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Physiology and Molecular Characterization of Microbial Communities in Oil Sands Tailings Ponds

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"Physiology and Molecular Characterization of Microbial Communities in Oil Sands Tailings Ponds"

by

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Abstract

In northern Alberta, mining operations to obtain bitumen from the oil sands generates large volumes of tailings. These are a mixture of sand, clay, water, organic solvents and residual bitumen that are deposited into old open pits, creating tailings ponds, where they are allowed to settle with the final goal of land reclamation. To speed up the sedimentation process, the addition of gypsum ($CaSO_4 \cdot 2H_2O$) is currently a management approach used bysome companies. This creates a deep watery mud line with very low oxygen permeability and enough sulfate to support the growth of anaerobic microbial communities. In this thesis work, the microbial physiology and communities associated with oil sands tailings ponds were assessed. Chemical, physiological, and molecular biology approaches were used to determine the key microbial processes (methanogenesis, sulfate reduction/oxidation), identify key substrates, and determine the dominant microbial community members in the anaerobic and aerobic zones of tailings ponds. Microbial community analysis showed that in the anaerobic zone of tailings, the sulfate-reducing/disproportionating bacterium *Desulfocapsa*. and the sulfide oxidizer/iron reducer *Thiobacillus sp*. are among the most prevalent organisms when sulfate is present. After sulfate is depleted, methanogenic Archaea become predominantly active and *Methanosaeta* and *Methanolinea* in association with *Syntrophus* dominate in the ponds, presumably interacting to biodegrade the available organic compounds. The residual naphtha components that constitute part of the tailings composition are the preferred electron donors in anaerobic zones (in comparison to naphthenic acids) based on enrichment culture studies. In naphtha-amended laboratory cultures, a variety of methanogens in association with *Thauera sp*. and *Desulfocapsa* sp. became enriched as the dominant organisms. Overall, microbial community composition as a function of depth in tailings ponds paralleled key

microbial processes that were measured (sulfate reduction and methanogenesis). In the aerobic surface water, other microbes with known metabolic capabilities to degrade hydrocarbon-derived compounds such as naphthenic acids were found. The results of this work also showed that operational changes to tailings ponds shift the microbial community structure and functions. For example, pond closure resulted in a shift from a predominantly methanogenic and sulfate-reducing environment to one dominated by putative hydrocarbon degraders, indicating a positive management outcome in microbial activity associated with pond closure.

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Dedication

To my children Fabian and Javier,

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
µCi	10^{-6} Ci, 1Ci= 3.7×10^{10} Bq
A	absorbance
ANME	anaerobic methanotrophs
AOM	anaerobic oxidation of methane
BTEX	benzene, toluene, ethylbenzene, and xylenes
CHAA	cyclohexaneacetic acid
CHCA	cyclohexanecarboxylic acid
CHPA	cyclohexanepentanoic acid
cpm	counts per minute
CT	consolidated tailings or composite tailings
DCM	dichloromethane
dsrB	dissimilatory sulfite reductase gene
EPS	extracellular polymeric substances
ERCB	Energy Resources Conservation Board
HS^-, H_2S	sulfide
Kan	kanamycin
LB	Lysogeny broth
MBH	Modified Bushnell - Haas salt solution
mbs	meters below surface
mcrA	α subunit of methyl coenzyme-M reductase
MFT	mature fine tailings
MLSB	Mildred Lake Settling Basin
mM	millimolar
MPN	most probable number
NA(s)	naphthenic acid(s)
NMDS	nonmetric muldimensional scaling

NTC	no template control
OD	optical density
OTU	operational taxonomic unit
PAH(s)	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction
qPCR	quantitative PCR
rRNA	ribosomal RNA
SEM	scanning electron microscopy
SOB	Sulfide-oxidizing bacteria
sox	sulfite oxidase genes
SRB	Sulfate-reducing bacteria
SRR	sulfate reduction rate
Ta	annealing temperature
TPW	tailings process water
TRO	tailings reduction operations

Chapter One: **Literature Review**

1.1 Oil sands tailings pond formation

1.1.1 Surface mining for bitumen extraction

Canada is home to the world's third largest oil reserve after Saudi Arabia and Venezuela [1]. This reserve is found mainly in north-eastern Alberta covering an area of approximately 142, 200 km^2 divided in 3 regions: Athabasca, Cold Lake, and Peace River [1]. With 170 billion barrels of proven bitumen reserve (proven reserve refers to a known mineral resource that can be recovered economically using existing technologies), only 3% of the total reserve (4, 800 km^2) can be surface mined because the bitumen is found very close to the surface, between 60 to 80 meters [2]. To date, approximately 715 km^2 of land has been disturbed for mining activity [1,3] [Figure 1-1].

Alberta's oil sands industry began commercial operations in 1967 pioneered by the Great Canadian Oil Sands, today known as Suncor Energy Inc., and later followed by Syncrude in 1978. Currently, more than 40 companies operate in the oil sands [4]. In 2010, Alberta produced 1.6 million barrels of bitumen per day mainly by *in situ* technology (SAGD: steam-assisted gravity drainage) and production is expected to increase to 3.5 million barrels per day by 2020 [1].

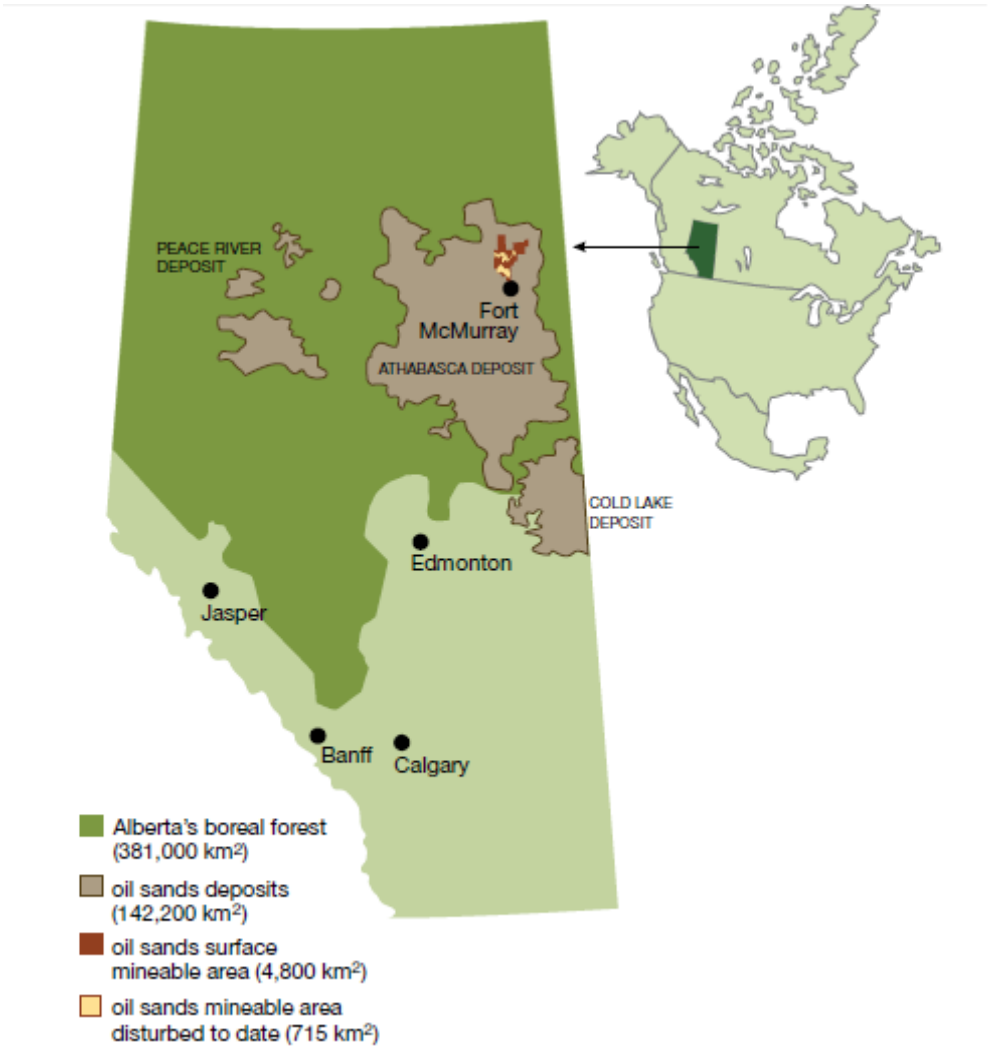


Figure 1-1 Map of Alberta showing the oil sands surface mineable area of 4, 800 km^2 of which 715 km^2 of land has currently been disturbed [1].

The oil sands are a mixture of solids, bitumen and water. The solids, or mineral content, which is the major component in the oil sands (85 wt %), varies in its composition but it is mainly composed of quarts, silts, and clays. The clay content is predominantely kaolinite, illite, illite-smectite, kaolinite-smectite, montmorillonite, and chlorite, with the first two being the most abundant in the Athabasca region [3,5,6]. An average of 12 wt % of bitumen can be found attached to these minerals together with water. The latter accounts for 3 – 6 wt % [3,7].

The open pit mining extraction method can recover approximately 88 to 95 % of the mineral-trapped bitumen [8]. The process involves using water, mechanical energy and chemicals such as sodium hydroxide [2,3]. The bitumen is separated from the sand, clays and other impurities by the Clark extraction method which uses hot water (79–93°C) and caustic soda [9,10]. This extraction procedure is possible due to the hydrophilic characteristic of the oil sands [3]. They consist of water-wet sand particles where the oil is found in the void spaces and not in direct contact with the mineral grain because it is surrounded by a thin layer of water [3,11]. For an average sand grain of 100 μm of diameter, a water film of 2.0 μm can be found [3,11].

The hot water treatment reduces the viscosity or thickness of the bitumen and the caustic soda helps the detachment of bitumen from the sand particles. Naphthenic acids (NA), naturally present in oils, including oil sands, act as surfactants and help separate the bitumen from the sand [3]. The mixing of oil sands and caustic hot water takes place in big drums and the slurry formed passes through a series of vibrating screens until the slurry is finally pumped into separation tanks. The primary separation vessel allows the

oil sand slurry to settle out into various layers with bitumen-containing layer situated at the top of the tank. The sand sinks to the bottom and together with excess water is pumped into a pipe which carries it to the tailings ponds. The described procedure is repeated with the middle layer from which another 2 to 4 % of bitumen can be extracted. In the final steps, diluents (e.g. naphtha) are added as a diluent to decrease viscosity and to help in the separation of the remaining bitumen from the sand. Once again, the slurry of water, sand and clay is carried through pipelines into the tailings ponds. This time, some of the naphtha is present in the slurry composition [3,11]. Figure 1-2 summarizes the whole process described above. Some operators (e.g., Shell Albian Sands) use aliphatic $C_5 – C_6$ as a diluent to help detach the bitumen from the sand in the extraction process [12].

This slurry is the so-called tailings: a mixture of water, clay, sand, and residual bitumen and hydrocarbon diluents. There are three main sources of tailings derived from the extraction process: (1) coarse tailings, (2) fluid fine tailings, and (3) froth treatment tailings [2]. The coarse tailings are mainly sand. Fine tailings are mineral particles less than 44 μm and froth accounts for only a small stream mainly composed of water, sand, fines, residual bitumen and solvents [2].

Due to the zero discharge policy, tailings are stored in settling ponds where the recycling of the water into the extraction process, and ultimately the restoration of the site, are the main goals [13,14]. Today, up to 90 % of the water is recycled, thus significant reduction of fresh water consumption for bitumen surface mining has been accomplished [4]. Typical ponds can comprise areas ranging from 2.5 to 25 km^2 and have an average depth of 50 meters below surface (mbs) [15,16].

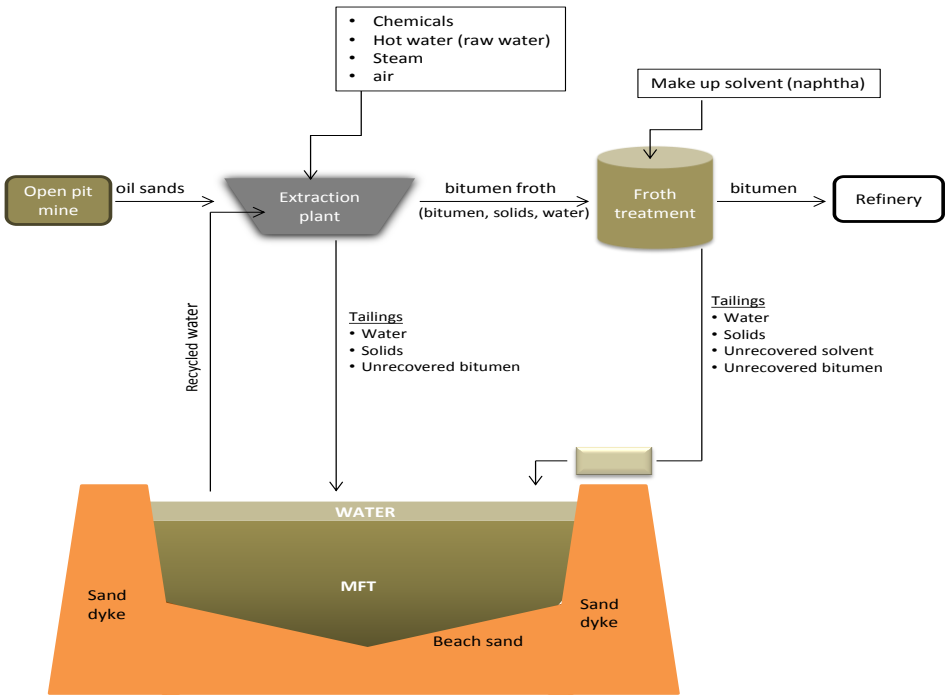


Figure 1-2 Bitumen extraction scheme by surface mining technology. The mined oil sands are transported to the extraction plant where bitumen is separated from the sand by the Clark alkaline hot water method. The tailings produced are transported through pipelines to the settling ponds where solids are allowed to settle so that the water can be recycled back into the extraction process.

1.1.2 Tailings pond composition

Tailings composition varies depending on the depth and age of the pond, on the source of the ore from which bitumen was extracted, as well as on the extraction process used [17,18]. In general, tailings have varying proportions of minerals, water, dissolved organic and inorganic salts, and residual organics including bitumen and hydrocarbon diluents. About 55 wt % solids are found in a typical tailings pond, of which 82 wt % are sand particles larger than 45 µm, 17 wt % are fines (< 44 µm or smaller), and 1 wt % is residual bitumen [3].

In the process of sedimentation, tailings ponds become stratified into 3 main layers: the rapidly setting sand particles that form a beach, an aqueous suspension of fine particles, composed mainly of silt and clay, and a clarified surface water layer also known as TPW (tailings processed water) or process-affected water (PAW) that contains total suspended solids, residual diluents, and bitumen [19]. The immediate contact of the fine particles in suspension to the TPW is known as the mud line. This line divides the pond into the surface, mostly aerobic (TPW) and the anaerobic zone (tailings).

The fines in the aqueous suspension are derived from the clay present in oil sands, thus their mineralogy is very similar: kaolinite (22 -76%), illite (7 – 10 %) and montmorillonite (1-8 %) [18]. Trace amounts of chlorite, quartz, iron oxides, and varying amounts of amorphous material can also be found [17,18]. This clay mixture forms a stable suspension that, after 2 to 3 years, settles into a fluid-like deposit called Mature Fine Tailings (MFT), made of about 30% solids and 65% water, and 5% bitumen. Under

current technology, the production of 1 m^3 of synthetic crude generates 6 m^3 of sand and 1.5 m^3 of MFT [14,20].

Approximately 12 barrels of water (1428 L) are needed to extract 1 barrel of bitumen from the oil sands but because much of the water is recycled, only 3 – 5 barrels need to be replaced by fresh water input [2]. In total, the tailings generated represent about 1.4 times the original volume of the oil sands [2]. In fact, current estimates of the total MFT inventory lies between 270 and 1000 million m^3, occupying an area of more than 170 km^2 in tailings ponds [2,4].

The MFT contains a substantial content of organic matter derived primarily from unrecovered bitumen. The bitumen content varies from one pond to the other. For instance, Syncrude's Mildred Lake Settling Basin (MLSB) may contain between 0.7 and 2.0 % of total mass whereas Suncor ponds could have between 0.3 and 5.0 % [17]. This organic matter was classified by Kasperski (1992) [18] into three categories: (1) residual bitumen, (2) soluble compounds, and (3) mineral-associated compounds. The residual bitumen (1) refers to the unrecovered bitumen from the Clark extraction process. About 10 to 15 % of the bitumen is trapped in the tailings sand and approximately 25 to 30 % is dispersed in the tailings [18]. The bitumen content in the tailings can range from 1 to 5 wt % depending on the depth where higher content will be found at deeper layers [18]. Soluble compounds (2) comprise those compounds that are dissolved in the water during the extraction process. In the water phase, these organic compounds can range between 100 and 120 $mg \cdot L^{-1}$ of which around 55 % are organic acids [18]. In the tailings fraction, these compounds are mainly polar, humic/fulvic acid-type compounds. Others like alkyl

phenols, polyphenolic aromatics, and polycyclic aromatic hydrocarbons (PAHs) are also found [18]. Finally, the mineral associated-compounds (3) include a variety of compounds composed of a mixture of fulvic and asphaltic acids probably fixed to clay particles through iron III linkage (Fe^{3+}) [18].

It is believed that the rate at which MFT naturally consolidate could take about 125 to 150 years to be completed [16]. One of the main reasons is due to the presence of approximately 3 % of ultrafines (colloidal phyllosilicate clays with dimensions < 0.3 μm) and the type of clay and its swelling capacity. The ultrafines form a gel-type structure with adequate internal volume capable of accomodating all of the water from the mature tailings [21,22]. Consequently, the continuous coarse solids that are carried into the pond become captured within this gel to finally produce the 30 wt % solids that are observed in this layer [21]. This structure is also closely dependent on the amounts of electrolytes and the types of anions present in the water [22]. In addition, the kaolinite and/or iron oxides present in the bitumen-clay interactions, together with the humic/fulvic acid compounds tightly bound to fines, in the presence of caustic soda, possess an enhanced negative surface charge which promotes dispersion of the particles, inhibiting their sedimentation and consolidation [3,6,23,24].

To overcome the slow consolidation rates, some oil sands operators (e.g., Suncor and Syncrude) have been using the non-segregating tailings technology by adding densification reagents such as calcium sulfate (gypsum) to the MFT and mixing it with sand, at a sand-to-fines ratio of approximately 4:1 [25]. Other operators, such as Shell Albian Sands, use other coagulants such as polyacrylamide [12]. The process is also known

as Consolidated Tailings (CT) or Composite Tailings [14]. The addition of Ca^{2+} as a flocculating agent markedly improves the settling characteristics [26]. The Ca^{2+} allows for the aggregation of fines due to an increase in the adhesion force between the fine particles [27]. About 1 kg of $CaSO_4 \cdot m^{-3}$ tailings are added to produce CT [3,28]. However, concerns with CT technology using gypsum are: (1) accumulation of Ca^{2+} in the recovered processed water can lower bitumen recovery efficiency [3], and (2) that sulfate (SO_4^{2-}) ions in the TPW can accumulate and under anaerobic conditions be biologically reduced to sulfide leading to the production of hazardous H_2S gas. Other coagulants can also be used but gypsum is more readily available as it is a byproduct of the oil industry [3].

Apart from diluents (e.g. naphtha) and bitumen, NAs can also be found in tailings ponds. These compounds, naturally present in the oil sands, are classically defined to be water soluble complex mixtures of alkyl-substituted acyclic and cyclic aliphatic carboxylic acids with the general formula of $C_nH_{2n+Z}O_2$, where *n* indicates the carbon number and *z* indicates the hydrogen deficiency due to ring formation. The *z* value divided by 2 gives the number of rings present in the compound [29,30]. However, high resolution analysis of NAs obtained from petroleum have shown the presence of pyrroles, thiophenes, and phenols. Some do not even have the carboxylic acid functional group but instead they contain heteroatoms such as sulfur and nitrogen [29]. These chemicals are solubilized during the bitumen extraction process [31] and as mentioned earlier, act as surfactants under alkaline conditions to help separate the bitumen from the sand [3]. NA can reach concentrations as high as 100 $mg \cdot L^{-1}$ in TPW [32-34]. They are known to be

toxic to a variety of aquatic organisms, algae, invertebrates, mammals and some microorganisms, thus representing a great environmental concern [35,36].

Finally, another important component in tailings ponds is microorganisms. Tailings are known to harbour an active microbial community [15,37-40]. These are presumably supported by the presence of the previously mentioned organic compounds and others such as asphaltenes, benzene, phenols, toluene, creosols, humic and fulvic acids, and PAHs. The ponds also contain calcium, sodium, chloride, sulfate, bicarbonate, and ammonia [19]. All these chemicals support the presence of syntrophs, fermentative bacteria, sulfate-reducing bacteria (SRB) and methanogens, which are abundant microbes in the anoxic layers. These groups of microorganisms are responsible for the methane and sulfide gas emissions that have been reported to evolve from tailings ponds [16,41,42]. Nevertheless, the surface water also sustains the growth of aerobic bacteria and algae that are dynamically related to the shallower anoxic layers of the pond. In general, despite the toxicity, tailings ponds contain a vast and interesting microscopic world that can be revealed using modern molecular biology tools. More details regarding microbes in tailings ponds will be discussed in section 1.2.1.

1.1.3 Tailings pond management and environmental concerns

Despite attempts to reduce the negative effects of the mining operations by improving tailings management and by finding new cleaner technologies, tailings ponds continue being the focus of attention by both the media and the Canadian government. On average, 375,000 m^3 of MFT per day are discharged into the settling ponds, considering that every cubic meter of synthetic crude generates 1.5 m^3 of MFT per day.

As these waste streams flow into the pond, the available oxygen is readily consumed by the existing microbiota. Subsequently, as layers of tailings are deposited, less oxygen is available due to the low permeability of the sedimented layers, thus resulting in mainly anoxic ponds [43]. Consequently, anaerobic Bacteria and Archaea tend to dominate these ecosystems from 1 to 60 mbs [37,41,44-47].

One of the main anaerobic bacterial groups in tailings ponds whose activity can lead to gas emissions is the sulfate-reducing bacteria (SRB). Due to the addition of gypsum, SRB become enhanced and favoured thus increasing the risk of hydrogen sulfide (H_2S) release into the atmosphere. However, given the slight alkaline pH of the tailings, most sulfide is present as HS^- which most likely precipitates as iron and other metal sulfides or pyrite (FeS_2) [16]. Moreover, the fraction of sulfide that is not precipitated could be potentially oxidized to sulfur and sulfate. Therefore, only a small fraction of H_2S emissions from CT deposits could be expected [44]. In anaerobic experiments with tailings, only 3% of the total amount of SO_4^{2-} reduced was detected as free sulfide [16]. If the pH in the pond becomes acidic, the metal sulfides would dissolve resulting in the generation of H_2S gas [16].

Another important group of microorganisms in tailings ponds are the methanogens. When sulfate drops to below 20 mg $\cdot$ L^{-1}, methanogens prosper, which can lead to the release of significant amounts of methane to the atmosphere [31,44]. Previous studies have shown that a typical pond can release a daily flux of 12 g of CH_4 per m^2 [46]. Since methane is a greenhouse gas and emissions can enhance the volatilization of lower molecular weight hydrocarbons, this represents a serious environmental concern [16].

Water management in tailings ponds may be an issue. The continual recycling of the water to the extraction plant can lead to an increase of dissolved ions that cause various operational problems, including poor extraction recovery together with scaling/fouling of piping and equipment [25]. On the other hand, as NAs become solubilized and concentrated in the TPW their persistence in the ponds becomes an environmental threat due to acute and chronic toxicity to various organisms [36,48].

Oil sands tailings ponds operators are enforced to comply with certain environmental rules and directives in order to lower the environmental impact produced by their operations. These sites are being continuously monitored for air and groundwater quality, as well as control of the volumes of tailings produced [4,49]. In February 2009, the Energy Resources Conservation Board (ERCB) developed the tailings management regulations Directive 074 [50]. This directive states that companies are required to reduce tailings and provide target dates for closure and reclamation of ponds. It also sets out timelines for operators to process fluid tailings at the same rate they produce them, in order to prevent the volumetric growth of the ponds [50].

Other operators search for cleaner technologies in order to comply with the established environmental targets. Such is the case of Suncor Energy Inc. which has recently started to implement a new technology for tailings management. The approach called the TRO (Tailings Reduction Operations) significantly improves the speed of tailings reclamation by converting fluid fine tailings more rapidly into a solid landscape. In this process, MFT is mixed with a polymer flocculant, and then deposited in thin layers over sand beaches with shallow slopes where it is allowed to dry in a matter of

weeks. The resulting product is a dry material that is capable of being reclaimed in place or moved to another location for final reclamation [51]. The closure of ponds with several layers of coke when they become full to capacity is another strategy followed by Suncor Energy Inc. [52].

Managing oil sands tailings is of great concern to Canada and has led to the development of the Oil Sands Tailings Research Facility (OSTRF), which is dedicated to collaborative and multi-disciplinary tailings research [25]. Others like the Oil Sand Tailings Consortium (OSTC) founded in 2010, collaborates on research and development related to tailings by reflecting the companies' commitment to socially and environmentally responsible operations. More recently, at the beginning of 2012, Canada's oil sands producers formed a new alliance, Canada's Oil Sands Innovation Alliance (COSIA), focused on accelerating the pace of improving environmental performance in Canada's oil sands.

In general, the Alberta government together with the industry and academic institutions continues to provide financial and other resources to improve the tailings management with the final aim of reducing the oil sands operational footprint.

1.2 Microbiology of tailings ponds

1.2.1 *Tailings ponds: a niche for microbial activity*

Despite the toxicity of oil sands tailings, the ponds harbor a varied community of microorganisms [37,45,46]. These were initially believed to come from the Athabasca river (source of fresh water for the bitumen extraction process by the Clark method) as

bacterial activity was found in the extraction streams discarded to the ponds, even those at high temperature [18]. However, a recent 16S rRNA gene-based study of the microbial population present in the Athabasca river and its sediment revealed that the microbial communities in fine tailings were distinct from those in the river and its tributary sediments [53]. These results may imply that tailings ponds harbor a unique microbial community that could have initially originated from the river, the pipelines, and the oil sands themselves; but once they reach to the pond, and as the pond ages, these organisms start interacting with each other (e.g. gene transfer), and start expressing metabolic genes depending on the substrate availability. Thus those microbes possessing better adaptability to tailings conditions will prevail.

It is thought that tailings microorganims may be predominantly found in the pond adhered to the clay or sand particles promoting tailings aggregation, thus contributing to densification [15]. In fact, species like *Pseudomonas*, *Thauera*, *Hydrogenophaga*, *Rhodoferax,* and *Acidovorax*, detected in the pond have the ability to grow in a biofilm structure, presumably attached to the mineral matter [38]. This arrangement allows microbes to survive the tailings toxicity and improves the absorption of the nutrients available.

The bulk of a tailings pond is mostly anaerobic. Hence, the majority of microbial activity is carried out by anaerobic Bacteria and Archaea. In the absence of oxygen, certain compounds like nitrate (NO_3^-), ferric iron (Fe^{3+}), SO_4^{2-} and CO_2 can be used as terminal electron acceptors [54]. These can be reduced by many organisms in a dissimilatory way to provide energy for their metabolism. Microorganisms are classified

based on the type of electron acceptors they use; hence, nitrate reducers use NO_3^-, iron reducers use Fe^{3+}, sulfate reducers use SO_4^{2-} and methanogens can use CO_2 [54]. The energy released from the oxidation of an electron donor using these compounds as electron acceptors varies. Each electron acceptor has different reduction potentials that make them more or less energy efficient based on their electronegativity. Therefore, nitrate reduction is the most energy-yielding followed by iron, then sulfate, then methanogenesis [54].

1.2.1.1 Sulfate-reducing bacteria (SRB) and methanogens

In some tailings ponds, SO_4^{2-} is readily available in fresh tailings due to the gypsum addition used to enhance tailings densification. The sulfate concentrations in tailings ponds typically decrease with depth [46], with the highest sulfate concentration found at the surface waters between 150 to 170 mg $\cdot$ L^{-1} , or as high as 576 mg $\cdot$ L^{-1} [55]. The use of SO_4^{2-} as an electron acceptor by SRB results in the production of HS^- at slightly alkaline pH values [54]. This sulfide release becomes a problem when its production exceeds the amount of metals available for the formation of metal sulfides or if the pH drops markedly in the pond . In addition, the amount of sulfate reduced depends on the bioavailability of the electron donors. SRB can be found in tailings at an average MPN (most probable number) of 10^9 $\cdot$ g of MFT for Suncor and Syncrude tailings [16,44].

As a pond ages, the sulfate levels in the anaerobic layers decrease and the pond becomes methanogenic [44]. A clear example of this is reflected by studies carried out in MLSB, the primary tailings pond managed by Syncrude Canada Ltd., where methane

was not detected in the first 15 years of operation. For these initial years the SO_4^{2-} concentrations were between 35 – 75 mg · L^{-1} for the deep layers and between 60 – 120 mg · L^{-1} at shallower depths [44]. However, by the mid-1990s, methane bubbles were visible on the surface of the MLSB and this corresponded with a drop in sulfate concentrations to values as low as 5 mg · L^{-1} in the year 1997 [16,44]. In 2000, Holowenko *et al.*[46] detected 10^5 – 10^6 methanogens and 10^4 – 10^5 SRB · g^{-1} of MLSB tailings. Because both types of organisms can compete for the same electron donors, with SRB catalyzed reactions being more energetically favorable, the presence of SO_4^{2-} can inhibit or delay the production of methane [46] [Reactions 1, 2, 3, 4]. This is true as long as electron donors are not abundant, in which case methanogens manage to sequester a portion of the electron flow even when SO_4^{2-} is present to support SRB [44].

Reactions catalyzed by SRB and methanogens

$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	$\Delta G = -152$ kJ · $reaction^{-1}$	[1]
$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$	$\Delta G = -47$ kJ · $reaction^{-1}$	[2]
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	$\Delta G = -135$ kJ · $reaction^{-1}$	[3]
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	$\Delta G = -31$ kJ · $reaction^{-1}$	[4]

Competition for H_2 is a very common feature in anaerobic environments. Hydrogen is usually readily produced and consumed, so its level remains relatively low in the natural environment. SRB consume H_2 more efficiently than methanogens thus maintaining concentrations too low for methanogens [Reactions 1, 3]. The same pattern

is observed when Fe^{3+} is available. Iron reducers can use H_2 thus decreasing its concentration to levels below the threshold for either SRB or methanogens [54].

Methanogens are strict anaerobes that produce methane when growing on H_2/CO_2, formate and other substrates like acetate, methanol, ethanol, isopropanol, methylated amines, and methylated sulfur compounds [56]. Some methanogens such as *Methanobrevibacter* sp. can only use H_2/CO_2, others like *Methanosaeta* sp. can only grow on acetate. Others are somewhat more flexible and can grow on H_2/CO_2 and formate like *Methanospirillum* sp. and *Methanobacterium* sp. or like *Methanosarcina* sp. that can grow on H_2/CO_2, acetate, methanol and a few other carbon compounds [56].

Methanogens rely on other bacteria, such as syntrophs or acetogenic bacteria, to degrade complex hydrocarbons to their simple substrates. SRB can also collaborate with the syntrophs to degrade hydrocarbons [57]. Overall, competition for substrates in the pond can lead to the inhibition of some groups of microorganisms like methanogens in the presence of sulfate [Figure 1-3]. However, because tailings can supply other electron donors for syntrophs, methanogenesis can still be active and methane can eventually evolve from the deep anaerobic zones towards the surface layers [44].

