacteria remained behind the total bacteria with a final $3.15x10^6$ copy numbers per gram. The growth of both total bacteria and sulfate reducing bacteria presents a good indicator that carbon is being assimilated by bacteria. Nitrate reducers remained in low numbers, with only 6.34 copy numbers per gram. The low numbers of sulfate and nitrate reducing bacteria indicated that non-specialized bacteria could grow in HiPOx pretreated oil sands tailings, but total bacteria dominated. The engineering significance to treat oil sands tailings with the HiPOx process shows that recalcitrant compounds can be decomposed into smaller biodegradable compounds that bacteria can uptake. The importance of applying HiPOx™ process to oils sands tailings is an engineering measure for a more effective treatment of oils sands tailings and it is strongly recommended to transform recalcitrant compounds into biodegradable ones. After the use of the HiPOx™ technology, biological treatments need to be explored to select the appropriate technology in order to couple these two treatment processes.

5. Relating chemistry with the numbers of bacteria present in the AAAO bioreactor found that the numbers of sulfate reducing bacteria agreed with a

decrease on sulfate in this scenario. COD and sulfate reduction process took place in this bioreactor and sulfate reducing bacteria were in high numbers, in the order of $4.55x10^5$ to $4.29x10^6$ copy numbers per gram. Total bacteria gave an estimate of the numbers and the growth of total bacteria in the order of $9.59x10^4$ to $4.68x10^6$ copy numbers per gram. It presents a good indicator that carbon is being assimilated by bacteria from the organic matter present in oil sands tailings by specialized sulfate reducing bacteria and total bacteria. This means that the chemistry observed (COD, SO_4, NA) is supported by the increase in numbers of sulfate reducing bacteria and the decrease of total bacteria. Under anaerobic conditions, sulfate reducing organisms were highly specialized and thrived in this environment.

6. Relating chemistry with numbers of bacteria present in the HTO bioreactor found that the presence of sulfate and total bacteria can carry COD degradation and sulfate conversion. For instance, the sulfate reducing bacteria ranged between $4.36x10^5$ to $3.15x10^6$ and total bacteria ranged from $7.21x10^6$ to $7.00x10^7$ copy numbers per gram. The reduction of NA under nitrate reducing conditions may be a process carried by high numbers of sulfate reducing bacteria, $4.36x10^5$ to $3.15x10^6$ copy numbers per gram and total bacteria, $7.21x10^6$ to $7.00x10^7$ copy numbers per gram. The advanced oxidation process along with the presence of approximately $1x10^7$ copy numbers per gram of total bacteria throughout the entire experimental setup showed that this pre-treatment allows the growth of total bacteria above specialized sulfate reducing bacteria, creating conditions for further biodegradation treatments. The degradation of target pollutants is mainly performed by either total bacteria or sulfate reducing bacteria in this scenario.

7. Bacteria identification and chemistry on the AAAO bioreactor. For the AAAO bioreactor the identified bacteria were *Acidovorax sp., Acidovorax ebreus*, uncultured bacterium from Desulfocapsa genus, *Acidovorax defluvii, Rhodoferax sp*., Beta proteobacterium from Rhodoferax genus, uncultured bacterium from Syntrophacea genus, *Cryobacteriuam psychrotolerans* and *Brachymonas petroleovorans*. In the AAAO bioreactor, COD and sulfate reduction were accompanied by the presence of sulfate reducing organisms such

as *Acidovorax, Desulfocapsa, Rhodoferax*. On the first 10 days, the community changed to only a few members of dominant bacteria, which are members of the *Syntrophacea, Cryobacterium, Desulfocapsa*, and *Acidovorax* genera which were responsible for the fast degradation of COD and sulfate. Finally, a particular dominant species was left, which consisted of *Brachymonas petroleovoras,* which grows slowly and was likely responsible for the final decrease in COD until the last measurement on day 72. Specialized bacteria can thrive in this environment and the bacterial community changes with time, giving more information on the identity of bacteria that can be found under anaerobic conditions in a bioreactor using MFT as its initial inoculum.