1.2.1.2 Iron reducing bacteria (IRB)

In tailings ponds iron comprises around 3% of the solids [44]. Iron can fluctuate between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms and their concentrations have been determined to be relatively low (~ 0.8 mg $\cdot$ L^{-1} in Syncrude tailings) [16]. The predominance of one form of iron over the other depends greatly on environmental physicochemical parameters such

as pH, oxygen concentration and redox potential [58]. Due to the slight alkalinity of tailings (pH ~ 7 - 8) and to the abundance of sulfate, one would expect to find iron as FeS or as FeS_2 [16]. The total number of iron reducers in Syncrude tailings were found in the range of 10^1 - 10^5 cells · g^{-1} of MFT as determined by MPN [44], lower than the numbers of SRB, NRB, and methanogens found in the same pond [16]. Different groups of bacteria have been studied for their ability to either reduce or oxidize iron compounds. Such is the case of *Thiobacillus denitrificans*, a strict autotroph that has been known for its ability to oxidize pyrite in the presence of nitrate in anoxic sediments [59] or *Acidovorax* sp. that can oxidize ferrous iron in the presence of organic acids such as acetate [58].

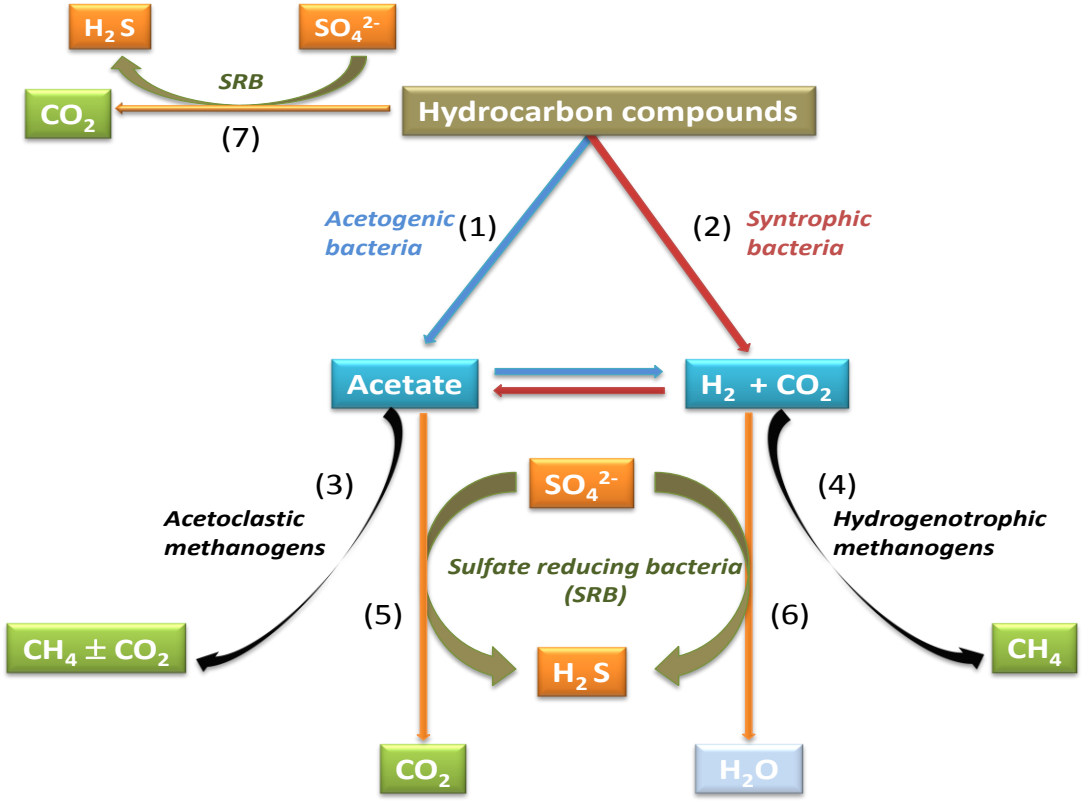


Figure 1-3 Schematic representation of the degradation of hydrocarbon compounds under anaerobic conditions. Oil hydrocarbons are attacked by (1) acetogenic bacteria to produce acetate or by (2) syntrophs to produce H_2, CO_2, and/or acetate. In the absence of electron acceptors acetoclastic methanogens are favored (3) but when sulfate is available SRB (5) compete with methanogens for acetate. Similarly SRB can compete for H_2 (6), but when no sulfate is available, hydrogenotrophic methanogens (4) utilize H_2 with the concomitant production of methane. SRB can also directly oxidize selected oil components with sulfate (7).

1.2.1.3 Nitrate reducing bacteria (NRB)

Other groups of microbes also detected in tailings ponds are the nitrate-reducing bacteria (NRB) . Nitrate has been found in Syncrude tailings concentrations of ~ 16 mg · L^{-1} . In a laboratory experiment, the NO_3^- disappeared after 36 days of incubation suggesting that it was converted to either N_2 or N_2O by denitrifying bacteria which were initially present in the tailings samples at a concentration of ~ 10^8 cells · g^{-1} of MFT [16], or converted into biomass.

A positive outcome of the presence of NRB in the pond is their ability to form biofilm and/or aggregates of clay particles that contribute to tailings densification [15,38]. Simultaneously, if nitrate were to be present in adequate proportions in the pond, this could help in controlling H_2S emissions, if any, in the same way that nitrate is used to control souring in oil fields [57].

1.2.1.4 Aerobic microorganisms in tailings ponds

Tailings ponds also support a group of aerobic and facultatively anaerobic bacteria that grow at the surface of the pond, where oxygen and other nutrients are available. However in earlier studies by Foght *et al.* [37], the microbiology of a Syncrude pond revealed that bacteria with known aerobic and facultatively anaerobic respiration were distributed from 0.5 to 12 mbs. Cell concentrations of 1.2 to 1.6 x 10^6 cells · mL^{-1} were detected with *Alcaligenes* and *Acinetobacter* being the most common genera found [37].

These bacteria may be growing at the expense of solubilized organic matter or may be playing important roles in utilizing the final products of the anaerobic microbes found at the anaerobic/aerobic interface. Such is the case of methanotrophs. This group of aerobic bacteria is capable of metabolizing methane as a source of carbon and energy [54]. It has been observed that the methane produced by methanogens travels to the surface of the pond as a bubble [45]. During its ascent, the bubble partitions other materials, carrying them to the surface. For instance, oxygen can be incorporated into the bubble enhancing the anaerobic nature of the deep layers of the pond [60]. Other gases, particles, oils, bacteria, volatile hydrocarbons, and surfactants can also be partitioned into the bubble, thus providing an important vertical transport mechanism [60]. Once the bubble reaches the oxic layers, it can burst and microbes inhabiting this area benefit from the nutrients released. For example, methanotrophs found in TPW potentially can use methane and convert it to CO_2, while sulfide-oxidizing bacteria (SOB) can use H_2S and convert it to sulfate. This interdependence may be beneficial as less methane and H_2S can escape to the atmosphere.

The oxidation of sulfur compounds by microorganisms is one of the oldest metabolic mechanisms known and it is a process that is found tightly related to other biogechemical cycles like carbon, oxygen and nitrogen in marine sediments [61]. SOB or SOP (Sulfur Oxidizing Prokaryotes), are mainly composed of aerobic lithotrophs or anaerobic phototrophs [62]. Apart from H_2S, these organisms can oxidize sulfur, sulfite, thiosulfate and various polythionates under alkaline, neutral, or acidic conditions with sulfate as their final oxidation product [62]. Aerobic bacteria with this activity include

Acidaunus, Acidithiobacillus, Aquaspirillum, Aquifex, Bacillus, Beggiatoa, Methylobacterium, Paracoccus, Pseudomonas, Starkeya, Sulfolobus, Thermithiobacillus, Thiobacillus, and *Xanthobacter* [62]. Anaerobic phototrophs include *Allochromatium, Chlorobium, Rhodobacter, Rhodopseudomonas, Rhodovulum* and *Thiocapsa* as the major genera described to date [62]. Members of the order *Sulfolobales* (Archaea), are also known to oxidize reduced sulfur compounds [63]. Two major biochemical pathways have been proposed to oxidize the sulfur compounds: (1) the sulfur oxidation pathways, and (2) the sulfite intermediate pathway [61]. The sulfur oxidation pathway (1), also called the *Sox* system, oxidizes sulfur compounds directly to sulfate without the formation of sulfite [54,61]. The sulfite intermediate pathway (2) can be observed in two different ways. The most common pathway is that employing the enzyme sulfite oxidase [54]. The other is via a reversal of the activity of adenosine phosphosulfate (APS) reductase, an enzyme present in the metabolism of sulfate by SRB [54].

As mentioned earlier, some aerobic bacteria may be metabolizing organic compounds dissolved in the TPW such as NAs. Several studies have revealed the NA degradation potential for indigenous tailings organisms [32,64-66]. For example, a mixed culture of species enriched from tailings such as *Pseudomonas putida* and *P. fluorescens* [64] and *P. stutzeri* and *Alcaligenes denitrificans* [67] has been found to degrade NAs. More details regarding NA biodegradation will be mentioned in section 1.2.2.

Independent of the metabolic processes ongoing at the surface of the pond, the presence of these aerobic microorganisms may fluctuate depending on the months of the year. Thus, during the wintertime, where a layer of ice tends to cover the ponds, these

populations may undergo a dormant state that can eventually be restored during the summer time.

1.2.2 *Sources of carbon for organisms living in tailings ponds*

As previously summarized, tailings are a mixture of water, mineral and organic matter that can support the metabolism of several living organisms. From eukaryotic microbes to Bacteria and Archaea, they all find a place in which to survive and/or co-exist. The specificity of electron donors used by microorganisms in tailings ponds is still somewhat unknown. However, based on the chemical composition of tailings, some assumptions can be made [44].

For instance, a small fraction of naphtha (less than 1%) is lost to tailings and incorporated into MFT, so this solvent could serve as a good source of electron donors. In fact, the biodegradation of various *n*-alkane hydrocarbons under methanogenic conditions has been previously demonstrated at the laboratory scale [41,47,68,69]. Some of the microbial communities present in the MFT are capable of utilizing C_6-C_{10} *n*-alkanes under methanogenic conditions, which supports the hypothesis that components from naphtha in oil sands tailings can sustain methanogenesis [41]. Experiments using BTEX (benzene, toluene, ethylbenzene, and xylenes) amendments at 0.05% have also shown that these compounds can be biodegraded under methanogenic conditions by tailings microbes [47]. SRB have also been shown to metabolize toluene, xylenes, ethylbenzene and naphthalene in other hydrocarbon-associated environments [70].

Other studies regarding the use of long chain alkanes by anaerobic organisms have also been carried out. Bacteria such as *Syntrophus* (a syntrophic bacterium), and *Desulfovibrio* (a H_2-utilizing SRB) have been found to be a part of alkane-degrading consortia and methanogenic Archaea like *Methanosaeta* (acetoclastic methanogens), *Methanospirillum* and *Methanoculleus* (methanogens utilizing H_2 and CO_2) have also been detected in alkane-degrading cultures, and are presumably involved in converting long chain alkanes to methane and CO_2 by a process called microbial alkane cracking [71]. More recently, the characterization of the microbial communities from tailings enrichments involved in methanogenic biodegradation of naphtha and its short-chain *n*-alkane (C_6–C_{10}) and BTEX components, revealed a syntrophic oxidation of hydrocarbons in oil sands tailings [69]. The presence of bacterial genera such as *Desulfotomaculum* and *Syntrophus/Smithella* together with the archaeal groups associated with *Methanosaeta* (in *n*-alkane and naphtha enrichments) and *Methanomicrobiales* (in BTEX and naphtha), suggests these organisms are involved as syntrophic partners in the degradation of these organic compounds [69].

Residual bitumen present in tailings should not be ruled out as a possible source of carbon in tailings. Studies by Wyndham and Costerton [72] showed that saturates and aromatic fractions from an Athabasca bitumen supported growth of some bacterial isolates. They revealed, by the use of microscopy, that bacteria adhere to the hydrocarbon surface making a bitumen-bacteria association with or without the formation of a glycocalyx. When no polysaccharide is needed, the bacteria can be found within a few micrometers of the bitumen surface or in channels penetrating the substrate [72].

Last, but not least, NAs are other carbon sources present in tailings that can support the growth of microorganisms. These natural compounds are extracted from the bitumen during the Clark extraction process. They are commonly found at the aqueous/non-aqueous interface of oil sands but because of the alkaline pH of tailings they are mainly present as water-dissolved naphthenate salts that end up in the tailings ponds [31]. As mentioned earlier, they are known for their acute or chronic toxicity to living organisms [36] and the degree of their toxicity has mainly been associated with their molecular weight and surfactant characteristics. The higher molecular weight compounds with increased carboxylic acid content will decrease hydrophobicity, thus becoming more bioavailable and accumulating in the cells [32,36]. Their surfactant properties are thought to disrupt the cell wall or membrane of the cells [73].

Despite the toxic nature of NAs, experimental evidence to date indicates that NA biodegradation is predominantly an aerobic process. Species like *Arthrobacter*, *Pseudomonas, Acinetobacter* and *Alcaligenes* have been previously reported for their ability to degrade NAs [74-78]. However, the persistence of NAs in tailings ponds is believed to be high. The ongoing recycling of the water back into the bitumen extraction plant tends to increase their concentration in the water, thus maintaining a steady concentration in tailings [31,79]. On the other hand, it has also been suggested that NAs could be derived from the action of microorganisms on the residual bitumen present in tailings ponds, thus contributing to the total NA content [80].

The mechanism under which biodegradation of NAs occurs is not well understood but in general their susceptibility seems to be related to their molecular weight and the

degree of the branched alkyl chains [81]. Low molecular weight NAs are more biodegradable than highly branched, high molecular weight compounds [32,82,83].

Several pathways have been proposed for the degradation of aliphatic and alicyclic carboxylic acids such as β-oxidation, combined β- and α- oxidation, and aromatization [81,82]. The β-oxidation pathway seems to be the preferred route by which several bacteria degrade these compounds [82]. For instance, the degradation of cyclohexanecarboxylic acid by species including *Acinetobacter anitratum, Alcaligenes faecalis* and *Pseudomonas putida* via β-oxidation has been observed [75,77].

In the anaerobic layers, the relatively high adsorption capacity of NAs to oil sands solids allows them to sink to the bottom of the pond where little biodegradation can be carried out. Even bitumen can act as trap for NAs, which would also contribute to their retention throughout the vertical tailings column [79]. Higher molecular weight compounds will be more strongly adsorbed to tailings particles than low molecular weight ones, and this can decrease the bioavailability of these compounds to the microorganisms [31]. It is not well known if NA can support growth in anaerobic environments. Previous studies under methanogenic conditions with commercially available, natural NAs (extracted from TPW), and surrogate NAs showed that methanogenic consortia present in a Syncrude tailings ponds did not utilize NAs as their main source of carbon [84].

Due to the complexity of the mixture of compounds in tailings, it is hard to determine which substrates are preferred as electron donors for the pond microorganisms. Also, the lack of available sources of nitrogen and phosphorus is thought to influence the rate at which degradation of these complex compounds occurs [37,66]. As in any given

ecosystem, the species that most successfully inhabit it, are those best adapted to grow on the nutrients and conditions available and tailings ponds are no exception [54]. An interdependent association between species where they all benefit from each other or compete for the same nutrients is expected.

1.2.3 Molecular biology techniques for unveiling microbes in tailings ponds

Although the characterization of microorganisms from the environment has been traditionally done by cultivation in synthetic growth media, this retrieves only a small fraction of species , estimated to be 0.1% or at most 10% of the total population [85]. This is most likely because commonly used laboratory media do not adequately mimic the environment in which most microorganisms grow. With the implementation of molecular biology techniques, microbiologists are able to detect the uncultivated microbes that make up a large fraction of the microscopic world. These techniques have allowed the discovery of microbial species that for years remained unknown.

Specifically, small subunit ribosomal RNA (16S rRNA) gene-based analysis has become a standard tool of molecular biology. This gene is an ideal marker biomolecule because it is universal to cells, it is highly conserved structurally and functionally and it is the central component of a complex translation apparatus in microbial cells. These genes can be amplified using “universal” primers or primers specific for different groups of microbes by the polymerase chain reaction (PCR). The PCR products can be further separated by clone library construction, Denaturing Gradient Gel Electrophoresis (DGGE), Single Strand Conformation Polymorphism (SSCP), Terminal Restriction

Fragment Length Polymorphism (TRFLP) or by pyrosequencing, among others. The separated product can then be sequenced, identified and arranged into phylogenetic trees that will help provide information on the possible role of these organisms in the environment, usually by identification of the nearest cultivated relatives.

Some difficulties when determining microbial diversity in oil sand tailings could be encountered, including the presence of microhabitats containing highly aggregating bacteria in clumps or "hot spots" [86]. Hence, effective sampling is a key issue when studying the microbial diversity in this type of ecosystem. Sometimes, the microbial populations could be underestimated resulting in high variability between replicates [87].

To target specific microorganisms based on function, specific primers are constructed, narrowing their selection. This approach is very important in linking community structure to activity. If these specific genes are detected, it is possible that the metabolic process is taking place. Most recently, the emerging powerful method of metagenomics or environmental genomics is capturing the attention of the scientific community. The two primary goals of this approach are to identify the organisms present in a sample by large scale sequencing and to identify what roles organisms have within a specific environment. This is facilitated by 454 sequencing, a large-scale parallel pyrosequencing system capable of producing billions of bases of sequences per day.

Overall, despite potential biases (such as low DNA yields [88] and the presence of PCR inhibitors [89], among others) molecular techniques are nowadays the most powerful

tool used to reveal organisms that are unable to grow in synthetic media that do nevertheless play important roles in the biogeochemical cycles of Earth.

Chapter Two: **Hypothesis and objectives**

Oil sands tailings ponds represent an environmental concern to Alberta and Canada. The large volumes of tailings that are generated daily, their high toxicity, and the hazardous gases potentially emitted to the environment, pose a threat to the flora and fauna in the area. In order to develop better tailings management strategies, it is important to understand the physiology of microorganisms that grow in these niches. For example, these microbes are the key players in sulfide and methane emissions but can pontentially also be used to remediate toxins in tailings ponds. Thus, the monitoring of these ponds over time would allow us to determine what microbes prevail and how they correlate with the chemical parameters and microbial activity observed.

It was hypothesized that changes to tailings pond management operations shift the pond microbial communities and activities, due to changes in selective pressures (e.g. available electron acceptors or donors) to which the microbial communities are exposed. For example, it is predicted that the addition of calcium sulfate (gypsum) or other electron acceptors would inhibit methanogenesis, pond aging would result in the increase of methanogen abundance and thus methanogenesis, and that pond closure (e.g., a scenario where no new tailings or treaments are added to a tailings pond) will shift the microbial community composition and activities (however, the outcome of pond closure is not known - e.g. does pond closure lead to positive or negative effects?). Thus, gaining information about the key microbial players and activities that occur in tailings ponds subject to different management strategies can help operators predict whether certain operations will lead to desired (e.g. biodegradation/bioremediation of tailings

chemicals) or undesired effects (e.g. gas emissions). The present research was thus aimed to study the physiology of two tailings ponds to gain insight into the key microbial processes and communities present. The ponds under study included an active pond (pond 6) and an inactive pond (pond 5) which we were able to monitor just before and after closure.

Specifically, the five major objectives of this thesis work were to:

1. Assess the microbial diversity in tailings ponds to determine the predominant communities as a function of depth **(Chapter 4)**

2. Assess the key physiological processes in tailings ponds (sulfate reduction, methanogenesis, sulfide oxidation) that can impact pond emissions and correlate these with the microbial community findings **(Chapter 4)**

3. Quantify the major microbes responsible for sulfate reduction, methanogenesis and sulfide oxidation in tailings using optimized qPCR analyses **(Chapter 6)**

4. Identify key electron donors (bitumen, naphtha, and naphthenic acids) that can be used by the endogenous anaerobic and aerobic microbial communities **(Chapters 5 & 7)**

5. Test for the use of nitrate as an alternate electron acceptor to control methanogenesis in tailings ponds and assess its impact on microbial community composition **(Chapter 7)**

Chapter Three: **Methods and Materials**

3.1 Sampling and origin of oil sands tailings samples

The tailings samples used in this work were collected by Suncor Energy Inc. and were collected as a function of depth. The samples were collected in autoclaved sterile Nalgene bottles, filled to capacity, and tightly sealed to prevent oxygen contamination. The samples were shipped to the laboratory by plane shortly after sampling. Upon arrival, the bottles were kept in an anaerobic glove bag (COY Laboratory products Inc.) containing an atmosphere of 90% N_2 and 10% CO_2. Subsamples of these were frozen at –80 °C in sterile, plastic 50 mL tubes for molecular studies. The rest remained in the anaerobic hood for chemical analysis and to prevent oxygen contamination. This research comprises the study of 4 years of monitoring (2008 – 2011) from 2 different ponds: Pond 5 (closed in 2010) [Table 3-1], and Pond 6 (active) [Table 3-2]. Tailings from both ponds 5 and 6 have been managed with the CT technology where MFT is combined with coarse sand and gypsum slurry to form a non-segregating material that releases water allowing a faster tailings consolidation.

Pond 5 is the first production-scale CT pond in the oil sands industry. It has been in use since 1995 and extends about 3 km north south and about 5 km east west occupying an area of approximately 15 km^2 [52]. After 12 years of tailings discharge, the pond reached its capacity (deeper than 50 mbs) and was decommissioned at the end of the year 2009 [52].

Pond 6 started filling in 2002 and in the spring of 2010 had already reached its capacity. At this time, the pond stopped receiving fresh tailings. Only CT tailings were

transferred into this pond. Therefore the pond has been considered “somewhat” active since 2010. It has an approximate depth of 41 mbs and it is capped with 1 m of water (personal communication, Dipo Omotoso, Suncor).

Table 3-1 Suncor Pond 5 sample information

Sampling Date	*GPS coordinates for samples*	*Site designation*	*Depths sampled (mbs)*
June 2009 (before closure)	57 0.4317N -111 32.46336W	P5S19A	1.8, , 2.4, 3.0, 4.6, 6.1, 7.6, 9.1, 10.7, 12.2, 13.7, 15.2, 19.8, 25.0, 29.0
July 2010 (after closure)	57 0.4317N -111 32.46336W	P5S19A	3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60, 63

Table 3-2 Suncor Pond 6 sample information

Sampling Date	*GPS coordinates for samples*	*Site designation*	*Depths sampled (mbs)*
October 2008	57 1.24812N -111 33.2466W	423 m away from P6S72A	0, 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18
June 2010	57 1.47426N -111 33.18378W	P6S72A	3, 6, 9, 12, 15, 18, 21, 22
July 2011	57 1.47426N -111 33.18378W	P6S72A	0, 3.5, 4, 7, 10, 13, 16, 18.5

3.2 Chemical techniques

3.2.1 Dissolved hydrogen sulfide concentrations in tailings

3.2.1.1 Preparation of reagents

Prior to sampling tailings samples a solution of DMPD (*N, N*-dimethyl-p-phylendiamine-dihydrochloride) was prepared as follows: 1.0 g of DMPD and 1.0 g of $Zn(CH_3COO)\cdot 2H_2O$ were dissolved in 50 mL of concentrated H_2SO_4 together with 950 mL of ddH_2O. This solution was dispensed (4.9 mL) in 15 mL test tubes followed by the addition of 5.0 mL of ddH_2O. A ferric chloride solution was prepared by mixing 5.0 g of $FeCl_3 \cdot 6H_2O$ in deionized water to a final volume of 20 mL. Standards of 0.5 to 15.0 µg of HS^- were prepared from a stock solution of $Na_2S \cdot 9H_2O$ at 1000 mg $\cdot L^{-1}$ in anoxic 0.01 N NaOH.

3.2.1.2 Assay procedure

This assay was always prioritized so as to prevent sulfide loss and it is based on the assay developed by Cline (1969) [90]. Therefore, it was done within the first 24 hours following tailings samples arrival when the bottles were first opened in the anaerobic bag. In the glove bag, approximately 10 mL of tailings were poured out from the sampling jars and transferred to 15 mL sterile centrifuge polypropylene tubes. The tubes were tightly capped and taken out of the anaerobic glove bag for centrifugation (1200 x g, 10 min, IEC Centra GP8R). The resulting supernatant (tailings water) was collected very carefully (100 µL) avoiding particles of oil and/or sand with a micropipette (GILSON®- pipetman®) and rapidly transferred into the test tube with DMPD and water

described above. Immediately after, 100 µL of the ferric solution were added. Tubes were vortexed and incubated at room temperature for 10 min to allow for color development and absorbance was read on a spectrophotometer at 660 nm against a blank [90]. When sulfide was present, the solution turned from light pink to blue. All spectroscopic measurements were taken using a UV-1800 UV-VIS spectrophotometer (Shimadzu). Sulfide concentrations were calculated based on the standard curve obtained.

3.2.2 Dissolved hydrogen sulfide assay method for SOB experiments

Sulfide determination for SOB experiments described in section 5.2.1 were done using the copper sulfide method [91].

3.2.2.1 Preparation of reagents

A solution of zinc acetate (24 g of $Zn(CH_3COO)_2$, 1.0 mL of 20% (w/w) CH_3COOH, $_dH_2O$ adjusted to 1.0 L); 1.0 L of diamine reagent (2.0 g of 4-amino-N,N-dimethylaniline, 600 mL of dH_2O, 200 mL of concentrated H_2SO_4); and 100 mL of iron alum solution (10.0 g of $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$, 2.0 mL of concentrated H_2SO_4, and dH_2O to complete 100 mL) were prepared.

3.2.2.2 Assay procedure

The samples (1.0 mL) from serum bottle experiments were obtained with a syringe and centrifuged for 30 sec (maximum speed, Eppendorf) and 125 µL of the supernatant were transferred into 25 mL vial that contained 2.0 mL of the zinc acetate solution. Distilled water (8.5 mL) and diamine reagent (2.5 mL) were swirled with the sample and

the zinc acetate and a brick-red-orange color was allowed to develop. Immediately after, 125 µL of iron alum solution was added and the mixture was vortexed and allowed to stand for 7 to 10 min at room temperature. In the presence of HS^- the mixture turned to a blue color. The mixture was adjusted to a final volume of 20 mL and OD was measured spectrophotometrically against a blank at 670 nm.

3.2.3 Anion concentrations (nitrate, sulfate, and acetate) concentrations by HPLC

The water phase obtained from the centrifugation step described in section 3.2.1.2 was also used for sulfate and acetate determination [92]. For some depths, the water content was not sufficient, thus more tailings sample was needed for centrifugation. The supernatants were then filtered using a 0.2 µm membrane filter (VWR). The filtrate was transferred into a clean 1.5 mL autosampler vial and then subjected to High Pressure Liquid Chromatography (HPLC) in a Dionex ICS-5000 system. Samples were loaded into an auto-sampler that used 25 µL of the sample for injection into the column. The anions were separated on a 4 x 250 mm column (IonPac®AS18, Dionex) with a mobile phase of water at a flow rate of 1 mL · min^{-1} (ICS-5000, Dionex). Standards were prepared at 100, 200, 300, 400, and 500 µM from a 1 mM anion stock solution (SO_4^{2-}, NO_3^-, NO_2^-, Cl^-, acetate). Nitrate and sulfate concentrations in laboratory experiments were prepared and analyzed in the same manner.

3.2.4 Water content

The water content in all tailings samples was measured following the ASTM standard [93]. A known weight of tailings was dried in an oven at a temperature of 60 +/– 5

ºC to a constant mass. The water loss was calculated using the mass of water and the mass of the dry specimen [Equation 1]. Duplicates of approximately 20 g of each tailings sample were analyzed.

Equation 1 Water content

W = Mw/Ms x 100 (expressed in %) where: Mw: mass of water

Ms: mass of oven dry specimen

3.3 Microbial activity measuremets

3.3.1 Sulfate reduction rates (SRR)

Sulfate reduction rates were determined for all samples collected at all depths using a method similar to that described by Ulrich *et al*. (1997) [94].

3.3.1.1 Preparation of reagents

Anaerobic concentrated HCl

Concentrated hydrochloric acid (HCl) was placed in a serum bottle, leaving a reasonable head space, capped and crimped followed by a continual flushing with N_2 gas for several minutes in the fume hood to make the acid anoxic.

Zinc acetate solution (10 %)

Boiled distilled H_2O (~ 100 mL) was bubbled with a flow of N_2 and allowed to cool. Zinc acetate ($Zn(C_2H_3O_2)_2$ (10 g) was added and once dissolved, the solution was

distributed in non-sterile serum bottles using a pipette. The bottles were capped and crimped and stored in the anaerobic hood.

Chromium – acid solution (Cr(III)-HCl)

A solution of Cr(II) (1 M) was prepared in HCl (0.5 N) in a volumetric flask. Separately, one third of a glass bottle was filled with mossy Zn and washed several times with HCl (0.5 N). Then the Cr/HCl solution was added to the mossy Zn and bubbled for 15 to 20 min with N_2 gas. After this time, the Cr solution turned color from green to crystal blue due to the reduction of Cr(III) to Cr(II). The solution was distributed using a glass pipette into serum bottles previously flushed (with N_2), capped and crimped.

Labelled sodium sulfate ($^{35}SO_4$) stock solution

A stock solution of 10 µCi· mL^{-1} of $^{35}SO_4^{2-}$ was prepared in distilled anaerobic H_2O from a stock solution of 1 mCi · mL^{-1} (Perkin-Elmer). The volume was enough to add 1 mL of radioisotope per serum bottle of 10 mL to have a final concentration of approximately 1.0 µCi· mL^{-1} in each experiment.

3.3.1.2 SRR technique

In the anaerobic glove bag, samples of tailings (5.0 g) were distributed into 60 mL serum bottles and capped with rubber stoppers. Then 5.0 mL of anoxic, autoclaved water was added with a syringe resulting in a total volume of approximately 10 mL. At this point, the sulfate concentration of the diluted tailings was determined by using HPLC [section 3.2.3]. For this determination, 300 µL of tailings were taken using a previously

flushed syringe. The diluted tailings were shaken and 1 mL of the radioisotope stock solution was added using a 1 mL syringe. Samples (100 µL) for each serum bottle were taken for counts per minute (cpm). This value represents the initial concentration of $^{35}SO_4^{2-}$. The samples were incubated in a shaker in the fume hood for 5 to 7 days. After the incubation time, 8 mL of tailings were transferred into clean anaerobic serum bottles that contained a 2.5 mL 10 % zinc acetate solution [Figure 3-1].

The samples were then acidified with 4.0 mL of Cr(II)-HCl and 4.0 mL of concentrated HCl. The mixture was incubated for 3 d on a slow shaker to allow the H_2S to be converted into its gaseous state and trapped in the zinc acetate traps. The amount of ^{35}S-sulfide collected in the zinc acetate trap (along with the remaining ^{35}S-sulfate in the tailings to ensure mass balance) was quantified by liquid scintillation counting on a BECKMAN LS6500. The SRR was calculated using Equation 2 [94] and expressed in nmol $SO_4^{2-} \cdot cm^{-3} \cdot day^{-1}$. Samples from each depth and sampling event were subjected to this technique.

Equation 2 SRR calculation

$$SRR = \frac{[SO_4^{2-}] \times (H_2{}^{35}S) \times \alpha}{(^{35}SO_4^{2-}) \times t}$$

where: SRR: Sulfate Reduction Rate

$[SO_4^{2-}]$: Sulfate conc. in tailings (nmol · cm^{-3})

^{35}S-H_2S: cpm in the sulfide trap

^{35}S-SO_4^{2-}: cpm in tailings

t: Incubation time (days)

α: discrimination factor for ^{35}S (1.06)

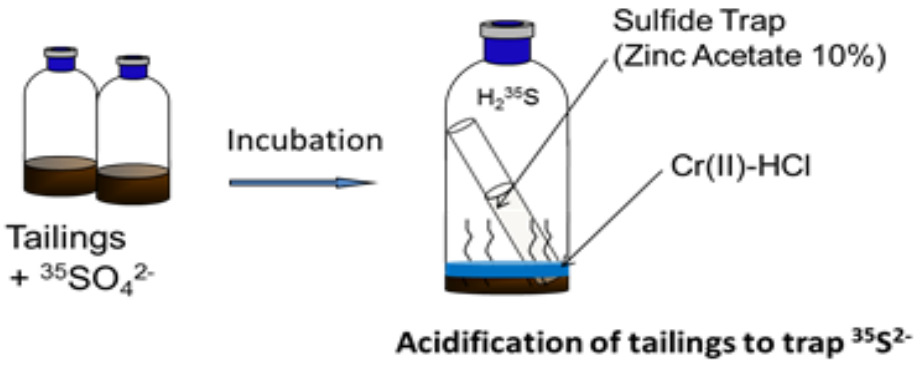


Figure 3-1 Schematic representation of the zinc acetate trap for SRR determination. Tailings are incubated with the $^{35}SO_4{}^{2-}$ for 5 to 7 days, then the tailings are acidified with a mixture of Cr(II)-HCl and the resulting $H_2{}^{35}S$ generated is trapped in the zinc acetate solution previously introduced into the bottle. The cpm in the sulfide trap is counted in scintillation equipment to determine how much of the original sulfate was reduced to sulfide.

3.3.2 Methanogenesis rates

3.3.2.1 Sample preparation

For samples collected from each depth, 20 mL of tailings were transferred into sterile, anoxic 60 mL serum bottles, capped and crimped. Some samples were autoclaved to create sterile controls. Samples were incubated at room temperature in the dark. Methane concentrations were determined in the headspace at day 0, day 12, and after 36 d of incubation. Rates were calculated based on methane produced after first 36 days in nmol $CH_4 \cdot cm^{-3}$ tailings $\cdot day^{-1}$.

3.3.2.2 Methane measurements by gas chromatography

Methane formation over time was monitored to determine rates in tailings samples and in enrichment cultures (different carbon sources and nitrate experiments) using a gas chromatograph (GC) [95]. Using a sterile syringe, preflushed with N_2/CO_2 (90/10), 0.2 mL of the incubation headspace was sampled and injected into a HP model 5890 GC equipped with a flame ionization detector maintained at 200 °C. Injections were carried out at 150 °C onto a packed stainless steel column (6 ft. 9 1/8 in., Poropak R, 80/100, Supelco) held isothermally at 100 °C. Methane amounts were determined based on calibration curves prepared from standards containing known methane concentrations.

3.4 Microbial community composition

3.4.1 DNA Extraction

For tailings samples, approximately 500 mg of sample from each depth was used for DNA extraction using the Fast DNA Spin Kit for Soil (MP Biomedicals) according to the manufacture's protocols. For samples collected from Pond 6 2008 only, the DNA was extracted using the same procedure plus skim milk powder (Fluka analytical) (40 mg · g of tailings $^{-1}$) to enhance DNA recovery from tailings [96].

For the water samples (surface water), 1 mL of sample was taken and DNA was extracted using Fast DNA Spin Kit (MP Biomedicals).

For laboratory enrichment cultures, 1 mL of culture fluid was typically sampled and DNA was also extracted using the FastDNA Spin kit (MP Biomedicals).

3.4.2 16S r DNA Pyrosequencing

The extracted DNA (typically ~ 2 ng·μL^{-1}) was subjected to polymerase chain reaction (PCR) amplification of 16S rRNA genes using 12.5 μL of 2xPCR Master Mix (Fermentas), 10.5 μL of nuclease-free water (Fermentas), 1 μL of genomic DNA (2 ng), and 0.5 μL of FLX Titanium amplicon primers 454TRA and 454T-FB (20 pmol μL^{-1}) for a 25 μL PCR reaction. These have the sequences for 16S primers 926f (aaa ctY aaaKga att gac gg) and 1392r (acg ggc ggt gtg tRc) as their 3′- ends. Primer 454T - RA has a 25 nt A-adaptor (CGTATCGCCTCCCTCGCGCCATCAG), whereas primer 454T-FB has a 25 nt B-adaptor sequence (CTATGCGCCTTGCCAGCCCGCTCAG).

Following PCR amplification (95 °C, 3 min; 25 cycles of 95 °C 30 s, 55 °C 45 s, 72 °C 90 s; 72 °C 10 min; final hold at 4 °C) with primers 454T-RA and 454T-FB, the PCR product was verified on a 0.7% agarose gel and purified with a QIAquick PCR Purification Kit (Qiagen).

For tailings samples where the concentration of the PCR product obtained was too low (< 5 ng · µL) or the presence of primer dimers appeared in the gel, a touch down PCR protocol was used. This protocol was as follows: 1 cycle of 95°C for 5 min for denaturation; 30 cycles of 30 s at 95°C, 30 s at 60°C, decreasing 0.5°C/cycle, and 30 s at 72°C. Then another 30 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C were carried out. Finally, the last cycle was for 10 min at 72°C. This protocol was used with individual PCR reagents from Qiagen.

PCR product concentrations were determined on a Qubit Fluorometer (Invitrogen), using a Quant-iT dsDNA HS Assay Kit (Invitrogen). PCR products (typically 20 μL of 20 ng·μL^{-1}) were sent to the Genome Quebec and McGill University Innovation Centre, where they were subjected to 10 PCR cycles with primers 454T-RA-X and 454TFB, where X is a 10 nt multiplex identifier barcode. The barcoded PCR products were analyzed by pyrosequencing, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). DNA from skim milk powder was extracted and amplified with the same protocol as used for the tailings pond samples in order to determine the organisms associated with this reagent.

3.4.3 Analysis of pyrosequencing data

The pyrosequencing data were analyzed by the Phoenix 2 pipeline [97]. Sequences with low-quality reads and potential chimeras were removed. The sequences thus filtered were used to generate operational taxonomic units (OTUs) based on consensus sequences of clusters obtained with differences of 3% and 5 % between them (clustering distance cut-offs). The OTUs were assigned taxonomically based on the SILVA 108 data set Small Subunit rRNA Database Release 108 (SSU Ref NR 108; http://www.arb-silva.de/no_cache/download/archive/release_108/Exports), a highly curated database for phylogenetic analysis.

The resulting reads at the phylum and genus levels were analyzed by selecting the most abundant OTUs with a cut-off of 3 % (e.g. those taxa that were present at > 3% abundance in the whole community). For environmental samples (tailings from ponds 5 and 6), the 3% cut-off was applied to the percent of pyrosequencing OTUs for each depth and to the average percent of pyrosequencing OTUs (except for the TPW samples where the average percent of pyrosequencing OTUs was not considered). For the enrichments, the 3 % cut-off was used based on the % of pyrosequencing OTUs of each particular experiment.

The taxonomic analysis was carried out at the phylum level to detect the microbial distribution at a high level, considering that some of the DNA sequences were not always assigned to a particular genus. However, for all the samples, the genus level was also

assessed because more detailed information (e.g. presumed microbial function) can be obtained at this level, thus allowing a better interpretation of our results.

Biodiversity studies were also carried out by the evaluation of the species richness and evenness distribution within each sample analyzed. Two types of biodiversity were evaluated (alpha and beta). The alpha diversity refers to diversity within a particular sample, and the beta diversity measures the diversity of several samples within a habitat. Within these types of diversities, biodiversity indices were considered such as the Shannon index, which measures evenness of the species in the community [98]; and the Simpson index, which focuses on dominance of species [99]. For the Shannon index, the higher the value obtained, the greater the number of taxa (higher biodiversity) and the more evenly they are distributed. The lower the Simpson index (the closer to zero) indicate that less diversity is found in a community as there is a dominance of some taxa over others for a particular sample. At the same time, the Chao index was also evaluated as this indicated the estimated total number of OTUs obtained [100]. Rarefaction curves and Non-Metric Muldimensional Scaling (NMDS) were also plotted which allowed us to visualize the microbial community richness and the relationship between the samples, respectively. The rarefaction curve plots the number of OTUs found as a function of the number of samples (# of reads). Therefore it generates the expected number of OTUs in a small subset of samples (# of reads) drawn at random from a large pool of samples (reads or sequences) [101]. Thus, a rich community would have a curve that initially has a steep slope but levels off as fewer new taxa are found per additional number of sequences analyzed, indicating that a reasonably sufficient number of samples were collected from

the environment. On the other hand, if the curves level off very quickly (at low number of samples (# of reads)), fewer taxa are obtained, this is an indication of saturation as fewer taxa are present. [101]. The NMDS plot shows grouping of closely related samples together within a two-dimensional space, whereas less closely related ones will be shown sparsely (farther apart from one another) in the space. Relational trees were also generated using the Bray-Curtis dissimilarity calculation which describes the dissimilarity between the structures of two communities (http://www.mothur.org/wiki/Braycurtis).

An overview of all of the samples processed for microbial community analysis and activity and enrichment studies from ponds 5 and 6 is shown in Table 3-3.

3.5 Microbial laboratory enrichments

The Dutchman Martinus Beijerinck (1851 – 1931) first proposed the enrichment culture technique. He proved that when providing specific nutrients to a natural sample, the growth of microorganisms with certain physiological properties can be promoted in a very selective way [54]. For this aspect of the research, enrichments of aerobic and anaerobic microorganisms were accomplished with the aim of studying particular microbial behaviour activity with selected carbon substrates including bitumen, NAs, and naphtha.

Table 3-3 General overview of all tailings samples used for microbial community analysis by 454 pyrosequencing and for the various experiments described in this thesis.

Pond	***Year sampled***	***Experiment / microbial community analysis***		
Pond 5	2009	natural environment		- Sulfide oxidation tests - NA isolates
	2010	natural environment		Nitrate enrichments
Pond 6	2008	natural environment (DNA extracted with skim milk powder)	Quantitative PCR (DNA extracted with skim milk powder)	
	2010	natural environment	Quantitative PCR (DNA extracted with skim milk powder)	Anaerobic carbon source enrichments
	2011	natural environment	Quantitative PCR (DNA extracted with skim milk powder)	

3.5.1 Incubation Media

A variety of different media were used to establish aerobic and anaerobic enrichments [shown in Tables 3-4 to 3-10].

3.5.2 Aerobic enrichments on naphthenic acids

One litre of TPW from Suncor Energy Inc. pond 5 (sampled on September 1, 2009) was filtered using a 0.2 µm filter to collect cells. Then the filter was soaked in a 50 mL sterile saline solution (0.85 % NaCl in water (w/v)) to allow the bacteria to detach from the filter into the saline solution. The cell suspension was used as the microbial inoculum by growing it in a basal salt medium (Bushnell-Haas, MBH medium) [72] described in Table 3-4 supplemented with the selected model NAs.

One percent inoculum from the original saline solution with the filter was added to 50 mL of MBH containing 100 $mg \cdot L^{-1}$ of a model NA. Cyclohexanecarboxylic acid, cyclohexaneacetic acid, cyclohexanebutyric acid, cyclohexanepropionic acid, cyclohexanepentanoic acid (all from Aldrich®), were each prepared as a basic solution (0.1 N NaOH) at a concentration of 6.0 $g \cdot L^{-1}$ that was used as the substrate stock solution (1.0 mL added to 50 mL medium). The inoculated flasks were incubated at room temperature for 28 d and growth was monitored using a UV 1800 spectrophotometer (at OD of 600 nm, Shimadzu).

Several transfers of these initial incubations were done (1% of inoculum) before isolating single cells in the solidified MBH medium (same as above but with 1.5% agar)

supplemented with its corresponding NA. Morphologically distinct colonies were isolated as pure cultures and a subset of each was identified by their 16S rRNA gene sequence after PCR amplification. More specific details about the isolates are described in Section 5.2.3.

Inocula for NA time course biodegradation experiments were prepared in lysogenic broth (LB) [Table 3-5] to obtain a high density of cells. The cells were washed with MBH medium and then transferred to the MBH medium amended with the particular model NA.

3.5.3 Aerobic medium for sulfide oxidation assays

For the sulfide oxidation assays, CSB-A medium [Table 3-6] was prepared and autoclaved in a Widdel flask. While the medium was still hot, 1 mL of trace elements [Table 3-9], 1 mL of selenite tungstate, 30 mL of 1 M $NaHCO_3$, and 3 mL of 1 M Na_2S were added. The pH was adjusted to 7.0. The medium was dispensed (60 mL) in 120 mL serum bottles sealed with rubber stoppers. Tailings from each depth (3 mL, refer to section 5.2.1) were added to the medium in the anaerobic hood. The sulfide concentration was measured using the copper sulfide method [91] [section 3.2.2] and adjusted when needed to 3 mM.

3.5.4 Anaerobic enrichments

For anaerobic enrichments, Pfennig medium [102] was used [Table 3-7, Table 3-8, and Table 3-9].

To prepare this medium, all the ingredients were mixed except for the sodium bicarbonate ($NaHCO_3$), and the pH was adjusted to 7.1 to 7.3. The medium was boiled to eliminate any oxygen followed by a bubbling of N_2/CO_2. $NaHCO_3$ was added to the medium once cooled, then the medium was distributed in flushed N_2/CO_2 serum bottles. Appropriate amounts of a 2.5 % solution of cysteine sulfide, (0.1 mL per 100 mL for nitrate-reducers, 2 mL per 100 mL for sulfate-reducers and methanogens), was added to each sealed bottle to serve as a reductant. Cysteine sulfide was prepared by dissolving 5.0 g of cysteine-HCl and 5.0 g of sodium sulfide in NaOH (0.1 N) under anoxic conditions. Details on each particular experimental set up are described under the Methods section of each chapter.

Table 3-4 Modified Bushnell - Haas salt (MBH) medium composition.

Component	***g/L water***
KH_2PO_4	1.0
Na_2HPO_4	1.0
NH_4NO_3	0.5
$(NH_4)_2SO_4$	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.02
$FeCl_3$	0.002
$MnSO_4 \cdot 2H_2O$	0.002

Table 3-5 LB (lysogenic broth) medium

Component	***g/L water***
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0

Table 3-6 CSB-A medium composition.

Component	***g/L water***
NaCl	7.0
KH_2PO_4	0.2
$MgCl_2 \cdot 6H_2O$	0.4
KCl	0.5
$CaCl_2 \cdot 2H_2O$	0.15
NH_4Cl	0.25
Resazurin (0.1 %)	2 to 3 drops

Table 3-7 General anaerobic medium composition

Component	***Amount / 100 mL water***
Pfennig I	5 mL
Pfennig II	5 mL
Wolin metals	1 mL
Balch vitamins	1 mL
Resazurin	0.1 mL (of a 0.1% solution)
$NaHCO_3$	0.35 g
(+/- electron acceptor)	
(+/- substrate)	

Table 3-8 Pfennig solutions

Component	***g per Litre***
Pfennig I	
K_2HPO_4	10.0
Pfennig II	
K_2HPO_4	10.0
$MgCl2\ 6H_2O$	6.6
NaCl	8.0
NH4Cl	8.0
$CaCl_2\ 2H_2O$	1.0

Table 3-9 Wolin trace metal solution

Component	***g per Litre***
EDTA	0.5
$MgSO_4$	3.0
$MnSO_4.H_2O$	0.5
NaCl	1.0
$CaCl_2.2H_2O$	0.1
$ZnSO_4.7H_2O$	0.1
$FeSO_4.7H_2O$	0.1
$CuSO4.5H_2O$	0.01
$Na_2MoO_4.2H_2O$	0.01
H_3BO_3	0.01
Na_2SeO_4	0.005
$NiCl_2.6H_2O$	0.003

Table 3-10 Balch vitamin solution

Component	***mg per Litre***
biotin	2.0
folic acid	2.0
pyridoxine-HCl	10.0
thiamine-HCl	5.0
riboflavin	5.0
nicotinic acid	5.0
DL calcium pantothenate	5.0
vitamin B_{12}	0.1
PABA	5.0
lipoic acid	5.0
mercaptoethane- sulfonic acid (MESA)	5.0

Chapter Four: **Microbial physiology and communities in oil sands tailings ponds**

4.1 Introduction

To characterize the microbial community composition and physiology in tailings, two ponds from Suncor Energy Inc. were studied over the years 2008 to 2011. Pond 6, an active pond, and pond 5, a pond that was initially active but was closed after a few years of continuous tailings discharge, (shown in Figure 4-1) were studied. Both ponds are located in Suncor's Lease 86/17 area (north of Fort McMurray, AB).

Suncor Energy Inc. is committed to a program of closure and reclamation of ponds 5 and 6 which have been receiving products from the oil sands mining operations since the mid 1990s – early 2000s, respectively. Pond 6 is currently capped with water and MFT are being withdrawn for the TRO treatment whereas pond 5 is being dewatered and capped with coke. The latter is scheduled for completion in 2019 [52]. These reclamation strategies have the final goal of decreasing the consolidation time of the MFT layers, therefore accelerating the landscape recovery times.

Ponds 5 and 6 have undergone the CT treatment where gypsum is continuously added to enhance densification rates of the tailings and around 90% of the water is recycled back into the extraction plant. Under current bitumen extraction technology, Suncor Energy Inc. uses naphtha as the organic solvent that helps in the bitumen detachment from the sand particles therefore the froth tailings discharged into the ponds contain some naphtha components.

As overviewed in this chapter, a depth dependent profile throughout the anaerobic zone (starting at the mud line) of each pond was analyzed from a chemical and microbiological point of view to have a better insight into the geochemical cycles currently ongoing and the microbial contribution to each. Finally, the effect of pond closure in pond 5 is discussed as a positive outcome of Suncor Energy Inc. tailings management operations.

A portion of this work (overviewing findings from Pond 6 2008 samples) was previously published in Environmental Science and Technology [55].

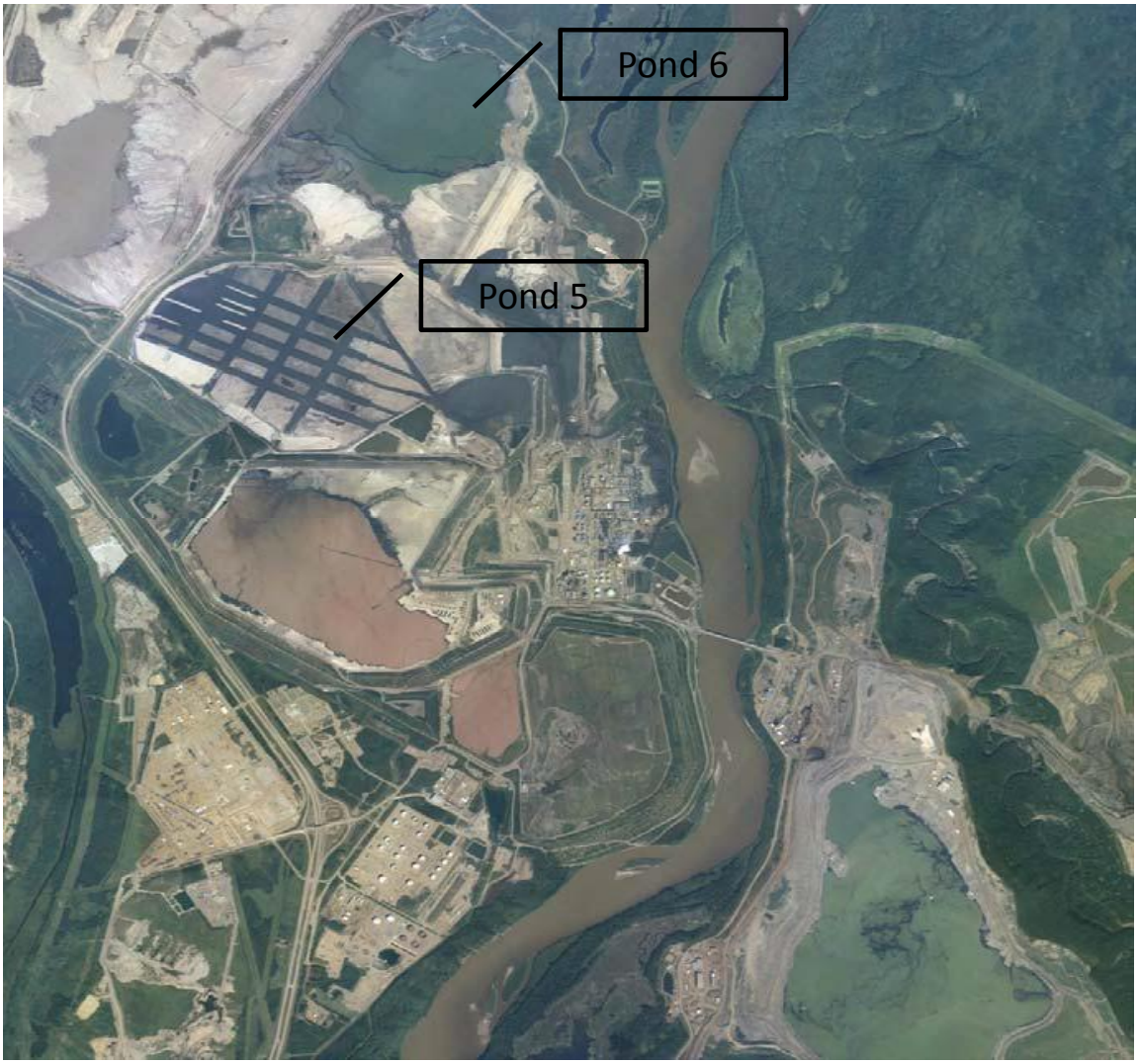


Figure 4-1 Aerial view of Suncor ponds 5 and 6. Image courtesy of Suncor Energy Inc.

4.2 Methods in brief

4.2.1 Pond 6 (active pond)

Our laboratory received samples from pond 6 over three years (2008, 2010, and 2011) from the coordinates listed in section 3.1, Table 3-2. The samples herein analyzed were taken from below the mud line, ranging from 3 to 18 mbs. Below 20 mbs, the MFT is highly consolidated, thus no samples could be taken. Samples were stored anaerobically or frozen and analyzed for a variety of chemical parameters, water content, microbial community composition, and microbial activities as described in sections 3.2, 3.3, and 3.4.

4.2.2 Pond 5 (inactive pond)

During the year 2009 and 2010, we evaluated the chemical parameters (sulfate, sulfide) together with anaerobic microbial activity (sulfate reduction and methanogenesis rates) to assess the effectiveness of the closure of pond 5. Samples were taken immediately after closure (2009) and a year after (2010). Sample coordinates are listed in section 3.1, Table 3-1. Samples were analyzed for a variety of chemical parameters, water content, microbial community composition and microbial activities as described in sections 3.2, 3.3, and 3.4.

4.3 Pond 6, an active tailings pond

Samples from tailings pond 6 from Suncor Energy Inc. were used for the microbial molecular and physiology studies of an active pond. The pond started filling in 2002 and in 2010 had already reached maximum capacity. It has an approximate depth of 41 m below the mud line, and it is capped with about 1 m of water. The pond is currently considered a somewhat active pond in that no fresh tailings are currently being incorporated into the pond but MFT is continuously drawn from pond 6 for MFT drying (Suncor's Tailings Reduction Operation). In the year 2011 some MFT from pond 2/3 were transferred to pond 6 (a one-time activity), which is an active pond that receives fresh froth tailings therefore it is rich in naphtha [Figure 4-2].

The pH of pond 6 ranges from 7.0 to 7.8 and the average temperature is about 20 °C year round. Only the surface water freezes during the winter months but underneath this layer the temperature remains stable at around 15 to 18 °C [37].

Pond 6 generally has no visible gas emissions except during the winter months, when occasional gas accumulated under the iced water cap can escape to the atmosphere when punctured (Suncor Energy Inc. personal communication).

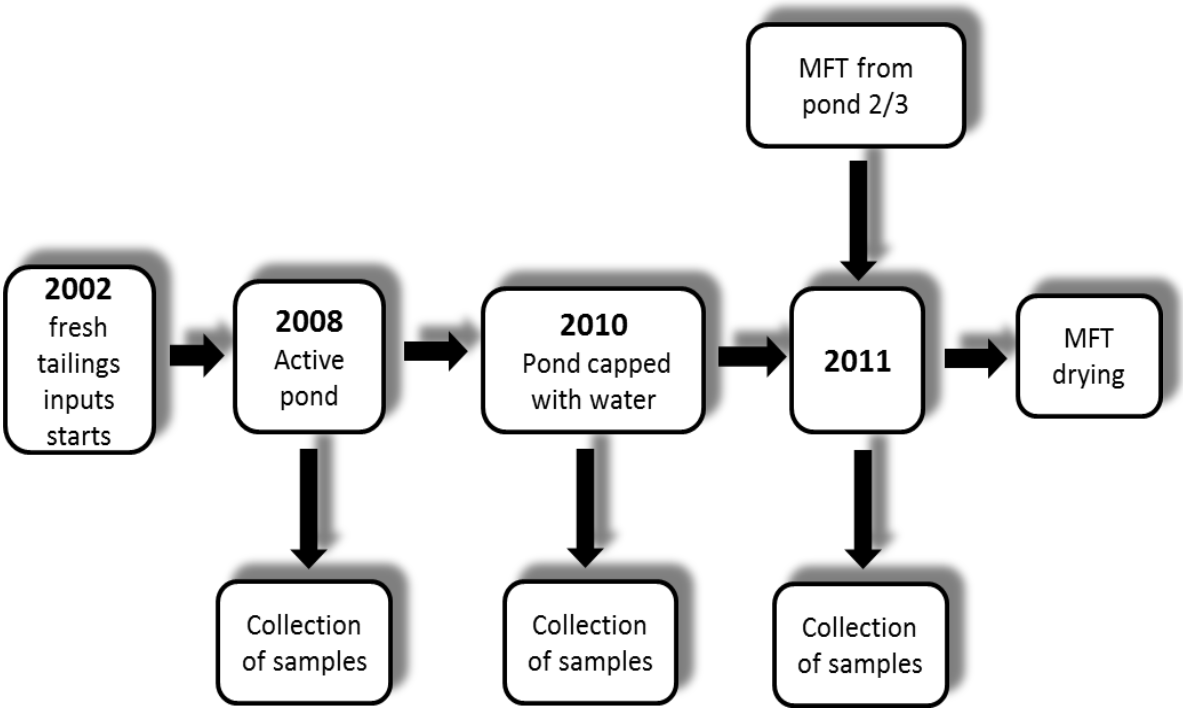


Figure 4-2 Flow diagram of Pond 6 management activities during the years 2008 to 2011. Based on personal communication with Suncor Energy Inc.

4.3.1 Results

4.3.1.1 Microbial community composition in pond 6

Phylum level analysis

The microbial community in pond 6 at the phylum level did not vary much from 2008 to 2011 [Figure 4-3 (A)]. The most abundant phylum present was Euryarchaeota. This group, which mainly included methanogens [54], was found in 2008 and 2011 to comprise approximately 40% average of the pyrosequencing OTUs but in 2010 this phylum almost doubled in abundance [Figure 4-3(A)]. The next most abundant phylum was Proteobacteria. This group is considered the largest and most metabolically diverse of all Bacteria [54]. It comprises a wide diversity of chemolithotrophic, chemoorganotrophic, and phototrophic species [54]. The proportions of pyrosequencing OTUs for Proteobacteria are very similar during the years 2008 and 2011, around 40% each, with relatively lower in abundance for the 2010 samples. Members of other phyla like Chloroflexi and Firmicutes were also detected in all the years sampled, but in fractions no higher than 10% of the average of pyrosequencing OTUs [Figure 4-3(A)].

Genus level analysis

At the genus level, pond 6 exhibited a diverse but constant microbial population during the three-year period studied [Figure 4-3(B)]. However, samples from 2008 showed a higher diversity of pyrosequencing OTUs (> 3% abundance) when compared with samples from 2010 and 2011 [Figure 4-4 (A)]. The extracted DNA from these tailings were dominated by methanogens, primarily by members of the acetotrophic

Methanosaeta genus. In particular, *Methanosaeta* is by far the most abundant methanogen genus in all the samples collected from pond 6 [Figure 4-3(B), Figure 4-4 (A, B, C)]. It was found disseminated throughout the pond but occasionally peaking in abundance towards the shallower depths [Figure 4-4 and Figure 4-5 (A)]. In addition, many other genera were present at > 3% of the total community abundance. These included other methanogens, *Methanolinea* and *Methanosarcina*, *Syntrophus* (a known syntroph), the S and Fe cycling bacterium *Thiobacillus*, the SRB *Desulfocapsa*, and several putative hydrocarbon-degrading bacteria (*Pseudomonas*, *Acinetobacter*, *Acidovorax*).

In the year 2010, *Methanosaeta* sp. were the most abundant at the genus level, reaching values ranging from 20 to 40% with no particular distribution pattern. For this same year a substantial reduction of *Syntrophus* sp. (decreased abundance from 3% to 0) and a 5-fold increase in *Methanosarcina* abundance was observed [Figure 4-3 (B)]. The second most abundant methanogen was *Methanolinea* [Figure 4-3 (B, C)]. This genus was found in all the tailings depths but in higher proportions in the upper mud line region (above 6 mbs) [Figure 4-5 (A)]. Other methanogens were also detected but in relatively low abundance, with the exception of *Methanosarcina* that was more abundant in samples collected in 2010 [Figure 4-5 (A)]. *Syntrophus* comprised the third most abundant genus for pond 6 [Figure 4-3 (C)]. The most prominent sequences at the genus level that are involved in sulfate reduction metabolism are affiliated with *Desulfocapsa*. For pond 6, SRB were always found in lower abundance than methanogens (less than 10 % of pyrosequencing OTUs), especially when the sulfate became limited. *Desulfocapsa*

sp. were found in all depths but mainly at the top, around 3 mbs, middle sections (12 mbs) or at the bottom, below 15 mbs [Figure 4-5 (B)]. Other genera like *Acinetobacter* and *Pseudomonas* also inhabited the pond but to a lesser extent. *Acinetobacter* was mainly present in 2010 and 2011, but *Pseudomonas* were found in all three years sampled [Figure 4-3 and Figure 4-4]. Other phyla refers to an average of 25 phyla whereas other genera refers to approximately 150 genera [Figure 4-3and Figure 4-4]For more information regarding the most abundant total microbial community members detected in all years sampled, refer to Appendix One:.

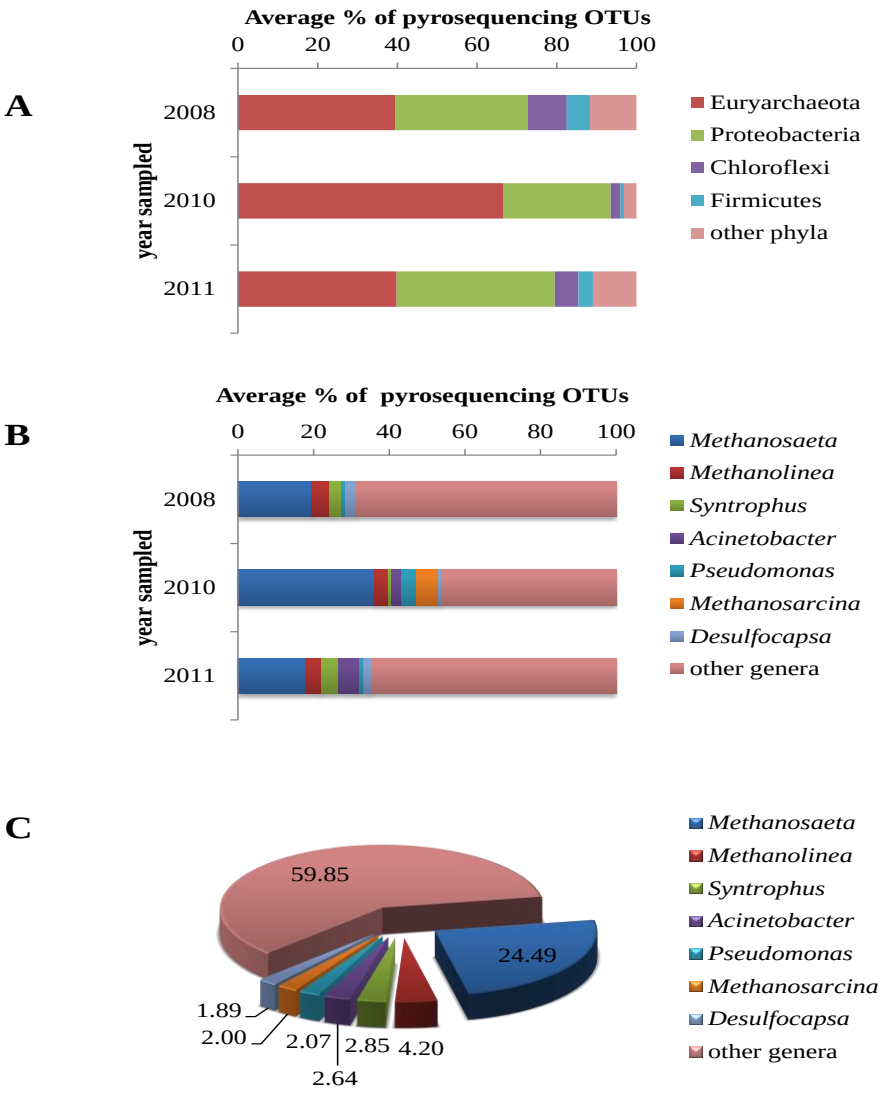


Figure 4-3 Most common microbial members found in Suncor pond 6 tailings at (A) phylum level, (B) genus level, and (C) total average of most common genera in the three years sampled. The selection was based on the average of the most common taxa for the three years sampled. Other genera and/or phyla refer to average % of pyrosequencing OTUs present at lower than 3% abundance in the total community.