8. For the HTO bioreactor identified bacteria were *Acidovorax sp.,* uncultured bacteria from Desulfocapsa genus, *Hydrogenophaga defluvii*, *Rhodoferax sp*., uncultured bacteria from Anaerolinea genus and *Acidovorax defluvii*. Bacteria present on the HTO bioreactor, such as *Acidovorax, Desulfocapsa* and *Hydrogenophaga,* correlates with the reduction of COD and sulfate. Over time, when sulfate was available, (70 mg/L), sulfate-reducers proliferated. These included uncultured members of *Desulfocapsa* and *Rhodoferax*. Before and after the nitrate was added, members of *Anaerolinea* (anaerobic) and *Acidovorax* (nitrate reducer) were present. The different bacteria present, shows the identity of bacteria that can be found in an HiPOx pre-treated oil sands tailing under anaerobic conditions in a bioreactor using MFT as its initial inoculum.

The identification of bacteria in the bioreactors showed that certain bacteria are capable of degrading hydrocarbons and sulfate, and shift their metabolisms to use nitrate as another electron acceptor. The identification of bacteria in this study corresponds to findings reported in other studies which used MFT or oil sands tailings water as their scope of study

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Chapter 4: Conclusions and recommendations

4 Conclusions and recommendations

At the start of this work, it was hypothesized that indigenous microorganisms from mature fine tailings (MFT) would biodegrade hydrocarbons. This hypothesis was based on the fact that the bacterial consortium is highly adapted to its environment which is rich in hydrocarbons, naphthenic acids (NA) and sulfate. It was also hypothesized that advanced oxidation processes would reduce NA concentrations, and change the composition of the remaining organic matter to predominantly short alkanes, which can be further biodegraded by native bacteria.

Using bioreactors inoculated with bacteria indigenous to MFT to treat oil sands tailings water presented an opportunity to investigate the aforementioned hypothesis in a laboratory setting, and to address the question of whether this kind of treatment offers an alternative solution to remediate oil sands tailings water.

This research showed that the chemical oxygen demand (COD) and sulfate present in fresh oil sands tailings pond water can be biodegraded using an anaerobic bioreactor spiked with bacteria indigenous to MFT. There is evidence of NA biodegradation in the reactors.

Treated oil sands with an advanced oxidation process (HiPOx) can mineralize and/or degrade NA into simpler and innocuous compounds. Further biodegradation of the remaining COD can be accomplished by anaerobic reactors, as evidence suggested on this research on the HiPOx treated OSPW bioreactor (HTO bioreactor). It would be useful the use of HiPOx process technology coupled with biodegradation to remove of the remaining biodegradable hydrocarbons. For further understanding and optimization of the biodegradation of NA is needed. More research is needed to determine if nitrate can help bacteria to biodegrade NA and to what extent this biodegradation can take place (i.e., if larger concentrations of NA are present). It is to be determined how much nitrate is needed to accelerate NA in this kind of reactor.

The use of molecular biology allowed us to obtain a complete picture of the microbial processes taking place in the bioreactors. The quantification of sulfate reducing bacteria (SRB) and nitrate reducing bacteria (NRB) populations, along with

the identification of species of bacteria in the reactors, support the idea that only a very few select species are present in MFT and are able to synergistically degrade organic matter in this environment.

The identification of specific bacteria that flourished in the acetic acid amended untreated and pre-treated oil sands tailings water gave specific information as to which organisms are the key players in the biodegradation process. To have identified bacteria before and after adding an external electron acceptor also gave information on which bacteria will dominate the community under each set of conditions.

Further research is needed to envisage the implementation of bioreactors to treat oil sands tailings water as a promising and feasible possibility. The following recommendations section outlines further research that could expand the understanding necessary to remediate oil sands tailing waters through the use of bioreactors.

4.1 Recommendations

It is recommended to use bioreactors made of a material, such as glass, which is impermeable to gases. The configuration of the bioreactors should be such that it can allow easy experimental setup, the addition of starting materials, and any other materials such as measuring probes and gas inputs.