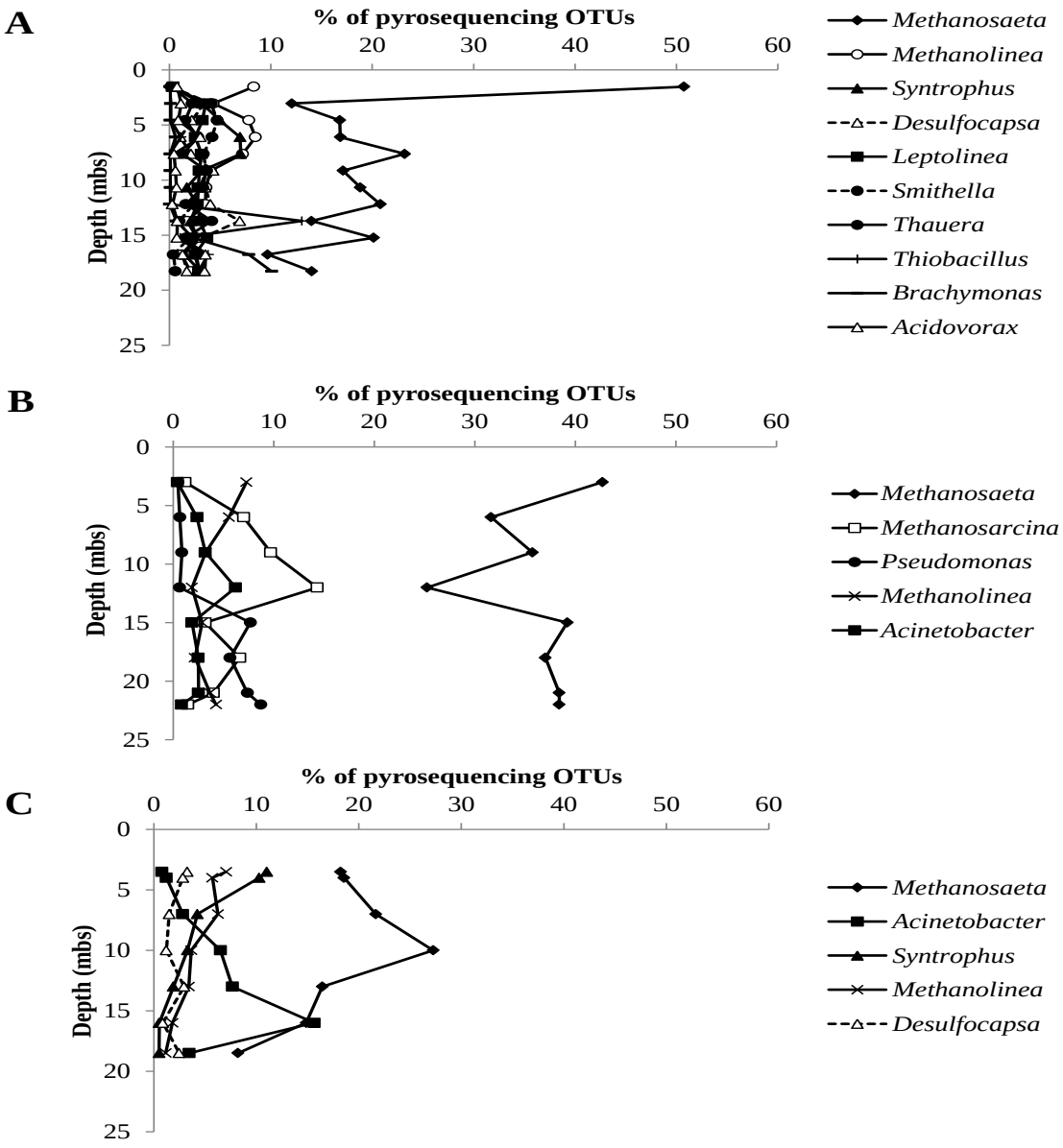


Figure 4-4 Depth dependent profile of most abundant percentage of pyrosequencing OTUs in pond 6 at the genus level (A) in 2008, (B) in 2010, and (C) in 2011. Notice that pond 6 samples collected in 2008 have the highest abundance of genera comprising > 3 % of the community.

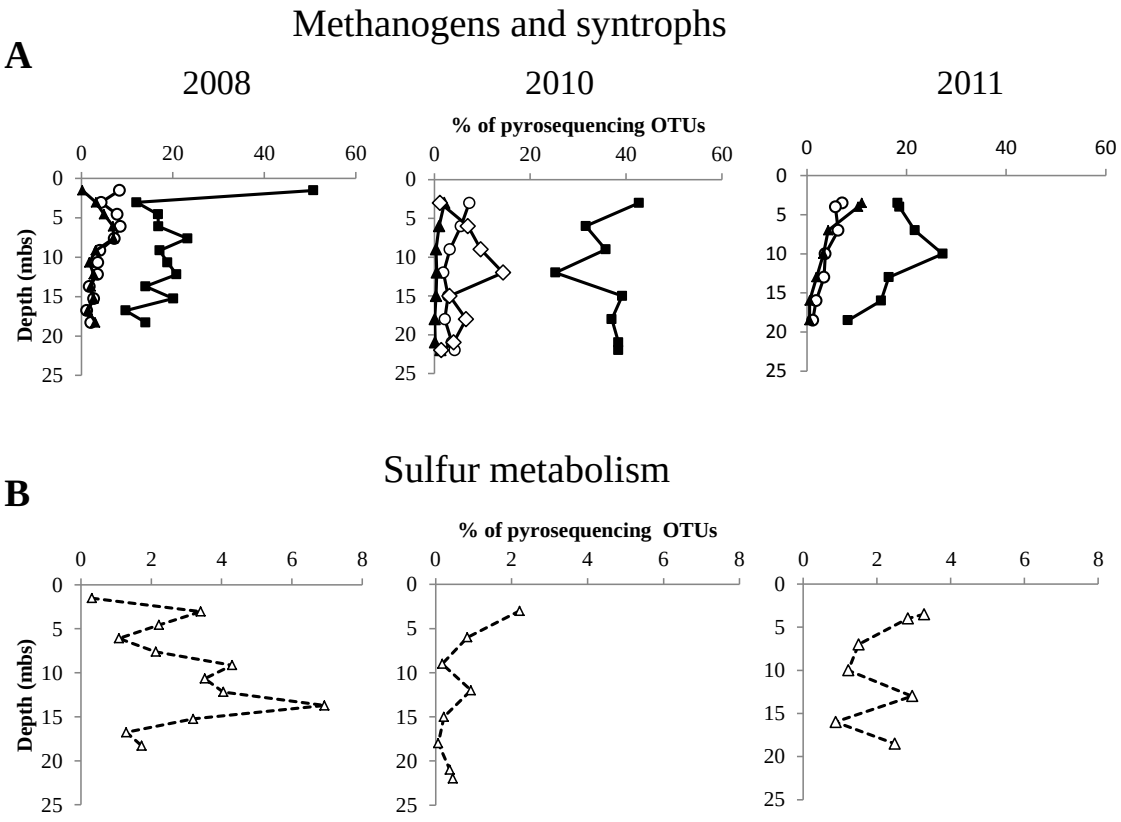


Figure 4-5 Distribution of selected microbial community composition at the genus level (as % of pyrosequencing OTUs) found in Suncor pond 6 sampled in 2008, 2010, and 2011 as a function of depth. (A) Methanogens and syntrophs: *Methanosaeta* (■), *Methanolinea* (○), *Syntrophus* (▲), *Methanosarcina* (◊); (B) sulfur metabolism, mainly *Desulfocapsa* (∆) as dominant SRB.

4.3.1.2 Relationship between pond 6 samples

From a microbial community point of view, pond 6 showed a relatively constant diversity throughout the years. The Shannon and Simpson indices for this pond suggest there is a somewhat diverse community (relatively high Shannon indices) with some dominance of some taxa over the other (Simpson's values closer to zero) [Appendix Five: Table 0-6], mainly dominated by *Methanosaeta* sp. [Figure 4-3 (B)]. The numbers of samples studied as a whole seem to have been enough to taxonomically characterize pond 6, as the rarefraction curves slightly turned into the saturation limit towards the end of the curve, we could assume reasonable coverage of the major communities [Appendix Six: Figure 0-1]. All samples taken in 2010 clustered together in the phylogenetic tree and that cluster was separate from the 2011 samples that also clustered together [Figure 4-6], which confirms the non-relateness between these two sampling periods. However, samples from 2008 seem to be more similar to 2011 as some of the depths from 2008 samples (from 3 to 9 mbs) are clustered with the 2011 samples [Figure 4-6]. The rest of 2008 samples are either not directly related to any of the sampling years (2010, 2011) or closely related to pond 6 2010 (2 mbs). NMDS analysis [Figure 4-7] shows that the microbial communities in samples collected at different times generally clustered together.

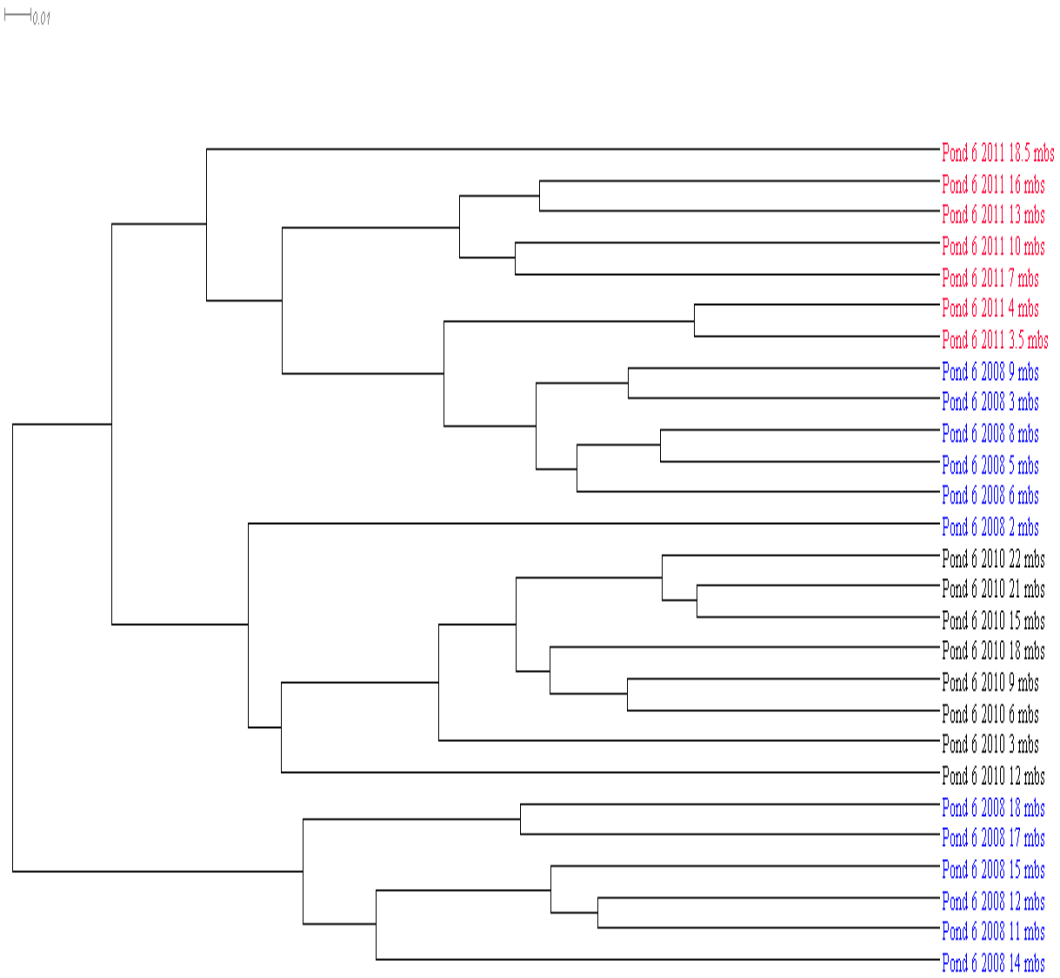


Figure 4-6 Relational tree clustering for pond 6 samples (2008, 2010, 2011). Bray-Curtis dissimilarity was used to calculate the distance.

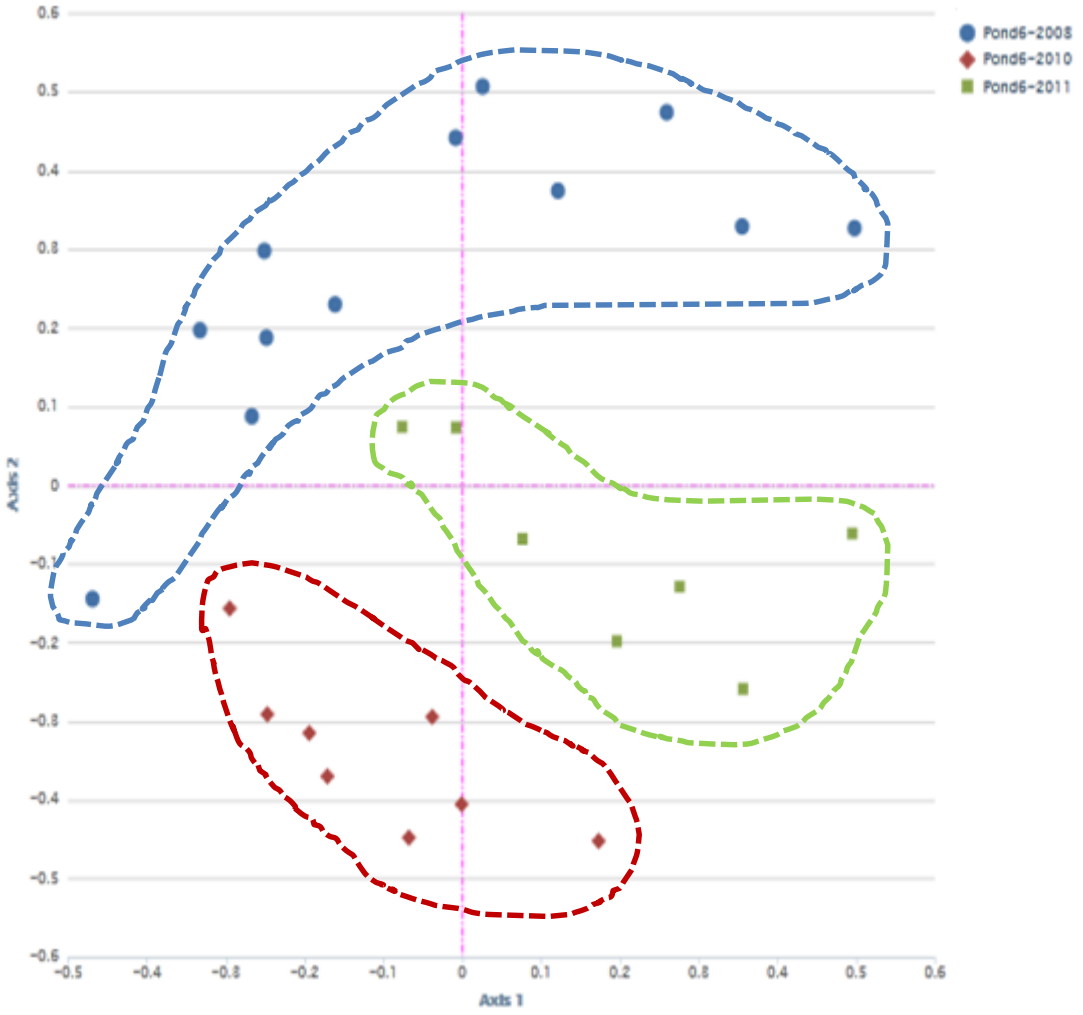


Figure 4-7 NMDS diagram showing all oil sands tailings samples collected from pond 6 in 2008 (●), 2010 (♦), and 2011 (■).

4.3.1.3 Microbial activity and chemistry of the pond

Over time, Suncor pond 6 showed a decrease in sulfate and sulfide concentrations [Figure 4-8(A, B)]. The sulfate concentration at the surface was highest above the mud line (shallower than 3 mbs) of the pond, especially in the 2008 samples where a maximum of 6 mM was measured. However, in 2011, sulfate levels decreased to around 1 mM in the surface water layer. Below the mud line, the highest sulfate values were always observed at deeper depths, below 14 mbs [Figure 4-8(A)]. The same was true for SRR where the maximum activities were measured either near the top of the anaerobic zone (near the mud line) or deep below the mud line. A marked average SRR decrease from 10.8 $nmol \cdot cm^{-3} \cdot d^{-1}$ in 2008 to nearly zero in 2010- 2011 was observed [Figure 4-8(D), Table 4-1]. Average sulfide concentrations also decreased from 2008 (~ 3 mM) to 2010-2011 (~ 0.4 mM). While the pond remained active (2008), the sulfide concentrations were relatively high throughout the depths. However, as the pond became less active (2010-2011), the highest concentrations of sulfide were found to be more towards the shallower depths of the pond, within the first 10 meters below the surface [Figure 4-8(B)].

Methane production had an average rate above 30 $nmol \cdot cm^{-3} \cdot d^{-1}$ for the years 2008 and 2011, however a four fold decrease was measured for the 2010 samples [Figure 4-8(C), Table 4-1]. For all the years sampled the highest methanogenesis rates were found fluctuating at depths shallower than 15 mbs [Figure 4-8 (C)]. The acetate concentrations also fluctuated but peaked at discrete depths above 15 mbs [Figure 4-8 (E)], particularly in 2010, where the highest acetate concentrations were detected at 3

mbs (~ 7.5 mM), and at 15 mbs (~ 6 mM) [Figure 4-8(E)]. The average acetate concentration found ranged between 0.065 to 1.8 mM over the sampled years. Overall, pond 6 had the highest activity between the mud line and 15 mbs which matches with the highest water content in the pond [Figure 4-8 (F)].

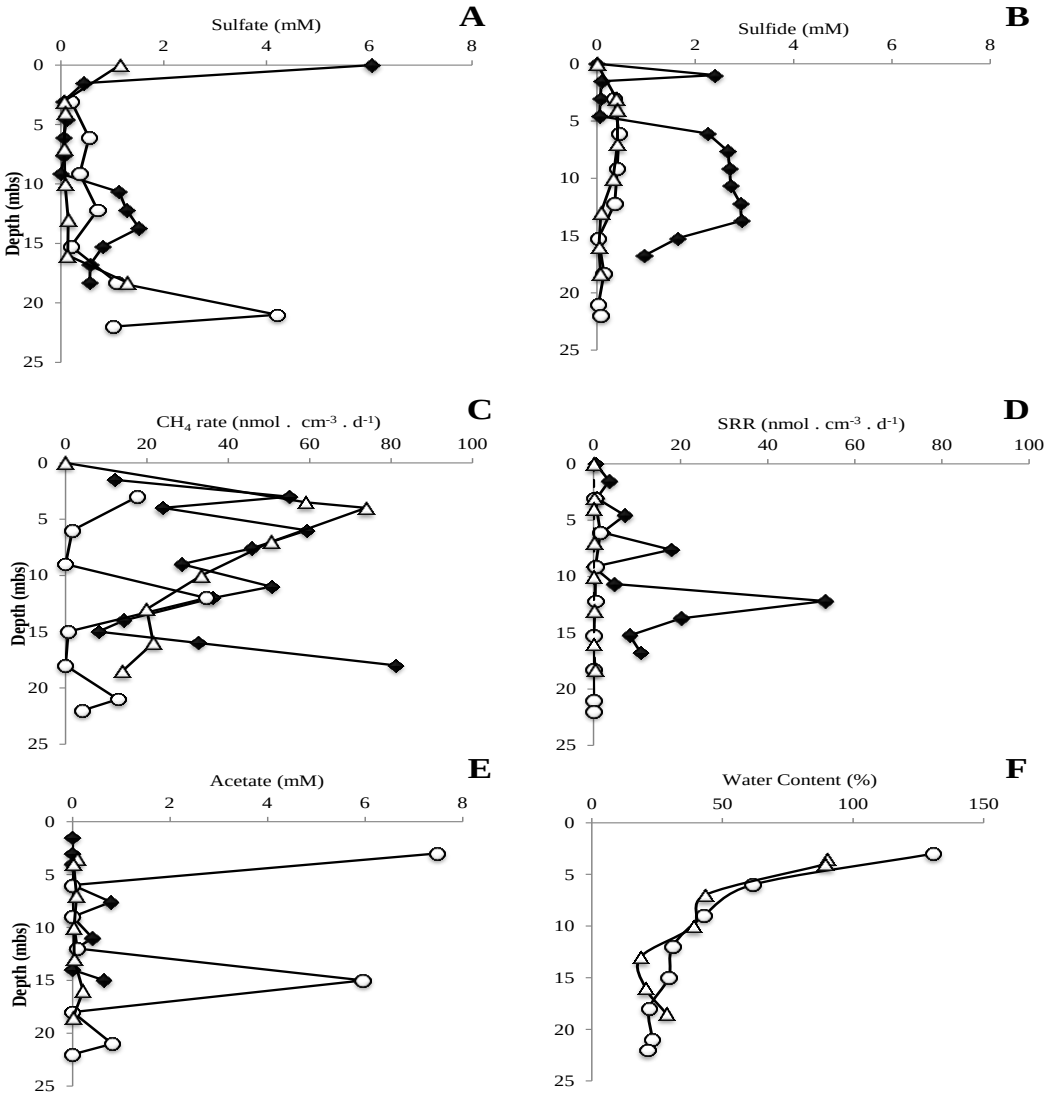


Figure 4-8 Chemistry and microbial activity in pond 6 determined during the years 2008 (♦), 2010 (○) and 2011 (∆). Depth dependent profiles of: (A) sulfate concentrations; (B) sulfide concentrations; (C) methanogenesis rate; (D) sulfate reduction rate; (E) acetate concentrations; and (F) water content (no water content measurement was performed for 2008 samples).

Table 4-1 Average SRR, methanogenesis rates, and putative methane evolution prevented in various samples collected over time in pond 6.

Sampling year	*Average SRR* *(nmol·cm^{-3}·day^{-1})*	*Average CH_4 production* *(nmol·cm^{-3}·day^{-1})*	*CH_4 prevented (%)*
2008	10.8	37.3	22.4
2010	0.4	9.0	3.9
2011	0.1	34.0	0.2

4.4 Effect of pond closure: Pond 5, an inactive pond.

Suncor's pond 5 is the first consolidated tailings pond being reclaimed in Alberta's oil sands region. It was decommissioned in December 2009 and it is being reclaimed in a three- phase period [Figure 4-9]. The first phase (2009 – 2012) involves the capping process, which is currently in its final season of construction. The second phase (2012 – 2019) includes drilling of wick drains into the pond through the coke cover to facilitate the in-situ dewatering operations. The third phase, which is planned for 2019, is the final land form and establishment of regional flora and fauna. An aerial view of how the pond looks in 2012 is shown in Figure 4-10.

Every year, this pond is monitored as a function of depth to determine the change in the physicochemical parameters over time and to evaluate the effectiveness of pond reclamation. Starting in 2009, we have received subsamples from different depths for microbial-based studies. This section describes the microbial community analysis and physiological studies carried out on samples recovered just after closing and approximately one year later.

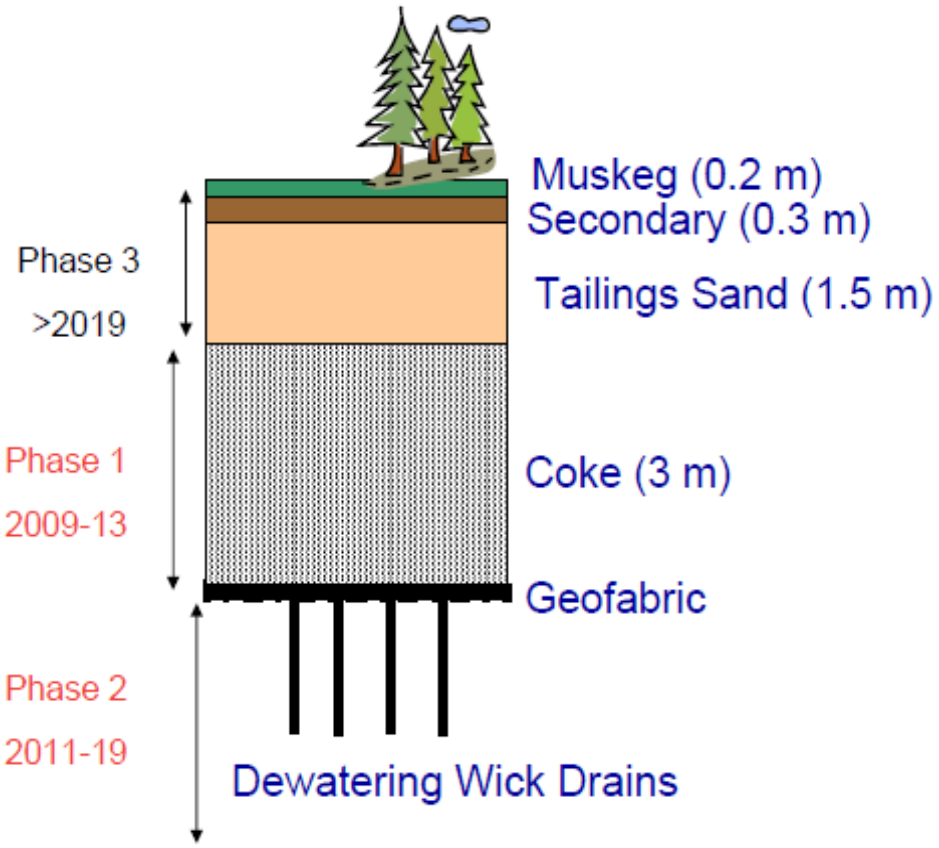


Figure 4-9 Concept of Pond 5 reclamation strategy. (Courtesy of Suncor Energy Inc.).

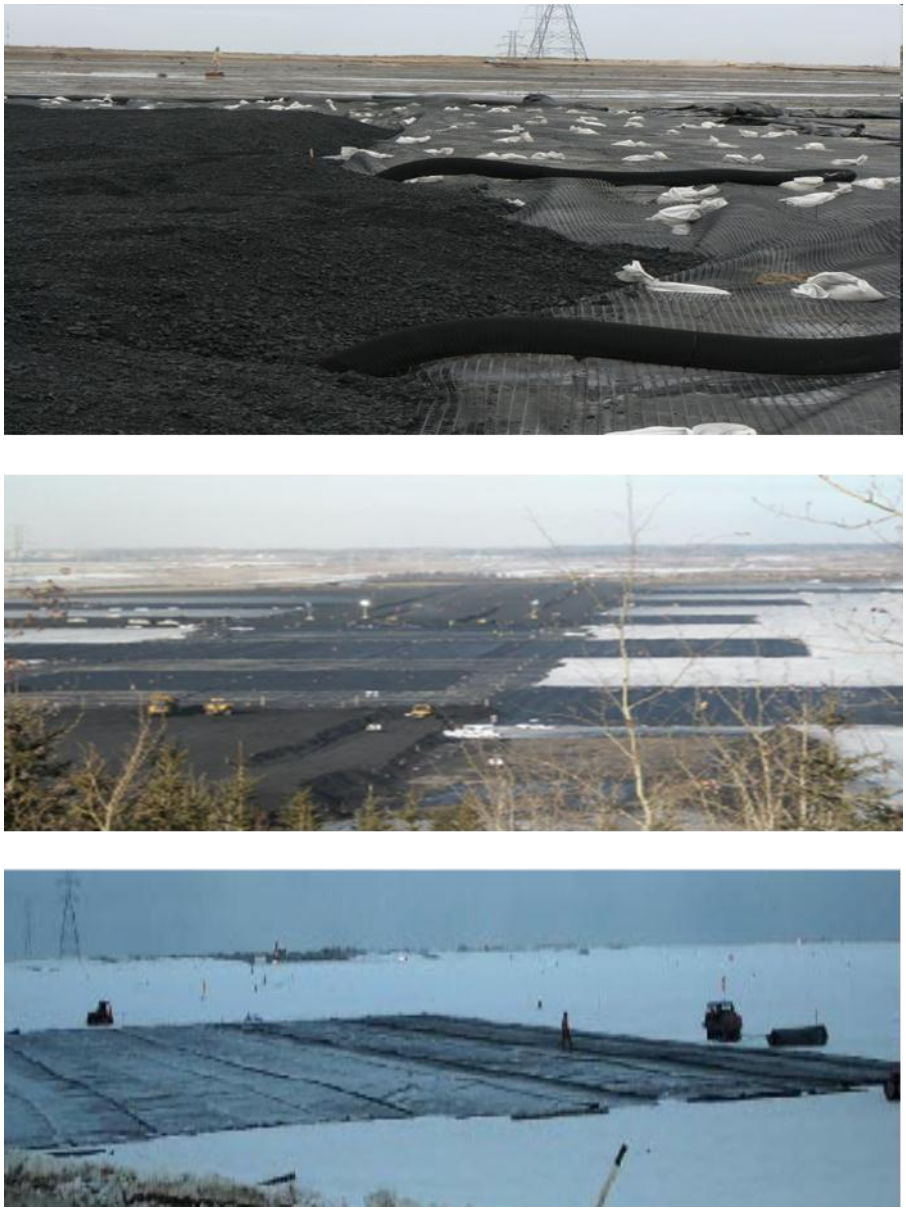


Figure 4-10 Views of pond 5 with geofabric and coke covering. (Image courtesy of Suncor Energy Inc.)

4.4.1 Results

4.4.1.1 Microbial community composition before and after pond 5 closure

Phylum level analysis

The average percentages of pyrosequencing OTUs at the phylum level are shown in [Figure 4-11(A)]. During the years 2009 and 2010, a high proportion of the average % OTUs belonged to Proteobacteria, comprising approximately 70% of the community OTUs. Second in abundance were Euryarchaeota. In 2009, members of this phylum reached an abundance of 26% while in 2010, a 12% abundance was observed. Other phyla like Chloroflexi and Firmicutes sequences were also present but at a lower percentage of pyrosequencing OTUs. Significantly, Firmicutes were more abundant in the samples taken in 2010, comprising 4.5% of OTUs [Figure 4-11(A)]. A year after pond closure (2010 samples) a slight increase in the abundance of sequences affiliating with the Proteobacteria was observed (up to 77%) while a decrease of the ones grouped under Euryarchaeota (to 12%) was observed. Other phyla refers to an average of 17 phyla [Figure 4-11(A)].

Genus level analysis

At the genus level, a marked difference in the relative abundance of organisms between the two sampling times was evident, shown in Figure 4-11(B). At the genus level, samples from 2009 were more dominated by the methanogen *Methanosaeta*, an acetate-using methanogen, whereas in samples from 2010, *Pseudomonas* sp., appeared to dominate the community identified [Figure 4-11(B)]. Other genera referes to approximately 99 genera in 2009 and 200 genera in 2010 [Figure 4-11(B)].

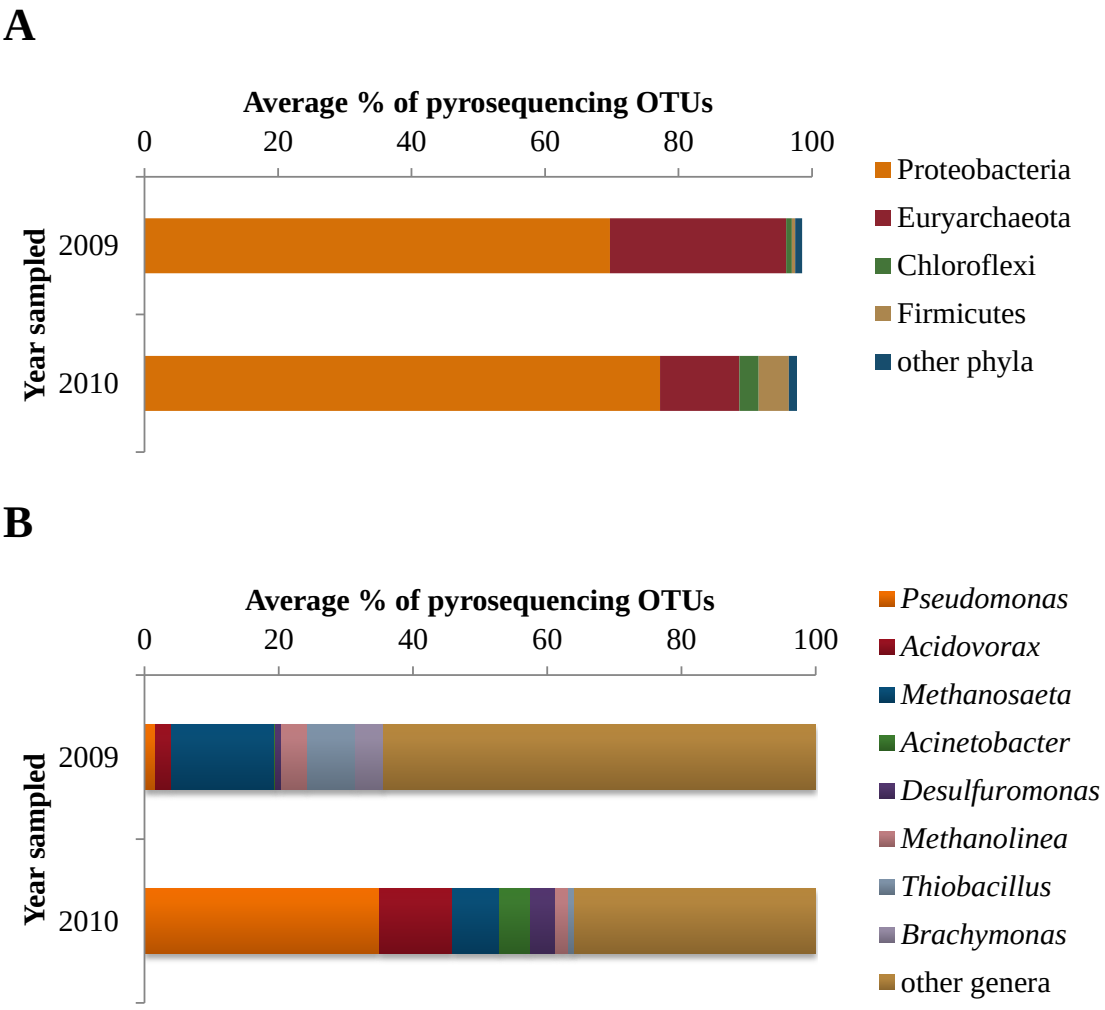


Figure 4-11 Average of pyrosequencing OTUs in pond 5 in 2009 and 2010 . (A) Phylum level, (B) Genus level. Other genera and/or phyla refer to average % of pyrosequencing OTUs lower than 3% of the total abundance of OTUs.

In 2009, *Methanosaeta* was found throughout the depths but was more abundant towards the shallower areas. For example, at 6 and 7 mbs, approximately 28% of pyrosequencing OTUs aligned with *Methanosaeta*. However, deeper into the pond, the abundance decreased to below 12 % [Figure 4-12(A)]. Although *Methanosaeta* was also present after the pond was closed, its abundance decreased reaching its maximum percentage at 3 and 18 mbs with 17 and 28 % of pyrosequencing OTUs, respectively [Figure 4-12(B)]. Another group affiliating within the Archaea that was present in relatively high abundance (average 3.9%) before the closure of the pond in the shallower depths was *Methanolinea*, a H_2/CO_2-using methanogen [Figure 4-11(B)]. However, in 2010, this genus decreased in abundance to less than 3% [Figure 4-11(B)]. Similarly, other genera like *Thiobacillus, Brachymonas,* and *Thauera* were relatively abundant before pond closure [Figure 4-11(A)] but scarcely found in 2010 with the exception of *Thauera* [Figure 4-11(B)]. *Acidovorax* and *Comamonas* were present in both years but at a higher abundance after pond closure [Figure 4-11(B)]. Specifically, at 51 mbs, *Acidovorax* reached its highest abundance, comprising 48% of the pyrosequencing reads [Figure 4-12(B)]. *Rhodoferax* was found in pond 5 in samples from 2009 at % pyrosequencing OTUs higher than 3%, but was only detected in samples from 2010 at a less than 3% abundance.

In summary, members of the genus *Pseudomonas* increased in abundance from 1.6 % in 2009 to 35 % in 2010, and *Acidovorax* increased 5 times in 2010 compared with the average % found in 2009. On the other hand, the abundance of *Methanosaeta* decreased from 15.4 % to 7 % [Figure 4-11(B)]. For more information regarding the microbial community composition detected in pond 5 please refer to Appendix Three:.

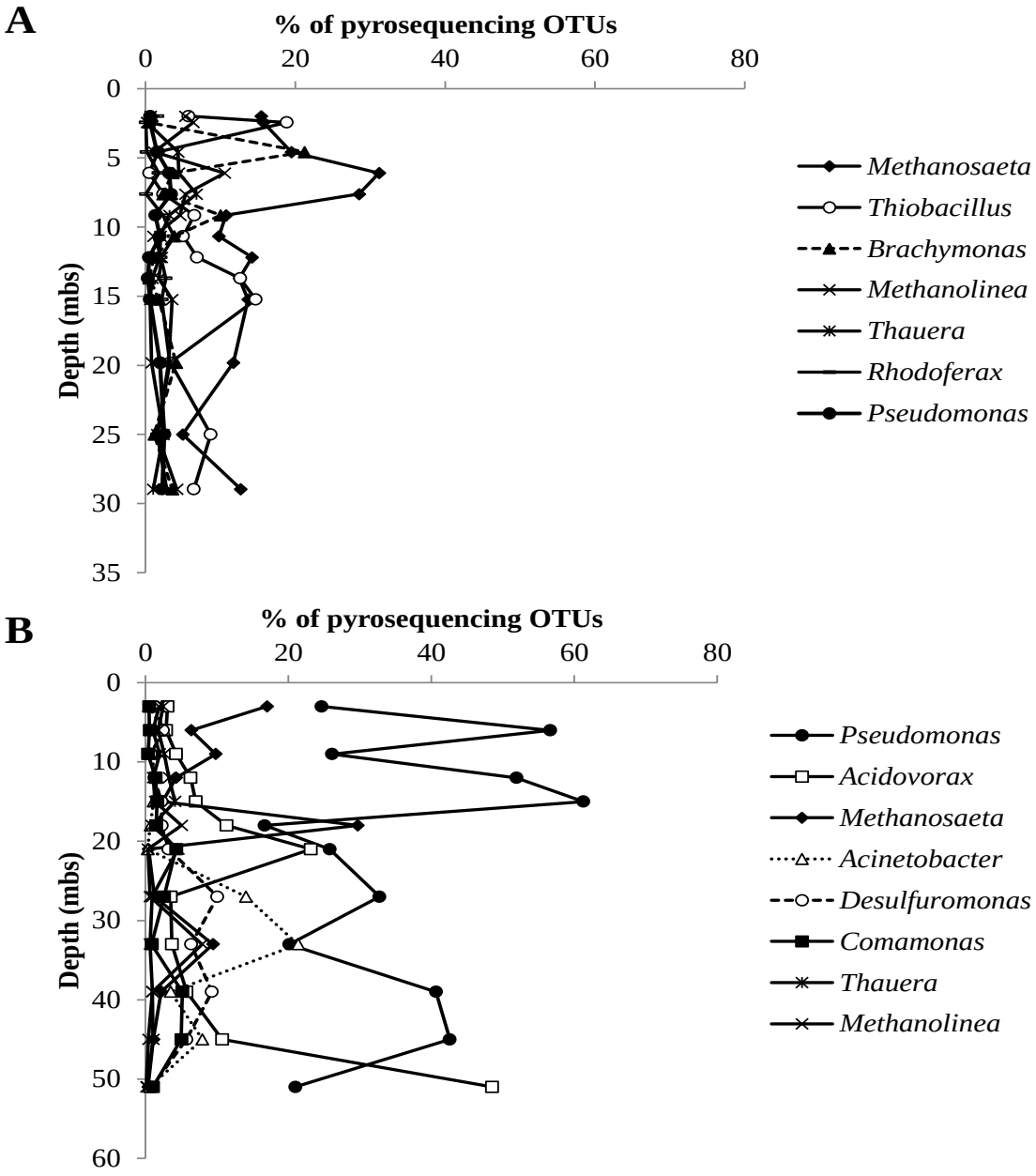


Figure 4-12 Depth dependent profile of most abundant OTUs (> 3% of the total community OTUs) in pond 5 at the genus level (A) in 2009 before pond closure, (B) in 2010 after pond closure.

4.4.1.2 Microbial relationship between the two sampling periods (before and after pond closure)

Both sampling times showed a somewhat diverse community composition, although samples from 2009 were richer according to higher values of Shannon indices [Appendix Seven: Table 0-7]. However, the low Simpson indices (closer to zero) inferred dominance of some taxa over others, which most likely refers to *Methanosaeta* in 2009 and *Pseudomonas* in 2010. The differences in their microbial composition due to a shift from a community dominated by methanogens to one dominated by putative hydrocarbon-degraders, make both sampling times very different. This was confirmed by the phylogenetic tree construction and NMDS plots where the communities from each sampling year grouped together in two separate clusters [Figure 4-13 and Figure 4-14]. The results are confident as the rarefaction curves showed curve lines reaching a plateau towards the end of the curve confirming that sufficient samples were taken from each period [Appendix Eight: Figure 0-2].

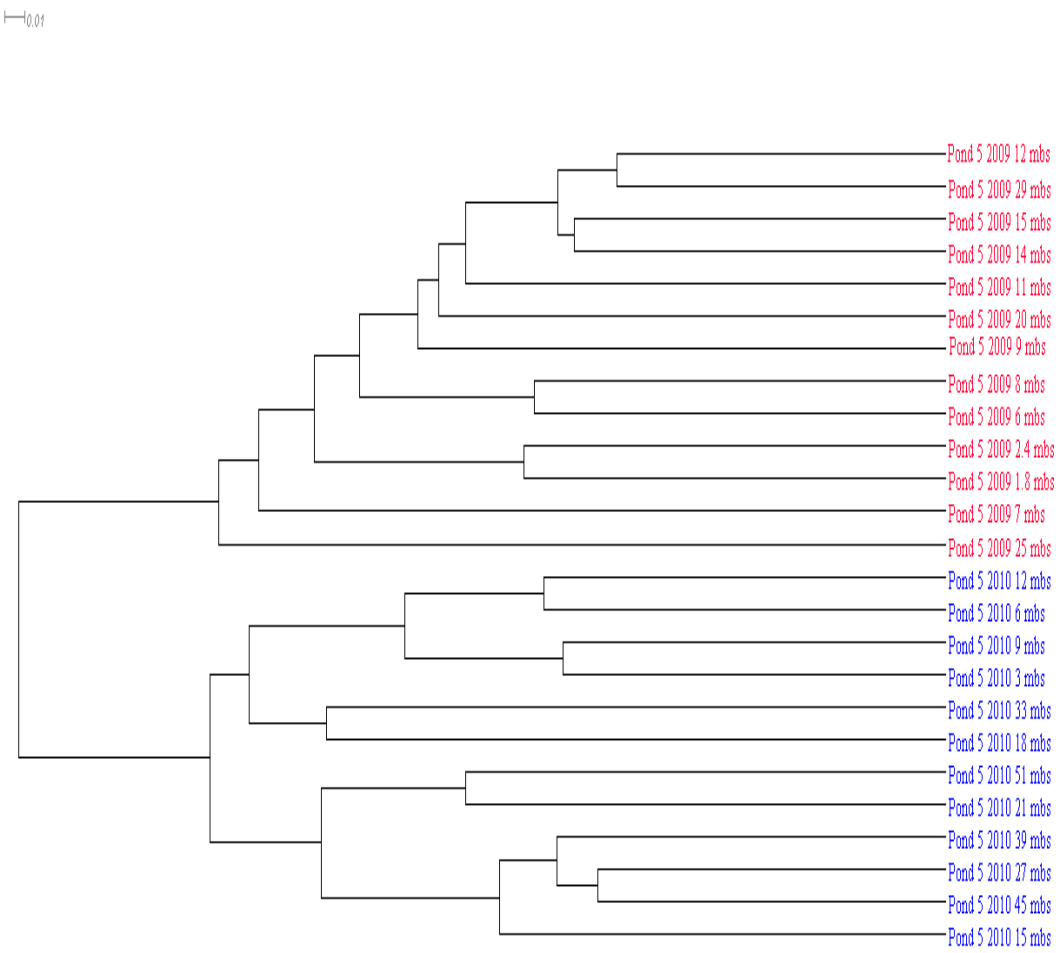


Figure 4-13 Relational tree clustering for pond 5 samples (2009, 2010). Bray-Curtis dissimilarity was used to calculate the distance.

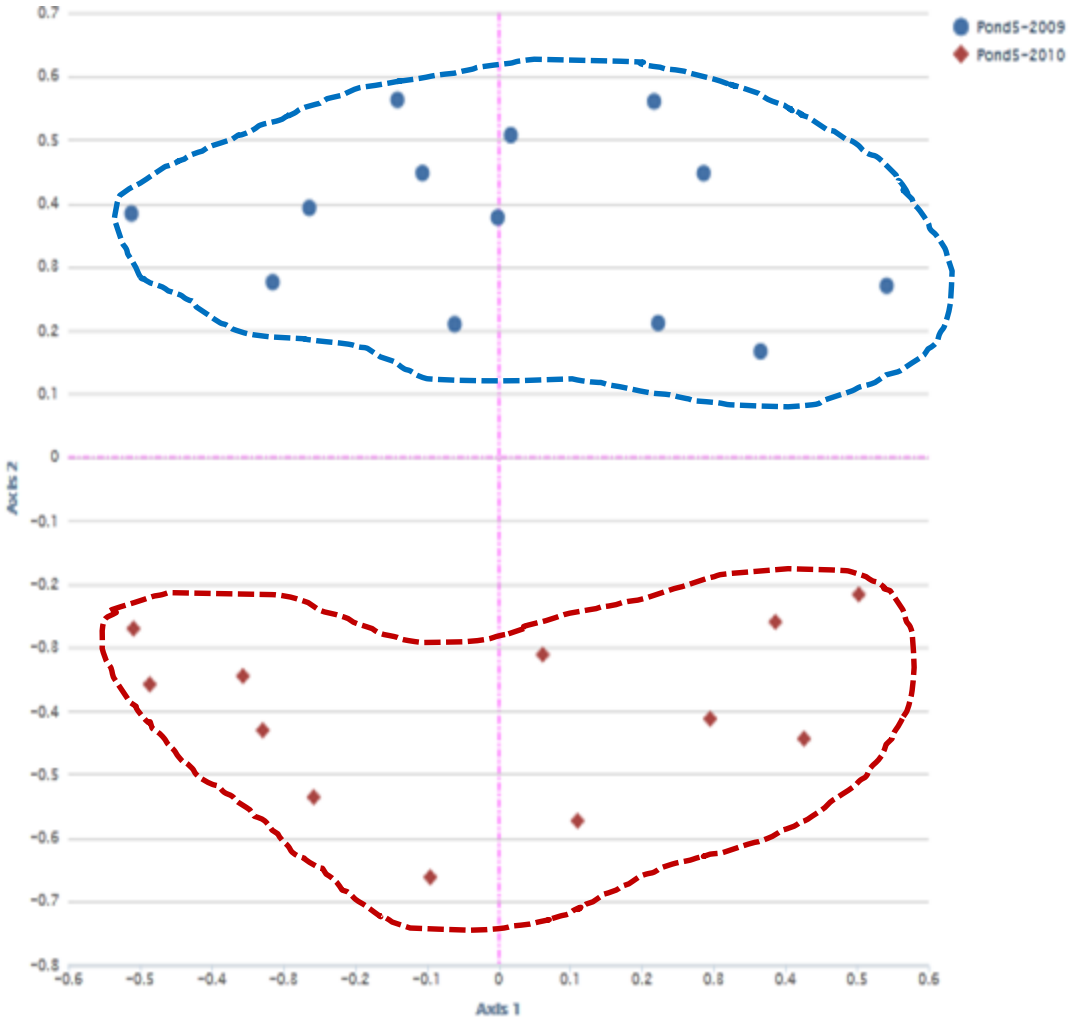


Figure 4-14 NMDS plot of the two sampling times in pond 5 to illustrate their statistical compositional difference. (●) pond 5 2009, (♦) pond 5 2010.

4.4.1.3 Sulfate-reducing and methanogenic activity in pond 5 before and after closure

Sulfate concentrations measured in tailings samples from pond 5 (2009 – 2010) were fairly low (0 - 0.5 mM) for the majority of the depths sampled, except near the surface of the pond (3 mbs). At this depth, samples collected in 2010 showed an increase in sulfate concentration compared with 2009. A maximum of 4.2 mM was observed in 2010 compared to 1.6 mM of sulfate initially detected in 2009 [Figure 4-15(A)]. Since sampling in 2010 was carried out deeper in the pond, high values of sulfate were also measured at increasing depths, averaging 1.5 mM. However, at these depths, no comparison can be made as no samples were received below 29 mbs in 2009 [Figure 4-15 (A)].

Sulfide concentrations in the pond were very low (almost zero) at both sampling times. But, similar to sulfate, the highest values of sulfide were found close to the surface of the pond (3 mbs). However, in contrast to sulfate, the sulfide values at 3 mbs decreased from 1.1 mM in 2009 to 0.07 in 2010 [Figure 4-15(B)].

The sulfate and sulfide profiles correlated with the microbial activity, as determined by methanogenesis rates and SRR in tailings shown in Figure 4-15 (C, D). The average methanogenesis rate decreased from 5.6 to 0.07 nmol $CH_4 \cdot cm^{-3}$ tailings$\cdot d^{-1}$ and SRR decreased from 16.6 nmol$\cdot cm^{-3}$ tailings$\cdot d^{-1}$ in 2009 to 3.5 nmol$\cdot cm^{-3}$ tailings$\cdot d^{-1}$ in 2010.

The microbial activity measurements also showed that the highest activities in the pond were found towards the surface (in the upper 10 mbs), corresponding to higher water content values [Figure 4-15(E)].

Although methanogenesis activity was not prevalent at deeper depths, there appeared to be a deep pocket of sulfate-reducing activity between approximately 50 – 60 mbs as measured in the 2010 samples [Figure 4-15 (D)], which correlated with the higher sulfate concentrations measured at this depth [Figure 4-15 (A)].

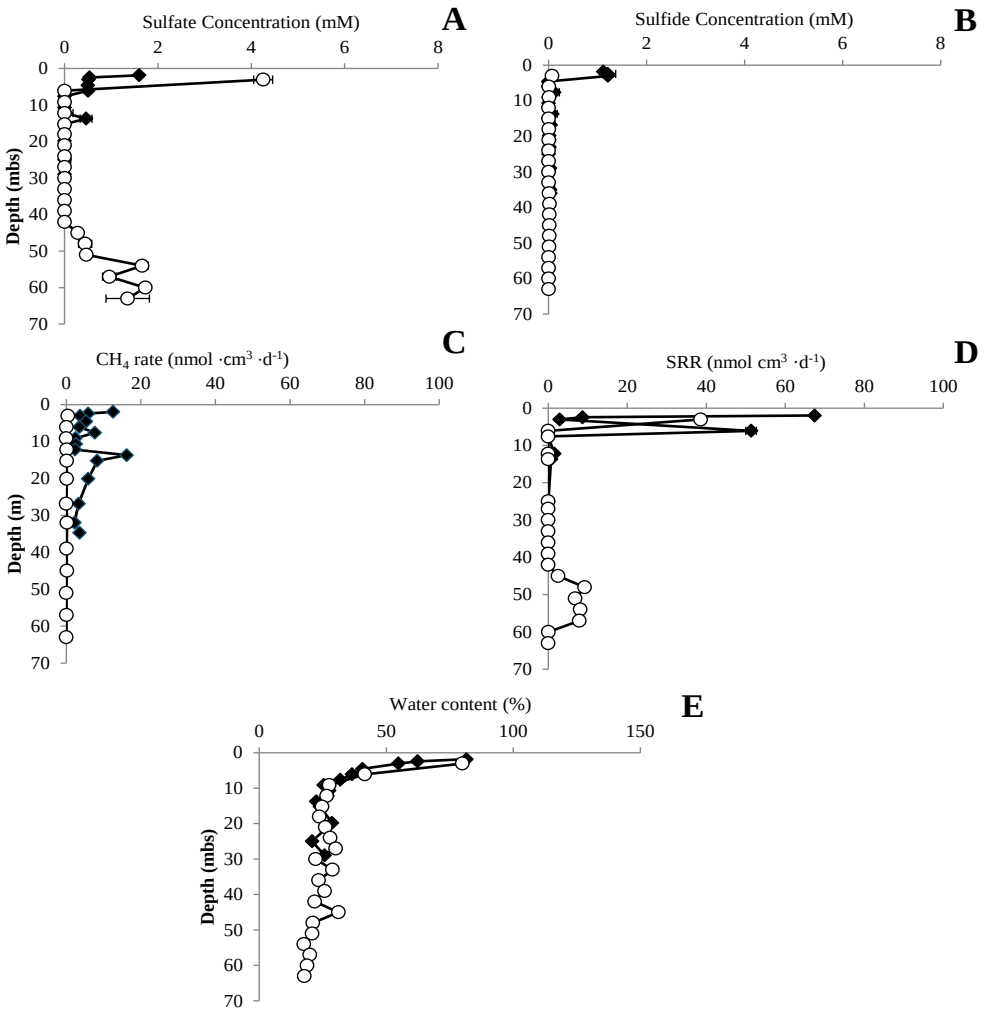


Figure 4-15 Chemical characterization and microbial activity measurements of pond 5 before pond closure in 2009 (♦) and after pond closure in 2010 (○). (A) Sulfate concentrations, (B) Sulfide concentrations, (C) Methane production rate, (D) Sulfate reduction rate, and (E) Water content. The error bars represent the standard error of two replicates.

4.5 Discussion

4.5.1 Physiological and chemical characterization of an active pond

Understanding how microbial communities behave in tailings ponds is a challenging task but is necessary in order to obtain baseline information and develop tools to direct current and future management strategies. Microbes enriched in the ponds can affect the tailings fate in many different ways. They can contribute to increased or decreased densification rates [15,44,45]; they can be very detrimental to the environment by producing harmful gases [45], or they can convert complex hydrocarbon compounds into less toxic substances [81].

In order to study the microbial community composition in an active pond, and to assess the correlation between these populations and the microbial physiology and chemistry in the tailings, a series of oil sands tailings samples from pond 6 as a function of depth were studied [Table 3-2]. It should be pointed out that since our microbial community survey was PCR-based, it likely suffered from PCR biases even though the primers used were designed to amplify a wide range of *Bacteria* and Archaea [103]. Hence, the survey presented here has its limitations. In addition, the DNA extraction protocol used for 2008 samples was carried out using skim milk powder which enhances the DNA detachment from clay particles therefore increasing the DNA recovery from our samples [96]. This could be the reason why samples from 2008 were more diverse than samples from 2010 and 2011[Figure 4-4], and that not all the samples within the year 2008 clustered together in the phylogenetic tree [Figure 4-6] or NMDS graph [Figure 4-7]. No skim milk powder was used for samples from 2010 and 2011.

In general, the microbial analysis (community structure and physiological experiments) as a function of depth confirms that there are at least two major metabolic processes currently ongoing in the tailings ponds: methanogenesis and sulfate reduction. Figure 4-16 shows a schematic diagram of the dominant processes ocurring in pond 6 (based on the work reported here), along with the changes in microbial community composition over time for pond 6. For each sampling year, many of the same taxa are present, however, the relative abundance of the taxa changed. The occurrence in time and space in the abundance of one taxa over another appears to depend on the availability of electron acceptors, fluidity of tailings, and management of the pond (e.g., fresh tailings input).

Overall, based on the three years sampled (2008, 2010, and 2011), microbial community analysis revealed that pond 6 is heavily colonized by methanogens, in particular *Methanosaeta* [Figure 4-4]. Penner and Foght [40] previously reported the dominance of *Methanosaeta* in two Syncrude tailings deposit samples (MLSB) indicating that acetoclastic methanogenesis could be the primary route for methane production in tailings ponds [40]. Methanogens are a unique group of anaerobic organisms that conserve energy during the production of methane. In general, they are capable of using specific compounds such as hydrogen, acetate, methanol, and methylated compounds but may also rely on symbiotic cooperation with other bacteria for energy from other carbon sources [104]. This type of cooperation, known as syntrophy, enables both organisms to energetically depend on each other to degrade complex substrates [105].

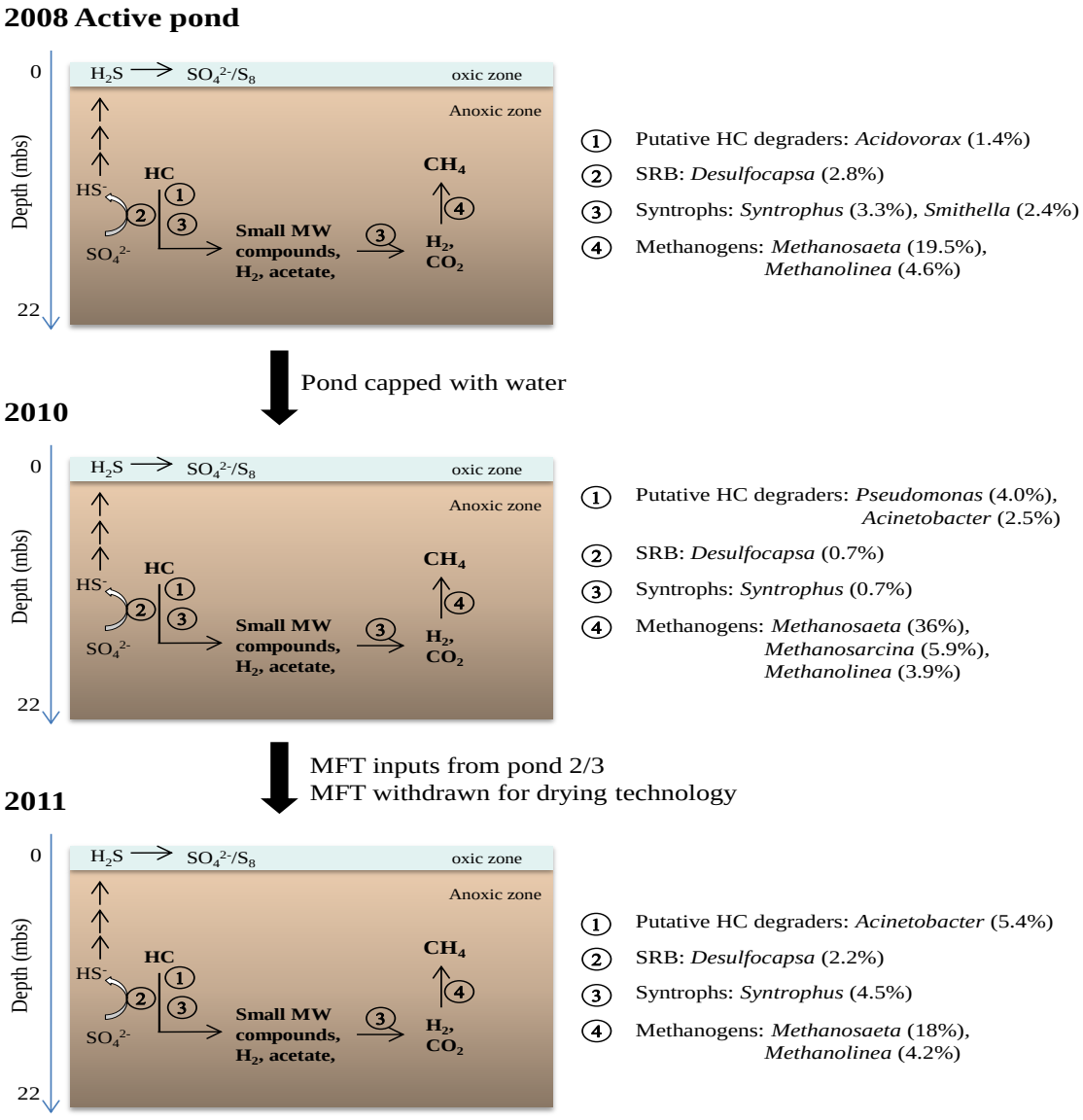


Figure 4-16 A schematic of the dominant microbial processes found in Suncor's oil sands tailings pond 6 based on data obtained in this work. Changes in the average relative abundances of the key microbial taxa over time are indicated on the right of each figure.

Indeed, the observation that *Syntrophus* (a syntroph thought to be involved in methanogenic hydrocarbon metabolism [106]) was among the most abundant bacterial genera in pond 6 aligns with the idea of syntrophy [Figure 4-3 and Figure 4-4]. In enrichments with MFT from Syncrude Canada Inc. amended with naphtha, the presence of both *Methanosaeta* and *Syntrophus* was observed [68,69]. The association of species belonging to these genera suggests their participation in *n*-alkane and/or BTEX biodegradation in MFT [68,69].

Members belonging to the genus *Methanosaeta* are obligate anaerobes and extreme metabolic specialists that use acetate as the sole source of energy and acetate as the sole carbon sources with concomitant production of CH_4 [107]. The methyl carbon of acetate is reduced to methane while the carboxyl carbon is oxidized to CO_2 [108]. The persistence of these kinds of methanogens in the pond may be due to the ability of most of the *Methanosaeta* species to grow in strong bundles of aggregated filaments [107], and to their coupling mechanisms with syntrophs to degrade complex organic compounds in different environments [69,109-112]. The low levels of acetate (usually less than 0.5 mM) in tailings also supports their growth due to their high affinity and low threshold for this organic compound [112,113] [Figure 4-8 (E)].

The increase in relative abundance of methanogens from 2008 to 2010 was not surprising as it was previously suggested that with age, tailings ponds have a tendency to become methanogenic [44]. The pond was already 8 years old in 2010 when it had reached maximum capacity and the input of fresh tailings into the pond ceased. This implied that no more SO_4^{2-} was available for SRB, as gypsum was no longer added; resulting in a

microbial population shift towards the methanogenic Archaea in the anaerobic zone [44,110]. However, it is not clear why a decrease in methane production rates was observed in 2010 [Figure 4-8 (C), Table 4-1]. However, one explation may be attributed to the increase in acetate concentration to above 4 mM [Figure 4-8 (E)]. The high concentrations of acetate could be inhibiting the dominant *Methanosaeta* sp. (who prefer acetate concentrations to be less than 1 mM), while enhancing *Methanosarcina* sp., that proliferate at higher acetate concentrations [104,109] [Figure 4-4 and Figure 4-5]. However, *Methanosarcina* sp. may not be present in the pond at high enough abundance to support methanogenesis at rates similar to that observed in 2008. In addition, a reduction in the abundance of *Syntrophus* sp. was also observed in samples from 2010 [Figure 4-3 (B)], suggesting that methanogenic activity decreased due to lower abundance of syntrophic partners able to utilize other carbon sources. Therefore, although the pond was closed in 2010, no more gypsum was added, and the % of methanogenic archaea increased, these organisms may not have been as active at the time of sampling. Interestingly, in 2011, the relative abundance of methanogens was similar to the year 2008. It was in this year (2011), that the pond was disturbed due to the implementation of the TRO technology. The disturbance included extracting MFT from pond 6, which probably accounted for oxygen incorporation into the pond with the concomitant inhibition of methanogens therefore resulting in decreased abundance in 2011 with respect to 2010 [Figure 4-3(A)]. Simultaneously, more gypsum-treated tailings from ponds 2/3 was added to pond 6 [Figure 4-2]. These fresh tailings inputs may be responsible for the increased methanogenic activity as more microbes (methanogens and syntrophs) were added to the pond. Figure 4-3 (B) supports this idea as the patterns for microbial distribution at the

genus level in 2008 and 2011 are very similar, with the exception of *Acinetobacter* sp., a putative hydrocarbon degrader [114], that increased its abundance from 0 to 5.4 % of average OTUs [Figure 4-3(B) and Figure 4-4(B, C)]. This could be due to the pond enrichment with naphtha coming from ponds 2/3.