As an initial parameter in the reactors' set-up, organic matter concentration should be less than 500 mg/L. This conservative value is recommended from the experimental acetic acid amended OSPW bioreactor (AAAO bioreactor). It is also important to closely take into consideration the initial sulfate concentration. The reduction of sulfate to sulfide can inhibit the growth of bacteria in the reactor, stopping the sulfate reduction and the degradation of organic matter. For the initial sulfate concentration, a good starting point would be 250 mg/L; in that way a complete sulfate reduction will take place without inhibiting the bacterial growth. The right concentration of organic matter and sulfate can play an important role in the biodegradation of both compounds by indigenous bacteria. The best ratio of organic matter and sulfate is to be determined in future studies.

This work has shown that sulfate reduction is the dominant process unless another external electron acceptor is used (nitrate). Future work may involve exploring

changing the concentration of sulfate or nitrate to understand the optimal concentration for biodegradation. Additionally, the use of nitrate needs to be optimized to improve the biodegradation of COD or NA present. Other types of reactors may be explored to compare the performance of the bacterial community in other circumstances, such as stress loads, addition of nutrients and lack of nutrients, or the evaluation of different electron acceptors.

The use of a method to measure and characterize naphthenic acids, such as ultra-performance liquid chromatography (UPLC) for selected samples, is also recommended. Electrospray may be a good alternative to characterize NA. It may be time consuming to perform exhaustive analysis and characterization through all samples, but a few selected samples may be enough to prove the mineralization or the decrease of NA on the system. Additionally, this characterization will provide information about what type of NA are present if degradation takes place, and what further technologies may be able to fully degrade NA (Hwang *et al.* 2013) .

Other genes may be used as markers to quantify the bacteria present, as a way to monitor what the organisms are transforming. An example would be genes such as mcrA, which show the methane generation or sox genes that prove the presence of sufite reductase, or other genes that prove the degradation of alkanes, cycloalkanes, and biphenyl among other possible degradation mechanisms that can further reveal unveil the bacteria degrading hydrocarbons (Ramos-Padrón 2013).

4.2 References: Chapter 4

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Appendix A: Sequences from AAAO bioreactor

U- Untreated, AAAO bioreactor

#-Band number

>U1

CNNTNAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCNGGCGGTCAA
CTTCACGCGTTAGCTTCGTTACTGAGAAAGTGAATTCCCAACAANCAGTTGA
CATCGTTTAGGGCGTGGACTACCAGGGNATCTAATCCTGTTTGCTCCCCACG
CTTTCGTGCATGAGCGTCAGTACAGGCCCAGGGGATTGCCTTCGCCATCGGT
GTTCCTCCGCATATCTACGCATTTCACTGCTACACGCGGAATTCCATCCCCCT
CTGCCGTACTCTAGCTATACAGTCACAAATGCAGTTCCCAGGTTGAGCCCGG
GGATTTCACATCTGTCTTATANAANCGCCTGCGCACGCTTTACGCCCAGTAA
TTCCGATTAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTT
AGCCGGTGCTTATTCTTACGGTACCGTCATGTACCCCCTTTATTAGAAGGAG
TCTTTTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCATCCTGCA
CGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCA

>U2

TGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCA
GGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGCCTCT
TTCTAATAAAGAGGGGTCATGACGGTACCGTAAGAATAAGCACCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATT
ACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGAGGTGAAATCCC
CGGGCTCAACCTGGGAACTGCCTTTGTGACTGCAAGGCTGGAGTGCGGCAG
AGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGA
ACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGCACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCAACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGT
GAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTG

>U3

TGAGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCGACGCCGCGTGGA
CGATGGAGGCCCTTGGGTCGTAAAATCCTGTCAGATGGAAAGAAATGTACA
GGTGTTAATATCGCCTGTATTTGACGGTACCATCAAAGGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTGTTCGGAA
TTACTGGGCGTAAAGCGCGCGTAGGCGGTTTGCTAAGTCAGATGTGAAAGC
CCTCGGCTTAACCGGGGACGTGCATTTGAAACTGGCAGACTTGAGTACTGG
AGGGGGTGGTGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATCGGGAG
GAATACCGGTGGCGAAGGCGACCACCTGGCCAGATACTGACGCTGAGGTGC

GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
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GCATTAAGTTCTCCGCCTGGGGAGTACGGTCGCAAGATTA

>U4

CAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTTCGTT
ACTGAGAAAGTGAATTCCCAACAACCAGTTGACATCGTTTAGGGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCA
GTACAGGCCCAGGGGATTGCCTTCGCCATCGGTGTTCCTCCGCATATCTACG
CATTTCACTGCTACACGCGGAATTCCATCCCCCTCTGCCGTACTCTAGCTAT
ACAGTCACAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGTCTT
ATATAACCGCCTGCGCACGCTTTACGCCCAGTAATTCCGATTAACGCTCGCA
CCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTA
CGGTACCGTCATGTACCCCCTTTATTAGAAGGAGTCTTTTCGTTCCGTACAA
AAGCAGTTTACAACCCGAAGGCCTTCATCCTGCACGCGGCATGGCTGGATC
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>U5

CAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTTCGTT
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CGGTACCGTCATGACCCCTCTTTATTAGAAAGAGGCTTTTCGTTCCGTACAA
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>U6

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GCCCTAATACGGCGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTAA
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TACTGGGCGTAAAGCGTGCGCAGGCGGTGATATAAGACAGATGTGAAATCC

CCGGGCTCAACCTGGGACCTGCATTTGTGACTGTATCGCTAGAGTACGGTAG
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ACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCAACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGT
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>U7

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GCCTTAATACGGTGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTAA
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TACTGGGCGTAAAGCGTGCGCAGGCGGTGATGTAAGACAGATGTGAAATCC
CCGGGCTCAACCTGGGACCTGCATTTGTGACTGCATCGCTAGAGTACGGTAG
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ACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCAACTGGTTGTTGGGTCTTAACTGACTCAGTAACGAAGCTAACGCGT
GAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTG

>U8

TAATCTTGCGACCGTACTCCCCAGGCGGTTCACTTAATGCGTTAGCTGCGGC
ACTGAGAGGGTCAATACCCCCAACACCTAGTGAACATCGTTTACAGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTACCCACGCTCTCGCGTCTCAGCGT
CAGTATAGGGCCAGAAAGTCGCCTTCGCCACCGGTGTTCCTCCTGATATCTA
CGAATTTCACCTCTACACCAGGAATTCCACTTTCCTCTCCCCTACCCAAGCT
GAATAGTTTCAAATGCACGTCCTGGGTTAAGCCCAGGGATTTCACATCTGAC
TTATTCAGCCGCCTACACGCTCTTTACGCCCAATAATTCCGAACAACGCTTG
CACCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTTT
AGCGGTACCGTCAAGCATGATGGATATTAGCCACCATGCATTTCTTCCCGCT
CGACAGAGCTTTACGGTCCGAAAACCTTCCTCACTCACGCGGCGTTGCTGCG
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>U9

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AGGCGGTTTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGC
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TCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
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>U10

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CACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTTT
GATGGTACCGTCAAATACAGGCGATATTAACACCCATACATTTCTTTCCATC
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>U11

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>U12

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>U13

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ACTGTATAGCTAGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGT
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CCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
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CAAGGTTG

Appendix B: Sequences from HTO bioreactor

T-treated, HTO bioreactor

#-Band number

>T1

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CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGTGCGCAGGCGGTTATATAAGACAGATGTGAAATCC
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AACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA
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>T2

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GGTGTTAATATCGCCTGTATTTGACGGTACCATCAAAGGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTGTTCGGAA
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>T3

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>T4

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>T5

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>T6

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>T7

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>T8

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>T9

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>T10

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