The second most common organism found in all samples from pond 6 (with the exception of samples from 2010), is the strict anaerobe, H_2- or formate-utilizing methanogen, *Methanolinea*. Species of this genus tend to grow in multicellular filaments, a very suitable morphological feature to enhance the nutrient uptake in CT ponds which could be somewhat challenging [54,115]. Based on our results, *Methanolinea* also seems to be closely metabolically coupled to *Syntrophus* as they showed a similar spatial distribution in the pond [Figure 4-5(A)]. As previously mentioned, *Syntrophus* can metabolize a wide variety of organic compounds including for example, benzoate and hexadecane [71,116,117]. Hence, a proposed cooperative mechanism in the ponds could be described in Equation 3 [71] using hexadecane as a model hydrocarbon. The decomposition of hexadecane by *Syntrophus* leads to the formation of hydrogen and acetate [Equation 3(*a*)] that is then cleaved into CH_4 and CO_2 for instance by *Methanosaeta* sp. [Equation 3 (*b*)]. Finally *Methanolinea* can then metabolize CO_2 and H_2 into more CH_4 [Equation 3(*c*)] [71].

Equation 3 Decomposition of hexadecane to methane by three groups of organisms

$$4\ C_{16}H_{34} + 64\ H_2O \rightarrow 32\ CH_3COO^- + 32\ H^+ + 68\ H_2 \qquad \Delta G = -\ 929\ \text{kJ} \qquad (a)$$

$$32\ CH_3COO^- + 32\ H^+ \rightarrow 32\ CH_4 + 32\ CO_2 \qquad \Delta G = -\ 385\ \text{kJ} \qquad (b)$$

$$68\ H_2 + 17\ CO_2 \rightarrow 17\ CH_4 + 34\ H_2O \qquad \Delta G = -\ 282\ \text{kJ} \qquad (c)$$

The spatial distribution of *Syntrophus* and *Methanolinea* mirrors the methanogenesis rate observed in pond 6 where the highest activity was mainly found towards the upper layers of the pond, near the mud line [Figure 4-8(C)]. Therefore, despite their low abundance with respect to *Methanosaeta*, the microbial activity suggests that *Methanolinea* sp. also plays an important role in methane production in the pond.

The relative abundance of SRB decreased over time as sulfate became less available due to pond closure. SRR assays confirmed this, as notable differences from 2008 to 2010 and 2011 were perceived [Figure 4-8(D)]. Sequences phylogenetically affiliated with the sulfur disproportionating bacterium *Desulfocapsa* seem to be the leading group of sulfate reducers in tailings pond 6 [Figure 4-5(B)]. Their abundance throughout the pond suggests that they can either be disproportionating oxidized inorganic sulfur intermediates such as thiosulfate and sulfite or carrying out the conventional sulfate reduction. Species of *Desulfocapsa* have been found to be metabolically diverse. For instance, *D. thiozymogenes* is able to grow as a sulfate reducer

on short chain alcohols while while *D. sulfexigens* cannot reduce sulfate despite having the complete enzyme machinery [118,119].

The origin of the high concentration of sulfate at the surface is not known, but some speculation can be made as an explanation. It is known that methane bubbles generated at lower depths of the pond can rise to the surface, scavenging particles, surface active materials, bacteria, and even oil, creating an important vertical transport mechanism [60]. Hydrogen sulfide produced by SRB at deeper depths for instance, could be partitioning with methane bubbles being transported to the surface where it can be oxidized back to sulfate either chemically or biologically. In fact, the neutral pH of tailings favor the presence of hydrogen sulfide as HS^- which has a tendency to precipitate as iron and other metal sulfides [16], therefore little sulfide will escape to the surface as gas. Further work to provide evidence to support this speculation is presented in Chapter Five:. Another explanation for the high sulfate concentrations at the surface could be that the sulfate added originally accumulates as more TPW is recycled and this remains in the surface water (e.g. as a dissolved solute) as no SRB activity typically occurs in aerobic environments.

Considering that 1 kg of gypsum per cubic meter of tailings is added to the pond, approximately 7 mM of sulfate would be expected to remain in the tailings if no SRB were carrying out sulfate reduction [3]. Interestingly, most of the sulfate was found to be consumed within the tailing pond 6, but at depths below 18 mbs, higher sulfate levels were measured [Figure 4-8(A)]. This could have different explanations: (i) as tailings consolidate, less water is available, therefore a reduction in the microbial activity

including the reduction of sulfate is expected; and (ii) at these depths, carbon sources are limited due to depletion of biodegradable compounds, leaving "untouched" the recalcitrant complexes, and as a result, lower sulfate reduction occurs. This together with the disproportionation of incompletely oxidized sulfur compounds (e.g., by *Thiobacillus* sp.) may explain some pockets of higher sulfate in the deeper zones. These pockets could also be the result of ground water filtration into tailings ponds, but this has not been proven so far.

As was previously suggested by Salloum *et al*. (2002) [16], the addition of gypsum to tailings contributes to a decrease in methanogenesis. Because the reduction of CO_2 to methane and the reduction of sulfate to sulfide both require 8 electrons, sulfate reduction by SRB will prevent the formation of an equivalent amount of methane. Thus, based on the SRR and methanogenesis rates observed in pond 6, we were able to estimate the approximate percent of theoretical methane that was not released to the environment due to the availability of sulfate [Table 4-1]. As the pond aged, however, less sulfate was available and therefore more methane could potentially evolve from the pond.

In conclusion, if maintained undisturbed, pond 6 has a high risk of becoming more methanogenic and methane bubbles may eventually be released from the pond. The microbial composition observed as well as the microbial activity measured indicated that such a pattern trend was occuring from 2008 to 2011. However, current management strategy being followed by Suncor Energy Inc., where MFT is being removed from this pond for the drying technology, could likely contribute to decreasing these risks.

4.5.2 Effect of pond closure

In an effort to lower the environmental impacts produced by oil sands mining operations, and to comply with the eco-friendly rules and directives enforced by the ERCB, Suncor Energy Inc. has started to engage in new tailings management strategies. In this regard, pond closure and the TRO technology are leading the way in the Athabasca area.

After a year of closure, pond 5 is already showing the positive outcomes of this management approach. Our results revealed that at the time of closure (2009), the microbial community present in the tailings pond was mainly methanogenic. Thus, high potential risks for methane release into the atmosphere would have been expected if no actions were taken. At that time, the most abundant organisms found were members of the genus *Methanosaeta*. Similar to what was found in pond 6 [section 4.3.1.1], species grouped under this genus are dominant in tailings ponds. The substrate availability (acetate), pH range and temperature, favors their development [40,111,120,121].

Interestingly, following a whole year of zero input of fresh tailings and dewatering of the pond, there was a shift in the microbial community from one dominated by methanogens (*Methanosaeta* sp.) to one dominated by putative hydrocarbon-degraders (*Pseudomonas* sp.) [Figure 4-11(B)]. This shift may have different explanations: (1) water stress to endogenous microorganisms due to pond dewatering, (2) toxicity and/ or bioavailability of carbon sources to methanogens as only the less biodegradable compounds are left, and (3) presence of oxygen in the pond due to Suncor's operation disturbances.

In the process of pond closure, water is at times being removed from the pond [52]. Operators have the final goal of a completely dewatered pond before the final capping is approved. Hence, only microbes that have a high tolerance to water stress may survive successfully under these circumstances. Such is the case of *Pseudomonas* species, a well-known genus of bacteria with the ability to survive under high water stress environments. *Pseudomonas* can be widely found in nature, as they are very well adapted to harsh conditions. In pond 5, sequences affiliating with *Pseudomonas* can be found throughout the pond, even at depths were water was less available [Figure 4-12(B)]. Their cell wall characteristics, as well as their ability to grow in a biofilm structure, make them very resistant to high toxicity and desiccation conditions. *In vitro* studies of direct culturing of oil sands tailings microbes showed *Pseudomonas* species as the predominant biofilm producer [38]. This capability for cell aggregation is mainly due to the production of extracellular polymeric substances (EPS) which are biosynthetic polymers of high molecular weight such as polysaccharides, proteins, nucleic acids, phospholipids; along with non-polymeric constituents of low molecular weight. These extracellular compounds are found surrounding the cells and are also key in the formation of flocs, sludges and biogranules [122]. This protective matrix can hold several times the microbe's weight in water thereby allowing the diffusion of nutrients to the cell wall [123]. In addition, some strains of *Pseudomonas aeruginosa* are known to produce lipases,stable enzymes under water restriction conditions that allow these bacteria to survive in low water environments [124,125]. Likewise, production of biosurfactants by *Pseudomonas* sp. seems to help in their survival when water is very low by regulating the hydraulic potential gradients [123]. Microscopy analysis of tailings by scanning electron microscopy (SEM)

has revealed the presence of microbial aggregates and the presence of EPS [15]. This suggests that microbial cells with such characteristics in tailings could potentially be bounded to clay particles with the concomitant formation of clay hutches. This feature is also thought to be contributing to tailings sedimentation over time [15].

In contrast to *Pseudomonas* sp., some methanogens are very sensitive to water stress [126] and oxic environments and could die after exposure to any of these . Nonetheless, there are methanogenic strains that have developed the ability to adjust and survive when long periods of complete dehydration or oxygen exposure is an issue. Such is the case of methanogens in wetland rice based cropping systems where cycles of flooding and drainage occur [127]. However, we do not think such is the case for tailings methanogenic organisms since the methanogenic community in the pond is not adjusted to these water stress cycles. Therefore, either water stress or oxygen incursion due to the introduction of the dewatering wicks into the pond may be a key point regarding a decrease in the methanogenic population [52]. Nevertheless, the continued presence of *Methanosaeta* sp. under these conditions may occur because they have the ability to form aggregates which in the long term protect them from extreme conditions such as water and oxygen stress .

Finally, the availability of organic compounds (e.g. carbon sources) in tailings becomes challenging for some microbes. In contrast, *Pseudomonas* are known to degrade a wide range of hydrocarbon compounds which under these conditions, especially if oxygen is available, could be very favorable. Furthermore, the ability of *Pseudomonas* to produce surfactants may allow them to have access to more complex compounds [128].

The lack of nutrients together with low water content (less than 20 vol %) may also explain the presence of sulfate pockets at deeper layers in the pond (below 50 mbs). As explained earlier, these factors could be affecting the SRR [Figure 4-15 (A, D)], contributing to an increase in the sulfate concentrations in comparison with shallower depths. The same pattern was observed in pond 6 (2010 samples) where below 21 mbs the water content also reached values below 20 vol% [Figure 4-8(F)], and high sulfate concentrations, compared with the rest of the anaerobic layers, were measured [Figure 4-8(A)].

In conjunction with *Pseudomonas*, other species could be playing important roles in the biodegradation of hydrocarbons in tailings ponds. The persistence of *Acidovorax* sp. both in 2009 and in 2010 suggests this. In particular, in 2010, *Acidovorax* sp. increased in population from 2.4 to 10 % of the average pyrosequencing OTUs [Figure 4-11(B)]. Many hydrocarbon contaminated sites have previously shown *Pseudomonas* and *Acidovorax* as dominant genera [114129]. These two together with *Acinetobacter* have known hydrocarbon degrading ability [114], including PAHs [130-134]. Furthermore *Acidovorax* species may also be involved in the oxidation of iron coupled to nitrate reduction [135]. Other genera with known denitrifying capabilities are *Thauera* [136], *Brachymonas*, and *Comamonas* [137]. All of them have also been previously found in hydrocarbon contaminated environments [138-140]. Although no nitrate was detected in tailings samples from pond 5, nitrite concentrations range from 1 to 2 mM (data not shown). The *Thiobacillus* genus detected in samples from 2009 and *Desulfuromonas* detected in 2010 samples, have members known to be involved in sulfur metabolism, and

may be carrying out this function in pond 5. The obligate chemolithotroph *Thiobacillus* can oxidize iron sulfides (pyrite) while using nitrate as electron acceptor [141]. They have been found to possess the enzyme sulfite cytochrome c oxydoreductase that oxidizes sulfite and produces sulfate [142]. On the other hand, *Desulfuromonas* sp. apart from degrading hydrocarbons, is a well-known iron- [114,143] and sulfur-reducer in anoxic sediments [144]. Since species known to be associated with iron metabolism are being detected in relatively high abundance in tailings, more research will need to be done in this area to better understand other metabolic processes apart from methane production or sulfate reduction in tailings ponds.

4.5.3 A comparison between active and inactive ponds

Comparing the two ponds studied we can conclude that each one is a unique ecosystem in terms of spatial distribution and type of organisms found. The phylogenetic relationship between the samples showed that pond 5 and 6 clustered separately, but within each pond the the communities within a given sampling time tend to be grouped together [Figure 4-17]. For example, although pond 6 was sulfate-reducing and methanogenic initially (2008), it appears to have shifted to being mainly methanogenic, with a prevalence of *Methanosaeta* and *Methanolinea* most likely coupled with *Syntrophus* species. The inactive pond 5 also showed a change in the microbial community composition within a year following pond closure, from being mainly methanogenic (dominated by *Methanosaeta*), to a community dominated by putative hydrocarbon-degraders such as *Pseudomonas* sp. and *Acidovorax* sp.

The microbial activity (e.g. rates of methanogenesis, SRR) for each pond generally decreased over time presumably due to a reduction of tailings input, particularly for the inactive pond. In pond 5, the anaerobic microbial activity was found close to the surface of the pond whereas for the active pond (pond 6) the anaerobic microbial activity is more homogeneously distributed throughout the depths sampled.

Unquestionably, tailings ponds harbour a wide variety of organisms that interact among each other in complex microbiological cycles. The physiological and community structure analysis carried out in these two ponds has given evidence of how tailings management (e.g. inputs, or lack of inputs that alter chemistry) influences the microbial population in these ecosystems. Thus, the strategy of dewatering and pond closing when tailings ponds have become methanogenic is a good approach for achieving the directives toward a safer environment in northern Alberta as our results suggest a community shift away from predominantly methanogenic to favor alternate microbial activities.

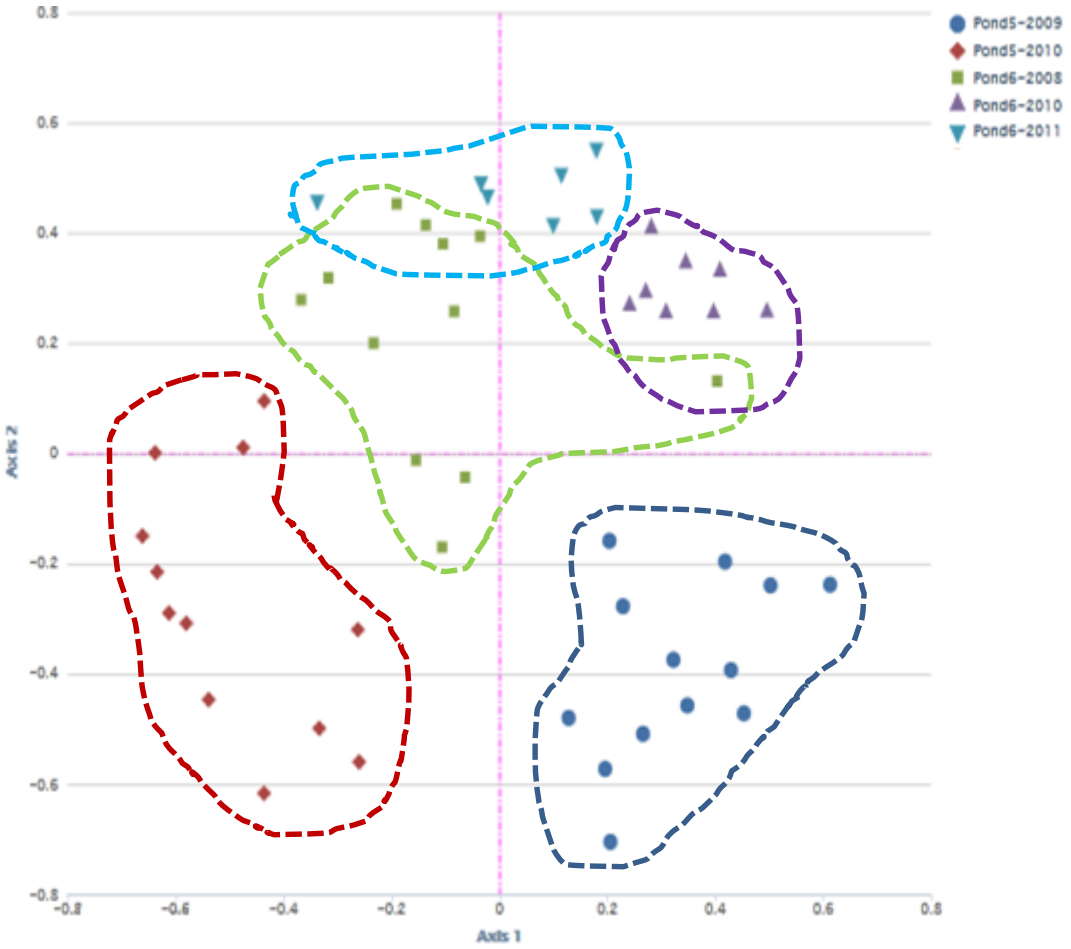


Figure 4-17 NMDS of all tailings samples collected (pond 5 2009, 2010, and pond 6 2008, 2010, 2011) showing how samples collected in the same year cluster.

Chapter Five: **Microbiology at the surface of tailings ponds**

5.1 Introduction

Tailings pond surface water, also known as tailings process water (TPW) is part of the post-process waste in the extraction of bitumen from the oil sands. As the tailings solids settle in the ponds, the pore water rises and can be reused in the extraction process. Although some chemical parameters may change due to the recycling of the water, in general, TPW are moderately hard (15–25 mg · L^{-1} Ca^{2+}, 5–10 mg · L^{-1} Mg^{2+}) with a pH (hydrogen ion concentration) of 8.0–8.4 and an alkalinity (a measure of the carbonate) of ~800–1000 mg · L^{-1} HCO_3^-. TPW also contains dissolved solids including sodium (~500 to 700 mg · L^{-1}), bicarbonate, chloride (~75 to 550 mg · L^{-1}), and sulfate (~200 to 300 mg · L^{-1}) and organic compounds such as bitumen, NAs, asphaltenes, BTEX, cresols, humic and fulvic acids, phenols, phthalates, and PAHs [19]. Trace metals have also been detected such as (in mg · L^{-1}): aluminum (Al) (0.07 – 0.5), arsenic (As) (0.06 – 0.015), cadmium (Cd), chromium (Cr) (0.003 – 2.0), copper (Cu) (0.02 – 0.9), iron (Fe) (0.8 – 3.0), lead (Pb) (0.04 – 0.19), Nickel (Ni) (0.006 – 2.8), and zinc (Zn) (0.01 – 3.2) among others [19].

The surface water of tailings ponds is most exposed to environmental changes. During the winter months, just the surface water tends to freeze and in the summer, it thaws completely allowing a more active microbial life. It has been speculated that three important microbial processes occur in the surface water of tailings pond: (1) oxidation of sulfide by sulfide-oxidizing bacteria (SOB) [62], (2) naphthenic acid biodegradation

[67,145,146], and (3) aerobic methane oxidation. In this chapter, only the first two processes will be addressed.

As was previously suggested (section 4.5.1) H_2S ions produced by SRB in the anoxic zone of the pond, may be transported to the surface by CH_4 bubbles [60]. Once the bubbles reach the surface, they burst releasing the H_2S , which is subsequently oxidized to S_8 (abiotically) or to SO_4^{2-} (biotically). To test this hypothesis, tailings waters and tailings from different depths of pond 6 sampled in 2008, amended with sulfide and exposed to air, were monitored for sulfide loss over time. Separately, surface water samples from ponds 5 and 6 were microbiologically characterized by 454 pyrosequencing. The results of these experiments are described in this chapter.

Naphthenic acids (NAs) are thought to be one of the sources of toxicity in TPW [36]. Their concentration ranges from 40 to 70 mg $\cdot$ L^{-1} but can occasionally reach as high as 130 mg $\cdot$ L^{-1} [32]. They are present in tailings ponds because they are solubilized during the hot caustic treatment used in the extraction of bitumen although they can also be the result of incomplete biodegradation of bituminous compounds once in the pond [80]. One of the most difficult challenges the oil sand industry faces today is the treatment of these NA-containing waters before they can be discharged [19]. Thus, devising mechanisms to eliminate or convert them into less toxic structures is of great interest to oil sands operators. Microbial degradation could be one alternative, as NAs have been previously proven to be biodegradable to some extent [31,34,65,67,81,82,145,147-150]. It is known from a handful of previous studies that the oxidation of these compounds can occur by β-oxidation of the aliphatic side chain and that their biodegradability depends on the extent

of alkyl side chain branching [81,145,151]. However, the detailed metabolic pathways through which biodegradation occurs have not been well described and it may vary depending on the microbes. As also described in this chapter, aerobic enrichments to cultivate and isolate bacterial NA degraders were established and a series of NA biodegradation experiments with pure bacterial isolates were carried out.

5.2 Methods

5.2.1 Sulfide oxidation experiments

For sulfide oxidation tests, 3 mL of tailings from different depths of pond 6 sampled in 2008 were added to sterile serum bottles containing 60 mL of a mineral salts medium (CSB-A medium, Table 3-6) amended with sodium sulfide (~3 mM starting concentration) [95]. Experiments were initiated by adding 45 mL of air, and sulfide concentrations were measured at time 0 and at 20 min intervals (up to 100 min) using the copper sulfide method [91] (described in section 3.2.2). Controls included sterile sulfide-containing medium and heat-killed tailings in sulfide-containing medium. Samples for all determinations were prepared in the anaerobic glove bag.

5.2.2 *DNA extraction and 454 pyrosequencing from tailings surface waters*

Microbial community analysis of TPW was conducted using 454 pyrosequencing following DNA extraction. DNA extraction of three water samples [Pond 6 2008, Pond 5 2009 (before pond closure), and Pond 6 2011, based on availability] were obtained using the procedure described in section 3.4.1 and microbial community composition was analyzed as described in section 3.4.3.

5.2.3 *Isolation and identification of naphthenic acid-degrading bacterial isolates*

Isolation and culturing of the isolates was described in section 3.5.2. The selected isolates were subjected to DNA extraction using a DNA extraction kit (Fast DNA Spin kit; MP Biomedicals) followed by PCR amplification of the 16S rRNA genes. The PCR reaction contained 25.0 µL of 2 x PCR master mix (Fermentas), 21.0 µL of nuclease-free water (Fermentas), 2.0 µL of extracted DNA and 1.0 µL of primers (27f/1392r) [152] for a PCR reaction totalling 50 µL.

Amplified products were visualized on 1% agarose gels, purified (Qiagen) and sequenced in both directions with the same primers used for the amplification (University Core DNA Services, University of Calgary). Sequences derived from the forward and reverse primers were manually aligned by overlapping the sequences, typically by ~100 bases. Putative identities were determined based on the nearest phylogenetic relative in the BLASTn (NCBI; http://www.ncbi.nlm.nih.gov/blast; default settings) or RDP II (Ribosomal Database Project II; http://rdp.cme.msu.edu; default settings) databases. The RDP II database was used to corroborate the identification obtained in the BLASTn searches.

5.2.4 *NA biodegradation time course experiments*

The inoculum used for the biodegradation experiment was prepared by growing the isolates on LB broth (described in 3.5.1,Table 3-5). The cells were washed and suspended in sterile saline solution until an approximate concentration of 1 x 10^8 cells · mL^{-1} was obtained (equivalent to the 0.5 tube on the MacFarland scale). To obtain a 1 %

inoculum in the biodegradation experiment, an aliquot from this suspension was then inoculated into Erlenmeyer flasks containing MBH medium plus a selected model NA at 200 mg·L^{-1}. The model NAs tested as growth substrates for the isolates included cyclohexaneacetic acid (CHAA), cyclohexanecarboxylic acid (CHCA), and cyclohexanepentanoic acid (CHPA). The NA stock solutions were prepared by dissolving 240 mg of the model NA in 40 mL of alkaline water to a final concentration of (6.0 g · L^{-1}). The stock solutions were filtered throught a 0.2 µm membrane filter (Millipore) and transferred to a sterile bottle. Sterile (no inoculum) and substrate free (no substrate added) controls were also established and monitored. All flasks were covered with a foam stopper to allow for sterile but aerobic conditions. The flasks were incubated on a rotatory shaker in the dark at 100 rpm and 30°C. Optical density (OD) at 600 nm was measured every day on a UV 1800 spectrophotometer Shimadzu. Subsamples from the culture (40 mL) were withdrawn as OD_{600} values increased. The samples were acidified with hydrochloric acid (HCl) to pH = 2 to stop the microbial growth, and then subjected to organic extraction.

Prior to organic extraction of the acidified cultures, 1 mL of octanoic acid was added as an extraction standard (100 mg · L^{-1}).

The acidified subsamples were then extracted with three volumes of dichloromethane (DCM) (15 mL each). The combined organic fractions were dried over anhydrous sodium sulfate and concentrated by rotary evaporation and under a stream of nitrogen to a volume of 100 µL. The samples were then silylated with 100 µL of *N,O-*

Bis(trimethylsilsyl)trifluoroacetamide (BSTFA) (Thermo Scientific, Waltham, MA) for 20 min at 60 °C.

Naphthenic acids and potential metabolites were analyzed using an Agilent 7890A gas chromatograph equipped with an HP-5 MS column (30 m 0.25 mm 0.25 µm film; Agilent Technologies) and an Agilent 5975C mass selective detector. The oven was held at 45 °C for 5 min, increased at a rate of 4 °C per min to 270 °C, and then held at this temperature for 5 min. The injector was operated in split mode (50 : 1) and was held at 270 °C. Naphthenic acid degradation was calculated by comparing the peak areas with the extraction standard, and metabolite identifications were either positively confirmed by comparing with the mass spectral profiles of pure compounds if available or tentatively identified by the mass spectral profiles.

5.3 Results

5.3.1 Potential for sulfide oxidation at the surface of tailings pond

Tailings samples from 0 to 9 mbs were used to determine whether sulfide oxidation in tailings ponds is due to chemical and/or microbial processes. Figure 5-1 shows typical results of the sulfide oxidation tests, where sulfide was oxidized almost completely within 80 to 100 min following the addition of air (~20% as O_2). This was observed both in the samples containing non-autoclaved ("live") tailings and in controls containing medium only or autoclaved tailings.

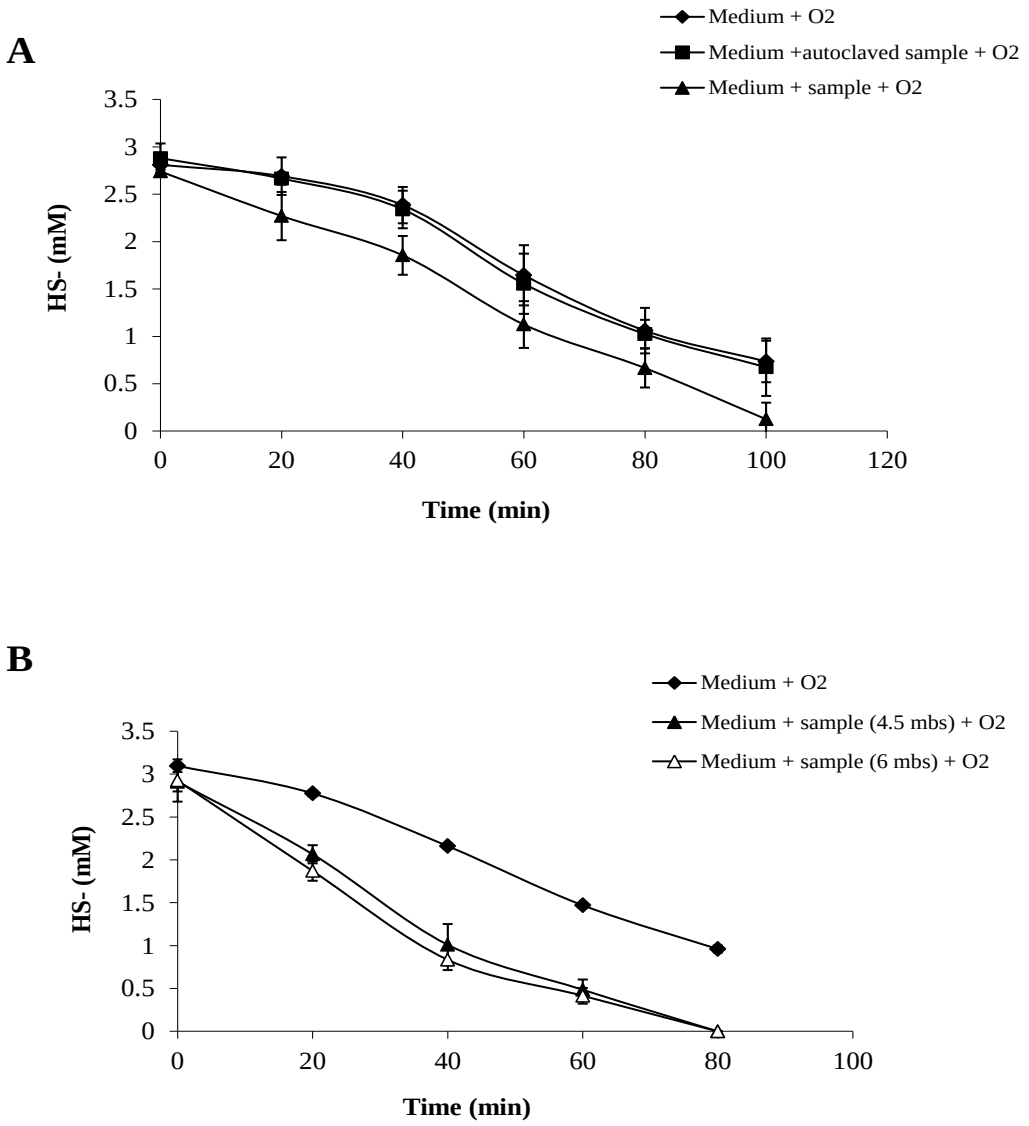


Figure 5-1 Sulfide loss over time in medium with 3 mM HS^- and live or autoclaved tailings. Controls with medium only were also included. Sulfide concentrations were measured following amendment with air. (A) Surface water from pond 6 2008, (B) tailings from pond 6 2008 from depths: 4.5 – 6 mbs. Error bars represent the standard error of two replicates.

5.3.2 ***Microbial community composition of surface waters***

Organisms affiliating with the phylum Proteobacteria dominated the water samples examined in this study [Figure 5-2 (A)], when all samples were pooled and averaged. Other phyla such as *Chloroflexi*, *Bacteroidetes, Actinobacteria, Cyanobacteria, Planctomycetes,* and *Acidobacteria* were also present but as lower percentages of pyrosequencing OTUs. At the genus level, differences in abundance and types of microorganism were observed among the pond water samples [Figure 5-2 (B)]. The pond 6 2008 sample was abundant in *Pedomicrobium* sp. whereas surface water samples for that same pond sampled two years later was dominated by *Porphyrobacter* sp. In the water sample obtained from pond 5, *Porphyrobacter* was also dominant together with *Flavobacterium* sp. Other genera like *Brevundimonas*, were also present in pond 5 2009. For the surface water, other phyla refers to approximately 16 phyla present with less than 3 % abundance whereas other genera refers to approximately 150 genera present in these samples at also low abundance (< 3%) [Figure 5-2].

Pond 5 2009 and pond 6 2011 seem to be microbiologically more related than the two pond 6 samples. Figure 5-3 shows these two samples clustering together. However, this could be due to the lack of diversity found in samples from pond 6 2008, corroborated by the low Shannon index when compared with the other two samples [Appendix Nine:Table 0-8].

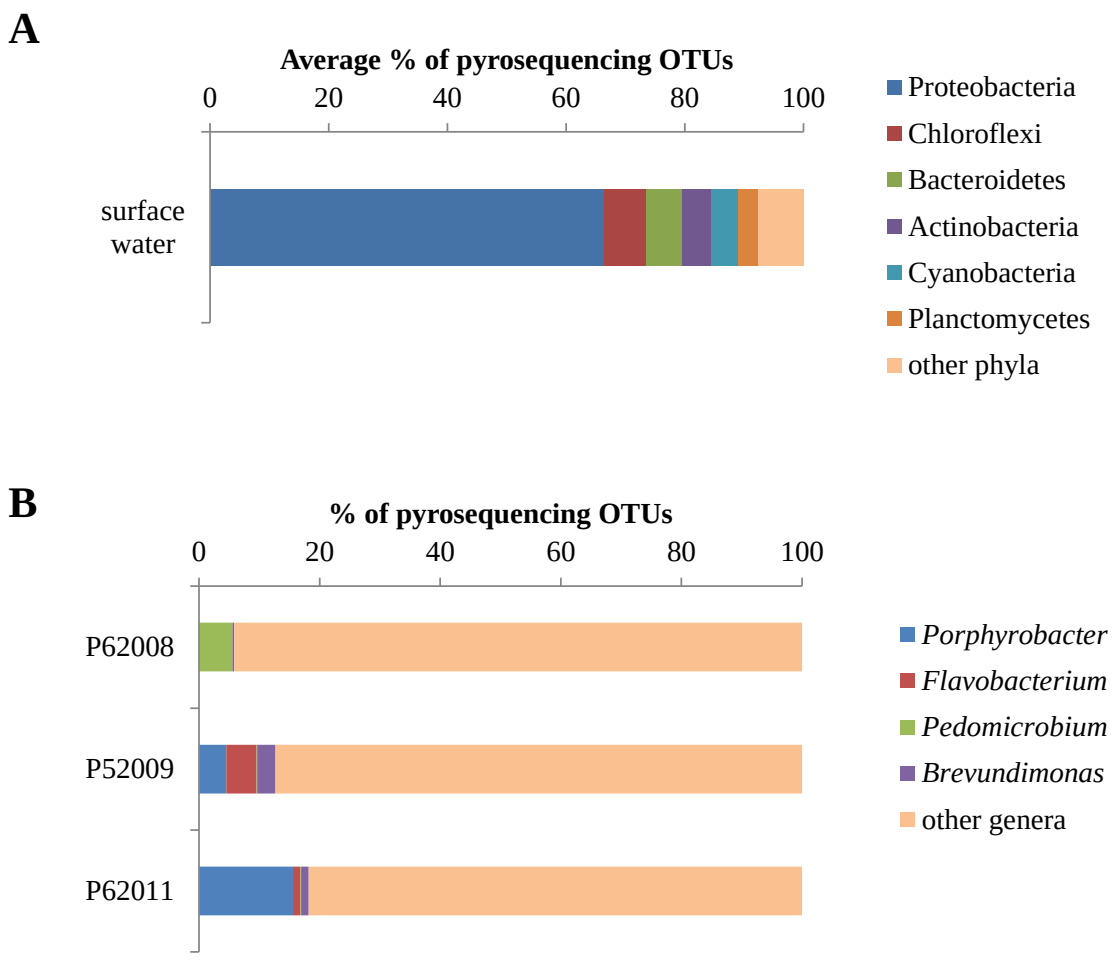


Figure 5-2 Most abundant pyrosequencing OTUs in tailings surface water from pond 6 2008, pond 5 2009, and pond 6 2011. (A) Average % of the pyrosequencing OTUs at the phylum level, (B) % of pyrosequencing OTUs at the genus level for the years 2008, 2009, 2010. The 'other genera' label indicates the genera that were present at less than 3 % abundance.

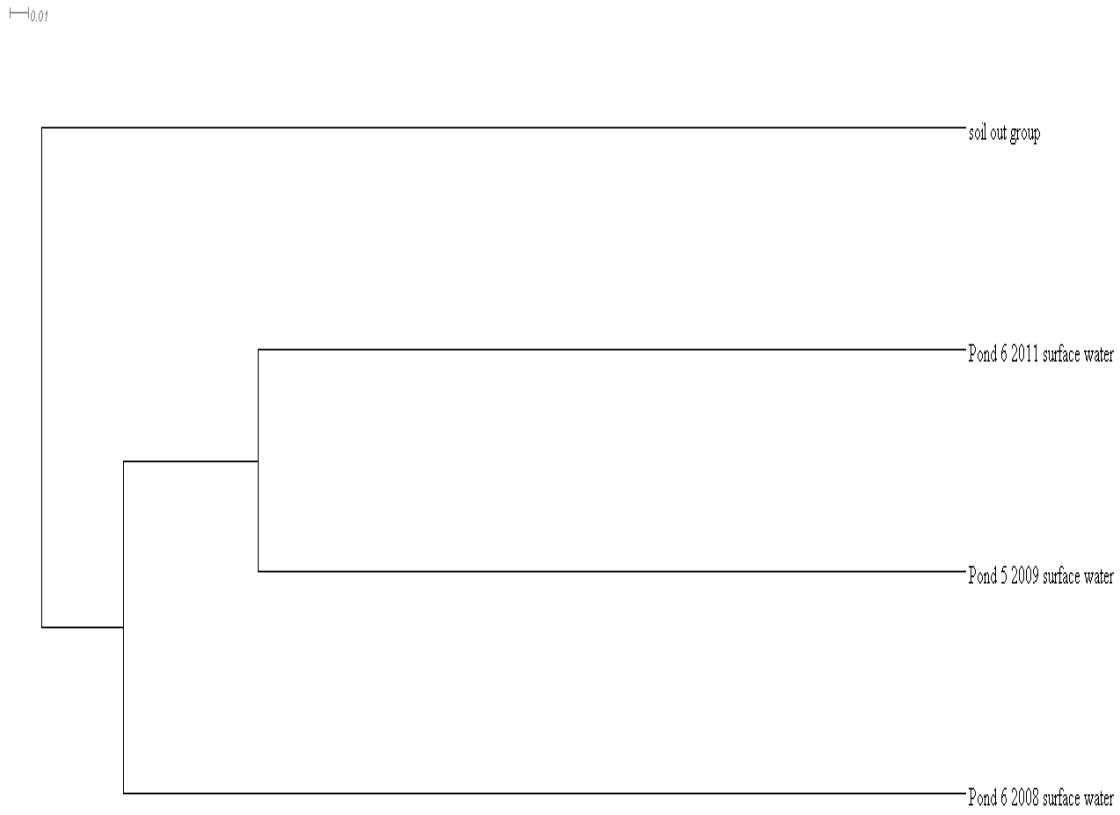


Figure 5-3 Relational tree for TPW samples (P62008, P52009, P62011). Bray-Curtis dissimilarity was used to calculate the distance.

5.3.3 Biodegradation experiments with NA isolates

Three bacterial isolates capable of growing on selected NAs were obtained: an *Acidovorax* sp., a *Xanthobacter* sp., and *Pseudomonas putida* strain ER28 [Table 5-1]. The blasted DNA sequences used for the isolate genus identification are listed in Appendix One:.

In the time-course biodegradation experiments, all the strains were found to biodegrade the model NAs effectively in less than 3 d[Figure 5-4]. All the strains showed a direct relationship between growth increase and degradation of the compound with maximum degradation during the exponential growth phase. *Acidovorax* sp. produced more biomass than *P. putida*. (e.g. higher OD_{600} was observed) but the latter had a faster growth rate. On the other hand, *Xanthobacter* sp. had variations in its growth measurements due to the formation of clumps or biofilm that adhered to the walls of the flask. The degradation of CHPA took slightly longer than CHAA and CHCA. In all cultures, no substrate was detected in the medium after 6 d incubation [Figure 5-4]. No growth was observed in substrate-free controls, and no substrate loss was detected in sterile controls (not shown).

Table 5-1 Surface tailings pond water isolates obtained and grown on model naphthenic acids

strain	*ID based on 16 S PCR BLAST*	*Model NAs*
ER10	*Acidovorax* sp.	Cyclohexaneacetic acid (CHAA)
ER19	*Xanthobacter* sp.	Cyclohexanecarboxylic acid (CHCA)
ER28	*Pseudomonas putida*	Cyclohexanepentanoic acid (CHPA)

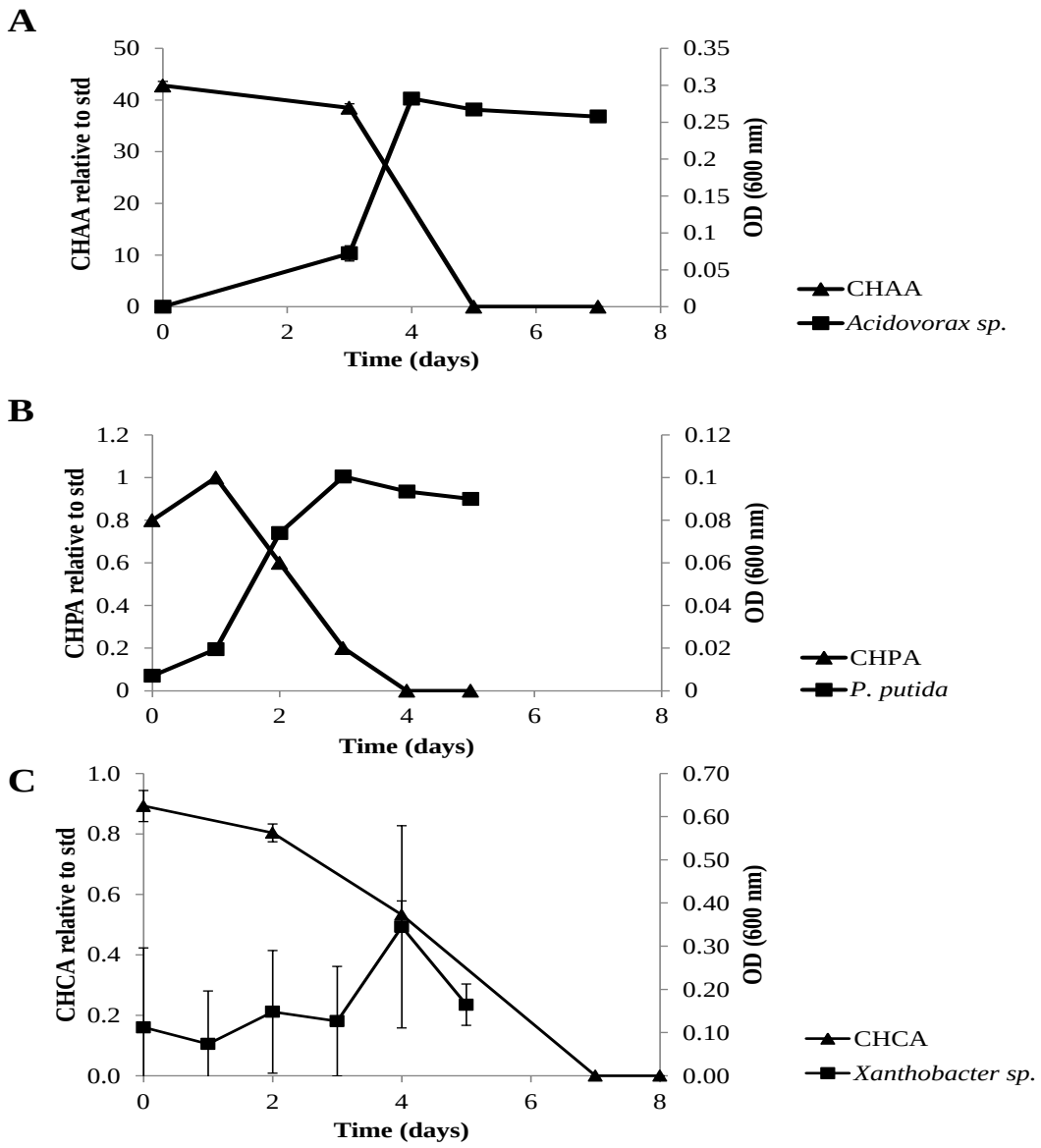


Figure 5-4 Time course biodegradation experiment of (A) *Acidovorax* sp. grown on CHAA; (B) *Pseudomonas putida* grown on CHPA; and (C) *Xanthobacter* sp. grown on CHCA. NA values are the GC-MS peak areas for the derivatized model NA relative to the peak area of the derivatized extraction standard octanoic acid. Error bars represent the standard error of three replicates.

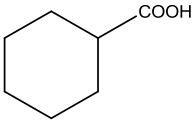
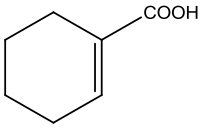
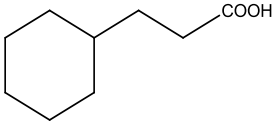
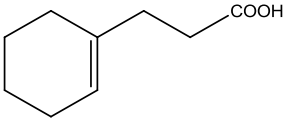
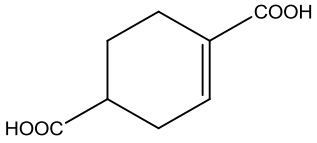
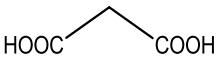
5.3.4 Naphthenic acid metabolite analysis

GC-MS analysis showed that after the third day of incubation several different metabolites were detected. Table 5-2 shows the structure of putative metabolites from CHPA degradation by *P. putida* and CHCA degradation by *Xanthobacter* sp.

Xanthobacter sp. metabolized CHCA to cyclohexene carboxylate; however no other metabolites were observed. For CHPA, different metabolites were detected suggesting a β-oxidation pathway for degradation [Figure 5-5].

Figure 5-6 shows the mass spectra and chemical structures of the putative metabolites identified during the degradation of CHAA. The *Acidovorax* sp. isolated was the only one of the three able to biodegrade CHAA [Figure 5-6]. Some metabolites were the same as those detected in the other cultures (e.g. cyclohexanecarboxylate and cyclohexenecarboxylate) but other new metabolites were detected [Figure 5-6], suggesting other mechanisms of biodegradation [Figure 5-7].

Table 5-2 Summary of metabolites detected from the degradation of CHCA by *Xanthobacter* sp. and CHPA by *Pseudomonas putida* growing on these substrates as their only carbon source. Compounds indicated with an asterisk were only tentatively identified due to lack of authentic standards.

Metabolite	***Chemical structure***	***Xanthobacter* sp. ER19**	***Pseudomonas putida* ER28**
Cyclochexanecarboxylic acid (CHCA)			✓
Cyclohexenecarboxylic acid		✓	✓
*Cyclohexanepropionic acid			✓
Cyclohexene propionic acid			✓
Cyclohexene 1,4 – dicarboxylic acid			✓
*3- carboxy-propionic acid			✓

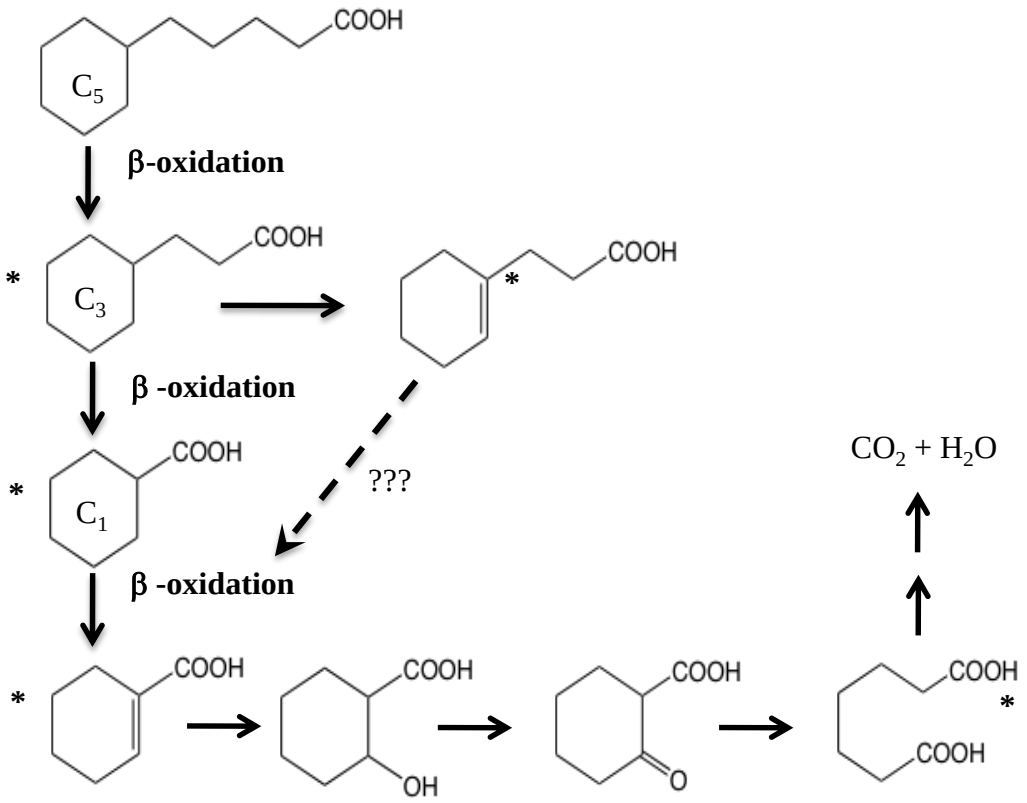


Figure 5-5 Proposed biodegradation pathway of CHPA via β-oxidation by *P. putida* strain ER28. Compounds indicated with an asterisk were only positively identified; others were only tentatively identified due to lack of authentic standards.

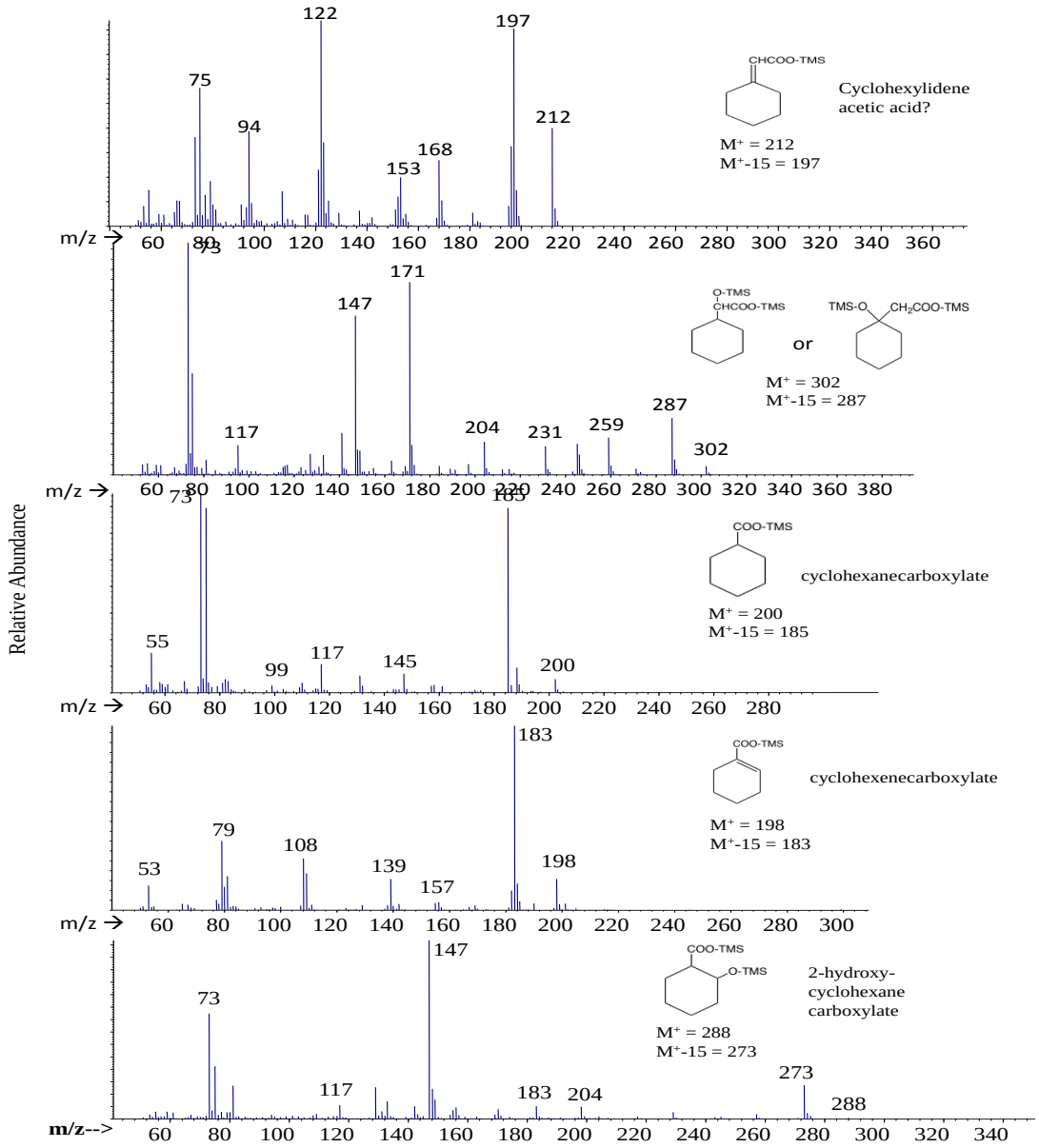


Figure 5-6 Mass spectra of metabolites detected (as silylated derivatives) after 3 d incubation of *Acidovorax* sp. strain ER10 with CHAA (100 mg · L^{-1}) as the sole source of carbon. The bacterium was grown on MBH, incubated in the dark at 30 °C and at 100 rpm. The metabolites were not detected in the sterile controls.

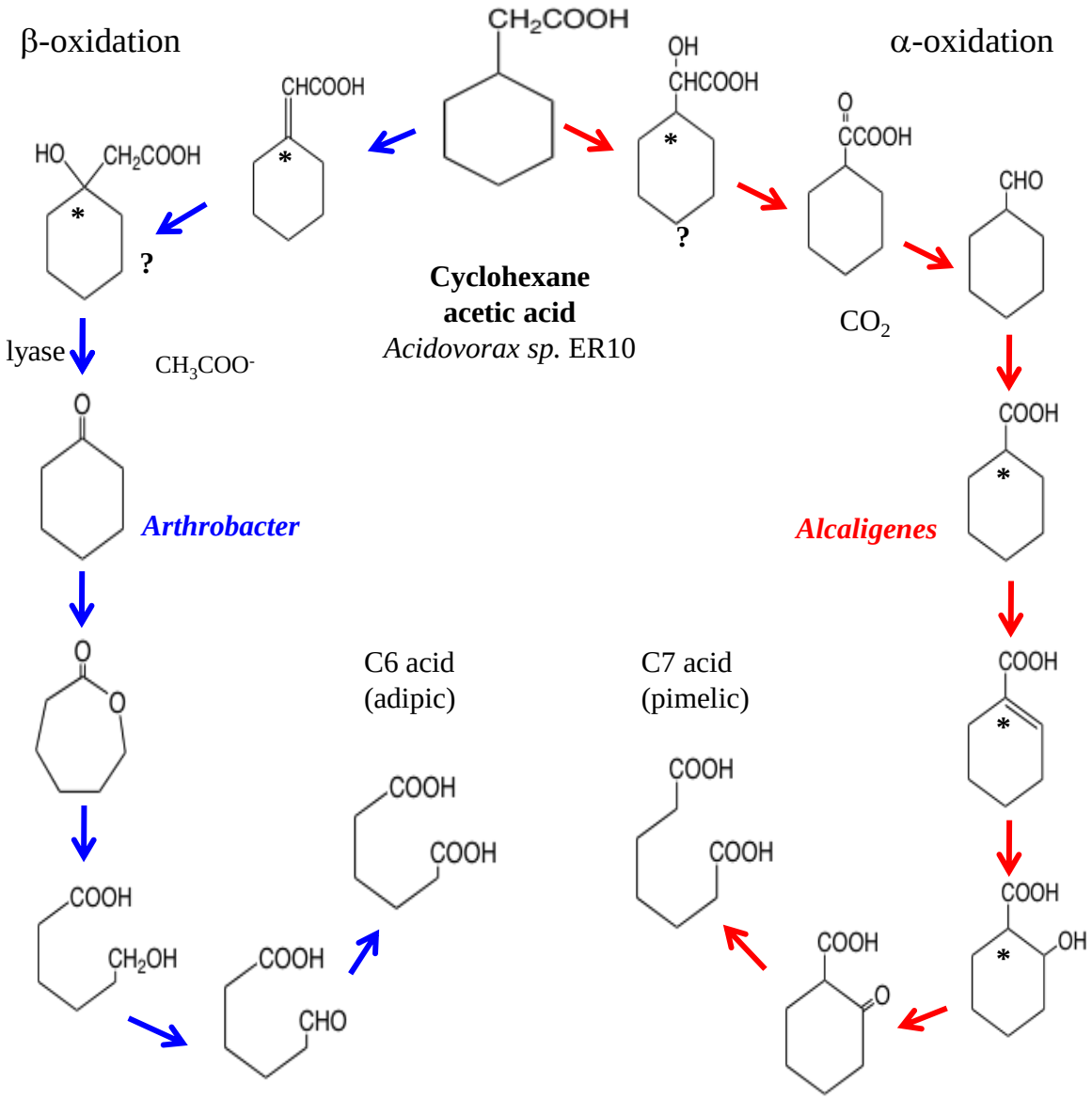


Figure 5-7 Proposed biodegradation pathway of model NA cyclohexaneacetic acid by *Acidovorax* sp. strain ER10. Compounds indicated with an asterisk were only tentatively identified due to lack of authentic standards. Pathways shown have previously been reported for *Arthrobacter* [153] and *Alcaligenes* sp .[78].

5.4 Discussion

Tailings pond surface water presents a different microbial community than the rest of the pond mainly due to differences in the water chemistry, low proportions of solids (i.e. less available reactive surface area), and the availabiity of oxygen as an electron acceptor. The high concentration of sulfate measured [section 4.3.1.2, Figure 4-8 (A)], and the aerobic nature of these waters allowed us to postulate that sulfate is the result of SOB activity. However, our results indicate that sulfide is mainly oxidized chemically when tailings are exposed to air. In samples from all depths tested, however, the sulfide concentration did decrease more rapidly and to a greater extent in the tests containing the "live" tailings than in the controls [Figure 5-1]. The 454 pyrosequencing results did not reveal a rich SOB population with the exception of samples from pond 6 2008 where ~5 % of the pyrosequencing OTUs comprised the budding-like bacteria *Pedomicrobium* [Figure 5-2]. Members of this genus have previously been reported to be involved in the oxidation of volatile sulfur-containing compounds, including H_2S [154]. Considering that pond 6 in 2008 was completely active and concentrations of sulfate were the highest in comparison with the rest of the sampling times, [2010, 2011, section 4.3.1.2, Figure 4-8 (A)], we can infer these bacteria may have been involved in the oxidation of sulfide to sulfate during this time. In addition, among the cultivation and isolation of NA degraders from TPW, some isolates of *Xanthobacter* have previously been reported to oxidize sulfide to sulfate via thiosulfate [155]. In TPW, species affiliated with this genus were found, ranging from 0.05 to 2.2 % of pyrosequencing OTUs. This suggests that if some H_2S escapes to the surface of the pond, some SOB activity could take place in the water layer. However, chemical sulfide oxidation is probably dominant.

Based on the identities obtained by pyrosequencing, we can infer that the majority of the microorganisms present in TPW are more likely to be involved in the degradation of NA and hydrocarbon-like compounds. In fact, in addition to *Xanthobacter* sp., a *Pseudomonas* sp. (isolated herein and also studied for NA biodegradation), appeared in the sequencing results from TPW of ponds 5 and 6 (data not shown due to percentage of abundance being less than 3 % of pyrosequencing OTUs). Other organisms like *Porphyrobacter* sp., that dominated TPW from ponds 5 and 6 in the years 2009 and 2011 respectively, have previously been detected in tropical land farm soils contaminated with oil waste [156]. Organisms in this genus are mainly found in association with algae and cyanobacteria accomplishing anoxygenic photosynthesis [157]. These species could well be co-metabolizing NAs with algae in TPW, as previous studies have shown algae in TPW with promising NA biodegradation capabilities [158,159]. The rest of the genera found in pond 5 and 6 during the years 2009 and 2011, are also related to the biodegradation of hydrocarbon like compounds including *Brevundimonas* sp. [160-162], *Xanthobacter* sp. [163], and *Pseudomonas* sp. [133,164,165].

It should be pointed out that TPW from pond 6 2008 did not show as much diversity as the other two surface waters sampled, probably due to DNA extraction biases. The DNA extraction protocol (FastDNA® kit) recomends that for liquid samples a volume of 200 µL with suspended cells of 10^9 bacteria should be used. However, for our TPW samples, the number of cells was not known (not measured) as they were environmental samples. It is possible that the number of organisms was lower than

recommended therefore more volume of water was initially needed to achieve the manufacture's recommendations.

Some previous work has demonstrated metabolic pathways by which NA may be degraded. For example, NA with odd-numbered fatty acid side chains (e.g. C_1, C_2, C_3) have been shown to be biodegraded by β-oxidation [150,151]. In particular, *Pseudomonas putida* has been previously reported as a NA-biodegrading organism [64,76,166]. The β-oxidation pathway seems to be the preferred route by which this bacterium degrades NAs [82]. This oxidation route involves the formation of new carboxylic acids with two carbons fewer than the parent compound [82].. Based on the metabolites detected in our experiments, the degradation of CHCA, by *Xanthobacter* sp., and CHPA by *P. putida*, both presumably occurred via by β-oxidation [Figure 5-5], confirming previous literature results.

Xanthobacter sp., although not previously reported as NA degraders, in general are characterized by their ubiquitous distribution in the environment, and metabolic versatility [167]. Some species like *Xanthobacter autotrophicus* exhibit chemolithoautotrophy, methylotrophy, short-chain hydrocarbon metabolism, and chlorinated hydrocarbon metabolism [168]. Others like *X. tagetidis* can grow as a chemolithotrophic autotroph on thiosulfate or as a heterotroph on thiophene – 2 – carboxylic acid, acetic acid and α-ketoglutaric acid, and as a mixotroph on a combination of thiosulfate and thiophene – 2 – carboxylic acid and/or acetic acid . Our observation that *Xanthobacter* is able to biodegrade model NA extends the known metabolic diversity of this genus.

In contrast to odd-numbered compounds, biodegradation of even alkyl chain NAs, such as cyclohexaneacetic acid (CHAA), does not occur by β-oxidation due to a blockage by the relative positions of carboxy group and cyclohexane ring [78]. The degradation of this compound needs either a lyase or α- oxidation process followed by a β-oxidation [78]. Two different pathways have been proposed, one in a marine bacterium *Alcaligenes* sp. [78], and the other in *Arthrobacter* sp. [Figure 5-7]. Neither of these organisms was isolated from TPW, but rather an *Acidovorax* sp. capable of degrading CHAA was isolated. In the CHAA – degrading experiments with *Acidovorax* sp., several metabolites were positively and tentatively identified. Based on the compounds detected, however, it could not clearly be deduced which pathway was being used by the *Acidovorax* sp. as putative metabolites from each pathway were detected [Figure 5-7]. Future work using labelled NA substrates or more detailed time course experiments may help further identify which pathway, or whether a novel pathway is being used by the *Acidovorax* sp. to degrade CHAA and related compounds. Nevertheless it is clear that the isolates obtained from TPW were able to transform the model NAs into other compounds, showing evidence of biodegradation. Although CO_2 production and/or molar mass balance were not measured, these isolates may serve as model organisms that can be used to further study the biodegradation of more complex NA. Although not directly a part of this thesis work, the isolates have now been sent for genome sequencing. From this, key genes and regulatory elements involved in NA biodegradation in general can be identified.

Chapter Six: **Targeting specific microbial functions in oil sands tailings ponds by quantitative PCR.**

6.1 Introduction

As demonstrated in previous chapters, the main microbial activities observed in tailings ponds involve the sulfur and the one-carbon (C_1) cycles. The addition of calcium sulfate as a tailings densification agent can promote sulfate reduction, subsequently producing hydrogen sulfide [3]. When sulfate concentrations are relatively low (~ 2 mM), methanogens start to dominate, making the production of methane a concern in tailings ponds [44]. *Desulfocapsa* sp./*Thiobacillus* sp. may be the key players in sulfur metabolism while *Methanosaeta* sp./*Methanolinea* sp. are the leading methanogens (Chapter Four). It was also seen that at some depths, there are "pockets" of sulfate that are not being reduced by SRB, probably due to the lack of biodegradable electron donors or possibly due to SOB metabolism potentially oxidizing sulfide to sulfate. To further characterize these microbial processes, additional measurements were carried out.

An approach that can be used to identify key microbes taking the lead in these processes, is by targeting specific genes that are known to participate in the sulfur oxidation/ reduction metabolism (*sox* and *dsr* genes) and methane production pathways (*mcrA* gene). Quantitative PCR (qPCR) can be very helpful to numerically estimate the distribution of microbial communities participating in these mechanisms [169]. In this method, fluorescenct dyes like SYBR green (Applied Biosystems) bind to the PCR products during thermal cycling. Increased fluorescence in every cycle is measured in the

extension step for each cycle of the PCR reaction. This is converted to the gene copy number of a given gene by comparing with a set of standards with known copy numbers.

For example, by targeting and quantifying the *dsrB* gene, we can estimate the distribution of SRB present in the pond more accurately than with the 16S rRNA gene [170,171]. Just to cite some examples, amplification of SRB genes has been targeted in produced waters from an oil field [169] and in a bioreactor simulating souring in a low-temperature oil reservoir [172]. Likewise, methanogenic Archaea [171,173,174], and sulfite oxidoreductases [175] have been monitored by qPCR by targeting the *mcrA* and *sox* genes, respectively. This chapter describes the results of targeting specific functional genes for key processes in oil sands tailings ponds (methanogenesis and sulfate reduction and sulfide oxidation) and compares them with other depth-dependent physiological measurements.

6.2 Methods

6.2.1 DNA extraction and selection of functional genes

For this study, genomic DNA extracted from all depths from pond 6 (2008, 2010 and 2011) was used as the DNA template for qPCR studies. Pond 6 is somewhat active therefore it is a good model to study and understand the distribution of particular genes in tailings ponds. For more details about pond 6, and DNA extraction protocols (with skim milk powder), please refer to sections 4.2.1 and 3.4.1. Table 6-1 lists the genes targeted in this study and their corresponding enzymes and metabolic activities.

Table 6-1 List of genes used for qPCR and their corresponding enzyme and metabolic activity.

gene	*enzyme*	*Metabolic pathway*
dsrB	dissimilatory sulfite reductase	Catalyzes the six-electron reduction of (bi)sulfite to sulfide, in dissimilatory sulfate respiration [176]
mcrA	α subunit of methyl coenzyme-M reductase	Terminal enzyme complex in the methane generation pathway. Catalyses the reduction of a methyl group bound to coenzyme-M with the concomitant release of methane [177]
sox	Sulfite oxidases*	Group of enzymes that catalyses the oxidation of sulfite or thiosulfate to sulfate [62,178]

* Biologically-produced sulfate in tailings ponds is produced by via sulfite by sulfite oxidases [Appendix Five:].

6.2.2 Primer design

The real time quantitative PCR primers for *dsrB*, *mcrA,* and *sox* genes were designed from sequences obtained from the metagenome of DNA extracted from tailings pond 6, sampled in 2008 and 2010 (unpublished data). For the metagenome, DNA was extracted using Fast DNA Spin Kit for Soil (MP Biomedicals) giving a total yield of 3 µg of DNA.

A list of the functional genes obtained for this metagenome was acquired through the Integrated Microbial Genome (img/mer) web page linked to the JGI website [https://img.jgi.doe.gov/cgi-bin/mer/main.cgi] through the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Twelve primer sequences were designed and optimized for qPCR [Table 6-2].

Table 6-2 Designed primer sequences to test for optional qPCR amplification of *dsrB*, *sox*, and *mcrA* genes based on the pond 6 (2008/2010) metagenome.

Sequence No.	*Primer name*	*5' primer sequence 3'*
1	DSR1F	5'- CAGGGYTGGRTMCACTGCCAYA-3'
2	DSR1R	5'- ACVGCRCCRCACATRTTCA – 3'
3	DSR2F	5'- CAGGGYTGGRTMCACTGCCA-3'
4	DSR2R	5'- GCRCCRCACATRTTCAGSCA-3'
5	SOX1F	5'-CGGTTACCCGCTGCGWCTC-3'
6	SOX1R	5'- TGRTGTGCGYATCTGTCT-3'
7	SOX2F	5'-GTTACCCGCTGCGWCTCRTSGT-3'
8	SOX2R	5'- GCGYATCTGTCTTCTGGGYCG-3'
9	MCR1F	5'-ATGCTNTWCGACCARA-3'
10	MCR1R	5'-ATRTTGTCNGTGTAKGCMGCGGT-3'
11	MCR2F	5'-GCTNTWCGACCARATCTGG-3'
12	MCR2R	5'-GTGTAKGCMGCGGTWGCRTA-3'

6.2.3 Primer optimization, cloning, and qPCR assay

6.2.3.1 Primer optimization

To optimize *dsrB* primers, genomic DNA from *Desulfovibrio vulgaris* Hildenborough was used. For the *mcrA* and *sox* genes, toluene enrichments under methanogenic conditions and a nitrate-reducing enrichment from souring bioreactors were used, respectively. Good quality primers were chosen based on the size and purity of the expected PCR product visualized on a 1.5 % agarose gel.

The primer optimization tested for different annealing temperatures (Ta) that yielded the single PCR product (e.g. most pure, correct size). The PCR mixture comprised 12.5 μL of 2xPCR Master Mix (Fermentas), 10.5 μL of nuclease-free water (Fermentas), 1 μL of genomic DNA (~2 ng) and 0.5 μL of the specific primer (20 pmol μL-1) for a 25 μL PCR reaction. The PCR program used is described in section 3.4.2.

6.2.3.2 TOPO TA cloning

The resulting PCR product from each amplification was purified using the QIA PCR purification kit (Qiagen), quantified using a Qubit Fluorometer (Invitrogen), and ligated into a TOPO PCR 2.1 plasmid vector at a 1:1 plasmid-PCR ratio. Cloning of the ligated product was achieved according to the manufacturer's protocol (Invitrogen). From each kanamycin (kan) LB plate spread with the product of interest, at least 6 white to light blue colonies were selected. The positive colonies were transferred to kan-LB broth for 24 h incubation followed by plasmid purification with Qiaprep Spin Miniprep kit (Qiagen). The plasmid was then PCR amplified with same primer set and PCR

program as previously described. The concentration of each selected plasmid was determined by fluorimetry and the gene copy number was calculated [179]. Quantitative PCR standard solutions were made with quantified plasmid. Stock standard solutions were serially diluted from 10^2 copies · μL^{-1} to 10^8 copies · μL^{-1} in a final volume of 200 µL. Each dilution was then PCR amplified to check for purity of the product.

6.2.3.3 Quantitative PCR assay

"Real-time" PCR was performed in a Rotor-Gene Q (Qiagen) quantitative thermocycler and the data were analyzed using Rotor Gene Q software (Qiagen). The reactions were performed in 0.2 mL PCR tubes for real-time PCR (Qiagen).The PCR samples had 12.5 µL total volume and contained 6.25 µL Reaction Mix, 0.3 µL each of forward and reverse primers (500 nM); 4.65 µL of qPCR grade water and 1.0 µL of genomic DNA. The real-time PCR reaction was subjected to the programs shown in Table 6-3 depending on the particular gene.

Software-derived standard curves were generated for *dsrB* (R^2=0.992, efficiency=0.9459, and slope=-3.459); *mcrA* (R^2= 0.993, efficiency= 1.105, and slope= -3.093); and *sox* (R^2= 0.980, efficiency=1.29, and slope= -2.776). Melt curve analysis was performed on all samples and no significant primer dimers were detected.

To rule out false positives, a no template control (NTC) was used as negative control. Skim milk powder DNA was also PCR quantified and subtracted from all the samples. The percentage of the functional genes normalized to the total 16S rRNA gene was calculated. All samples, standards, and NTC were done in triplicate.

Table 6-3 real time PCR program for each particular gene

gene	***PCR program***
dsrB	95 °C for 3 min; 30 cycles of: 95 °C for 30 s; 55 °C for 45 s: data acquisition
mcrA	95 °C for 5 min; touchdown decreasing 1 °C every cycle for 40 cycles [95 °C for 30 50 °C for 45 s; 57 °C for 60 s]: data acquisition
Sox	95 °C for 10 min; 40 cycles of: 95 °C for 30 s; 57 °C for 45 s: data acquisition
16S rRNA	95 °C for 10 min; 40 cycles of: 95 °C for 10 s; 60 °C for 30 s: data acquisition

6.3 Results

6.3.1 Functional gene primers selected and qPCR

Table 6-4 lists the set of primers selected and their corresponding optimized annealing temperature (Ta) and amplicon size in base pairs. The copy number obtained for each sample was converted into gene copies per gram of wet tailings.

The genes targeted (*dsrB*, *mcrA*, *sox*) were detected in all the tailings depths evaluated, although *dsrB* and *mcrA* were more abundant [Figure 6-1]. Comparing the 3 years of sampling, pond 6 2008 is more dominated by communities that contained the *dsrB* genes whereas in the pond 6 2010 samples the *mcrA* gene is more dominant throughout the pond [Figure 6-1, Table 6-5]. The proportions of *dsrB* and *mcrA* for pond 6 2011 are relatively close [Table 6-5]. Interestingly, *sox* genes were relatively low in abundance and not present in the surface samples. Pond 6 2008 had a very homogeneous distribution of *dsrB* and *mcrA* in the pond [Figure 6-1(A)] with gene copy numbers per g of tailings averaging 4.7×10^7 and 2.8×10^7, respectively. Similarly for pond 6 2010, the average gene copy numbers per g of tailings were 3.8×10^7 for *dsrB* and 8.1×10^7 for *mcrA*. Pond 6 2011 appeared more stratified, with the greatest gene copy numbers at the top and bottom of the of the MFT layers [Figure 6-1(B)]. For these samples, the average gene copy number per g of tailings were 9.6×10^7 and 7.1×10^7 for *dsrB* and *mcrA* genes, respectively.

Table 6-4 Set of primers selected for functional gene study in tailings and their optimal annealing temperature (Ta), amplicon size, and copy number obtained from each reference organism.

Set of primers	*Ta (℃)*	*Amplicon size (bp)*	*Copy number*
dsr1f/dsr2r	55	145	5.4×10^9
sox2f/sox2r	57	94	1.1×10^{10}
mcr1f/mcr1r	50	90	1.0×10^{10}
16S rDNA 341f/534r	60	193	7.2×10^8

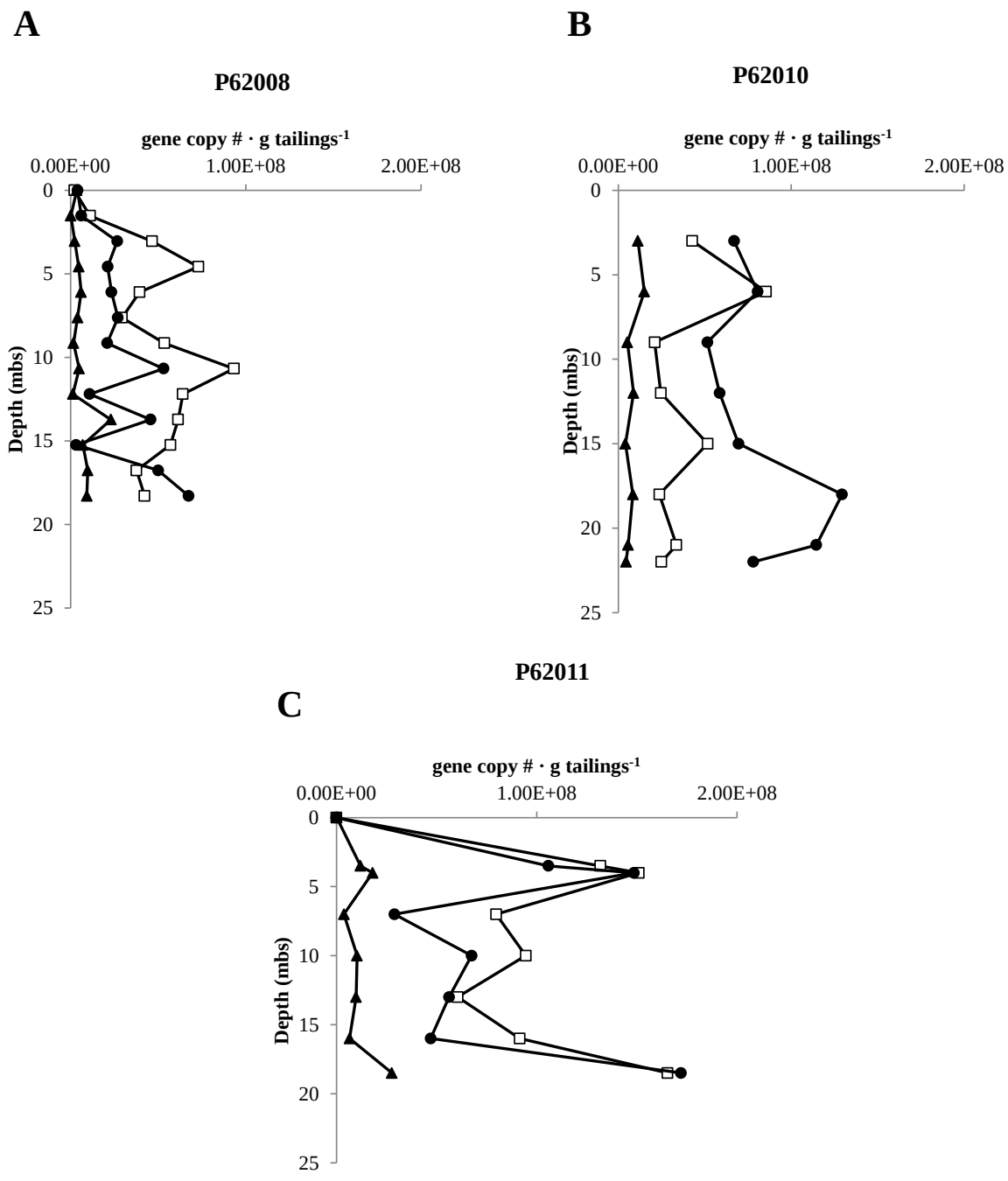


Figure 6-1 Gene copy number of specific genes (*dsrB*, *mcrA*, *sox*) per gram of tailings, quantified in tailings samples from pond 6. (A) Sampled in 2008, (B) Sampled in 2010, and (C) Sampled in 2011. (▲) *sox*, (●) *mcrA*, and (□) *dsrB*.

The percentage of each functional gene contained in relation tothe total amount of 16S rRNA genes quantified, shows that out of the total 16S rRNA gene, pond 6 2008 and pond 6 2011 have higher numbers of *dsrB* genes with respect to *mcrA* [Table 6-5]. For pond 6 2008, the abundance of *dsr* genes is almost double that of of *mcrA* but for pond 6 2011, less of a difference is observed [Table 6-5]. On the other hand, in pond 6 2010 the proportion of *dsrB* genes with respect to *mcrA* is the opposite wherein *mcrA* gene copy numbers are double those of *dsrB* genes. *Sox* genes are the least abundant for all the samples studied [Table 6-5], with gene copy numbers averaging 10^6 and 10^7 for all the tailings samples, one to two orders of magnitude lower than for the *dsr*B and *mcrA* genes.

Table 6-5 Average percent of functional genes normalized to the total 16S rRNA genes. These values are obtained by dividing the copy number of each particular gene by the copy number of the 16S rRNA for each depth, and then averaging the values of all depths for each pond.

Pond sample	*dsrB(%)*	*mcrA(%)*	*Sox(%)*
P62008	51.2	28.5	5.9
P62010	7.9	16.1	1.6
P62011	70.0	56.8	8.4

6.3.2 Correlation of functional genes with microbial activity and chemistry composition as a function of depth in the tailings samples.

The pattern of *dsrB* gene numbers measured in the pond 6 2008 samples correlates well with the SRR and abundance of *Desulfocapsa* as a function of depth [Figure 6-2(A, B, C)]. For example, all three measurements show evidence for elevated sulfate reduction from 12 to 15 mbs. Microorganisms containing the sulfite oxidases (Sox) were also highly detected at these same depths but appeared to correlate most closely with *Thiobacillus sp.* and the SRR profile [Figure 6-2(B, D, E)].

Rates of methanogenesis showed a fluctuating distribution throughout the pond for the 2008 samples [Figure 6-2 (G)]. This pattern positively correlated with the *mcrA* gene quantification as a function of depth [Figure 6-2 (F, G)]. However, the *mcrA* gene quantification profile did not closely parallel any particular taxon of methanogens [Figure 6-2 (H, I, J)]. Nevertheless, the *mcrA* gene depth-dependent profile is consistent with the abundance of different taxa at different levels of the pond; *Methanosaeta* and *Methanolinea* likely contribute to CH_4 production at the upper depths [Figure 6-2 (H)], *Methanospirillum* and *Methanosarcina* in the middle [Figure 6-2 (I)], *Methanomethylovorans* and *Methanobacterium* contribute to CH_4 production in the deeper zones [Figure 6-2(J)].

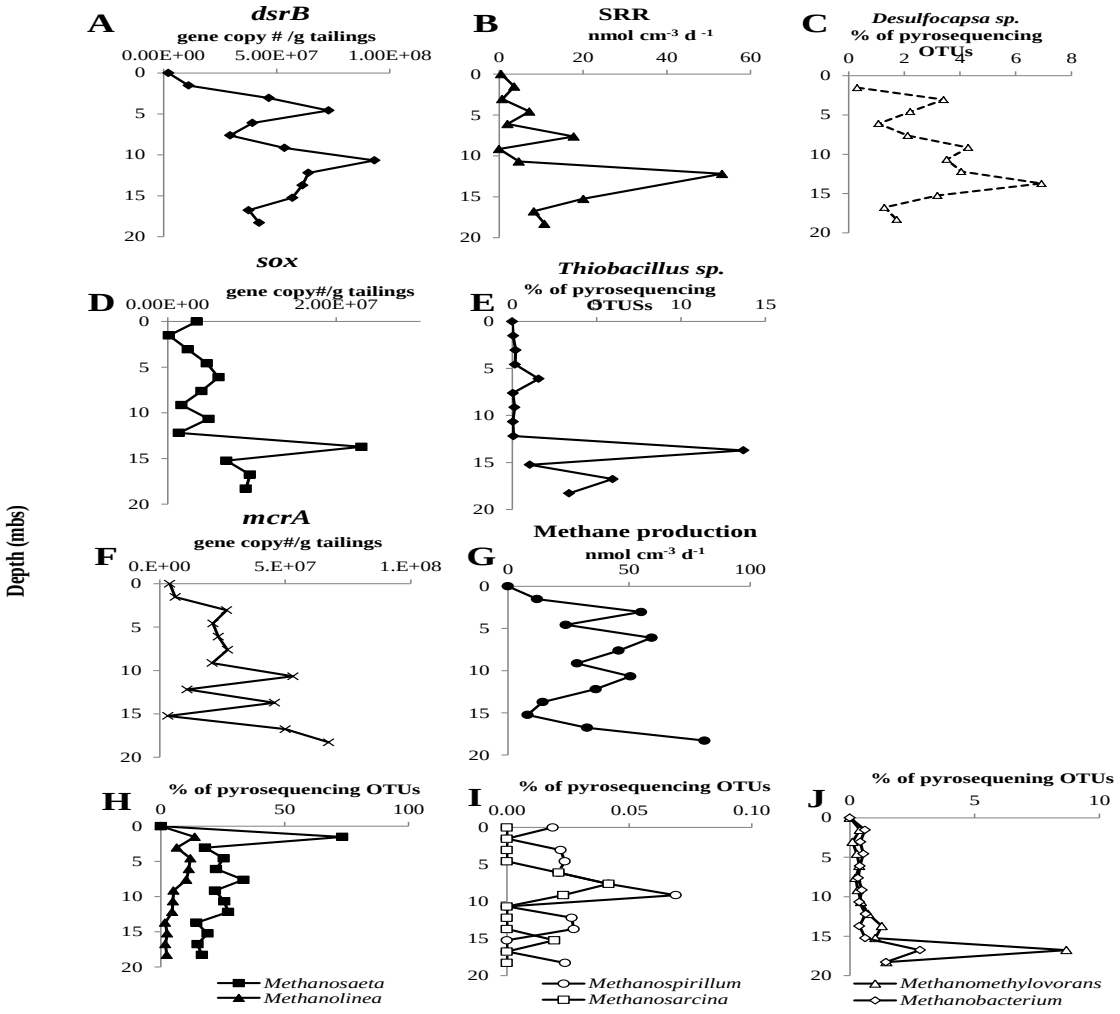


Figure 6-2 Correlation between qPCR of the functional genes (*dsr*B, *mcr*A, *sox*), in pond 6 2008 with the microbial activity and key microbial genera detected by 16S rRNA gene sequencing. (A) *dsr*B gene, (B) SRR, (C) % of abundance of *Desulfocapsa* sp., (D) *sox* gene, (E) % of abundance of *Thiobacillus* sp., (F) *mcr*A gene, (G) methane production rate, and (H,I,J) % abundance of methanogen taxa present in pond 6 2008 samples. Error bars obtained from three replicates are on the order of 10^2 for the PCR data analyses (not visible).

Correlation analysis for the pond 6 2010 and 2011 samples were similar to that obtained for pond 6 2008. For example, the abundance of *Desulfocapsa* sp. mirrors the SRR and the copy number for the *dsrB* gene [Figure 6-3 (A, B, C,) and Figure 6-4 (A, B, C)], while the % abundance of *Thiobacillus* sp. matches with that of the *sox* gene and SRR [Figure 6-3 (D, E) and Figure 6-4 (D, E)]. For pond 6 2010, sulfate reduction and sulfite oxidation potential is near the more shallow depths of the pond [Figure 6-3 (B, D)]. However in these samples, the *sox* gene profile does not correlate as closely with *Thiobacillus* sp. abundance [Figure 6-3 (D, E)] as was observed in the pond 6 2008 profiles [Figure 6-2 (D, E)], although % abundance of *Thiobacillus* sp. is also comparatively low. Further, in the 2010 samples, the *mcrA* gene copy numbers also did not correlate well with the rates of methanogenesis or the % abundances of the dominant methanogens.

For pond 6 2011, sulfate reduction and sulfite oxidation processes are more towards the top and bottom of the pond [Figure 6-4 (A, B, C, D, E). For the pond 6 2011 samples, the *sox* gene copy numbers closely paralleled the % abundance of *Thiobacillus*, especially at the deeper depths [Figure 6-4 (D, E)] (compared to the 2010 samples). The *mcrA* gene copy numbers closely aligned with % abundance of the dominant methanogen (*Methanolinea* sp.) and the methanogenesis rates, especially at the shallower depths [Figure 6-4 (F, G, H)].

Overall, the *dsrB* gene profiles aligned well with SRR and SRB abundance in all years sampled. The *sox* and *mcrA* genes closely mirrored microbial taxa and/or activity in teh 2008 and 2011 samples, but did not correlate as well with these other measurements for the 2010 samples.

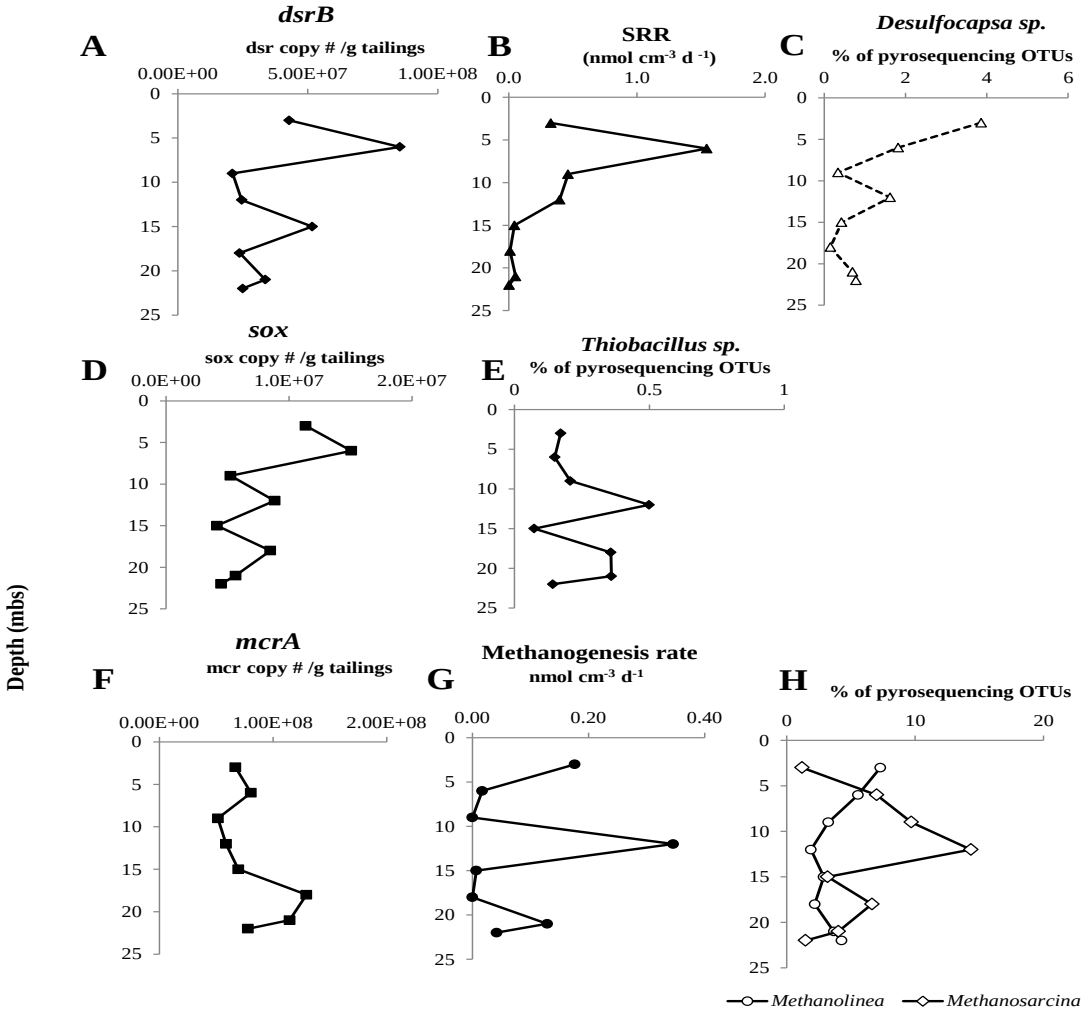


Figure 6-3 Correlation between qPCR of the functional genes (*dsrB, mcrA, sox*), in pond 6 2010 with the microbial activity, and key microbial genera detected by 16S rRNA gene sequencing. (A) *dsrB* gene, (B) SRR, (C) % abundance of *Desulfocapsa* sp., (D) *sox* gene, (E) % abundance of *Thiobacillus* sp., (F) *mcrA* gene, (G) methane production rate, and (H) % abundance of methanogen taxa present in pond 6 2010 samples. Error bars obtained from three replicates are on the order of 10^2 for the qPCR analyses (not visible).

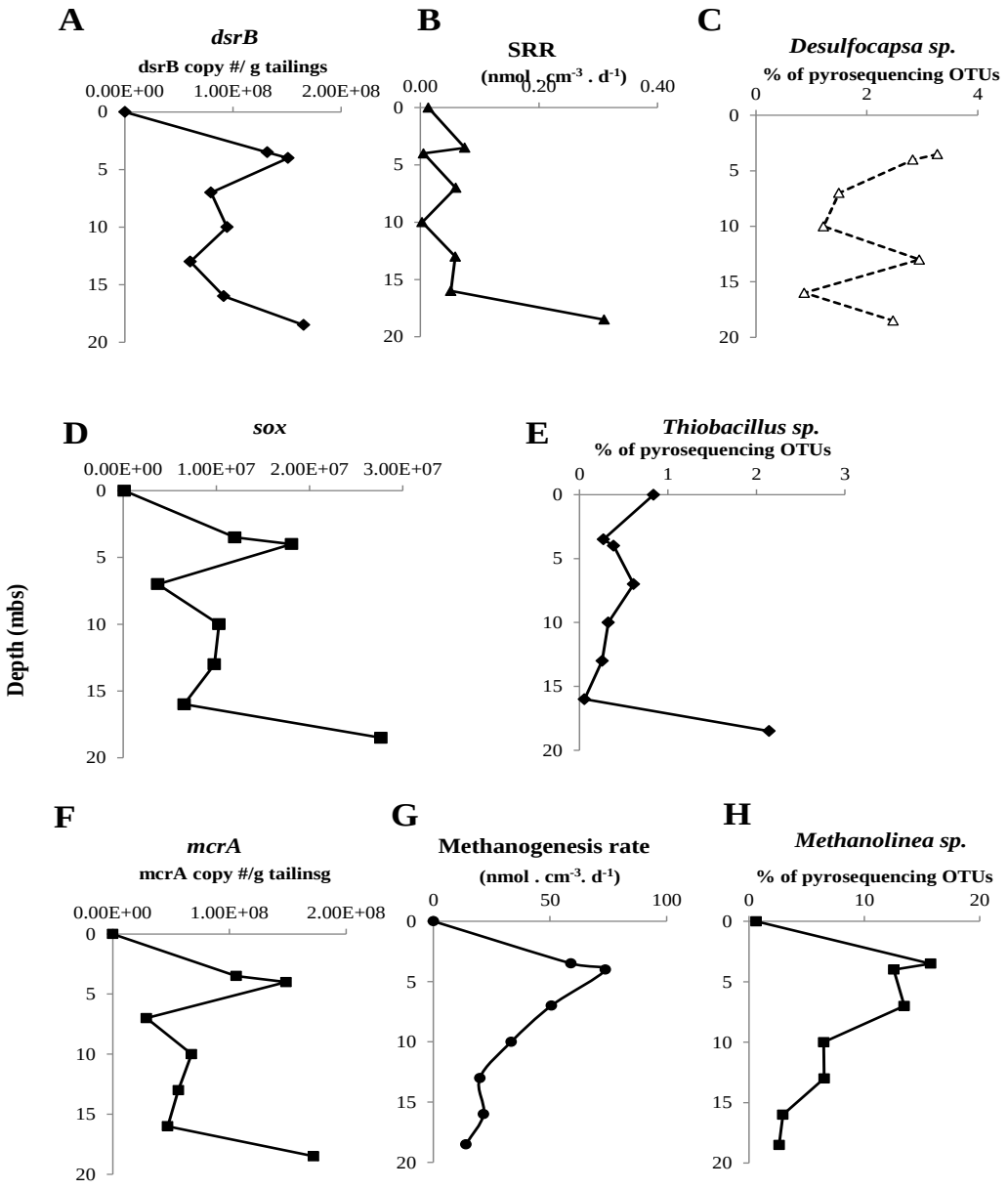


Figure 6-4 Correlation between qPCR results of the functional genes (*dsrB, mcrA, sox*), in pond 6 2011 with the microbial activity, and microbial genera detected by 16S rRNA gene sequencing. (A) *dsrB* gene, (B) SRR, (C) % of abundance of *Desulfocapsa* sp., (D) *sox* gene, (E) % abundance of *Thiobacillus* sp., (F) *mcrA* gene, (G) methane production rate, and (H) % of abundance of *Methanolinea* sp. Error bars obtained from three replicates are on the order of 10^2 for the qPCR data analyses (not visible).

6.4 Discussion

Molecular approaches based on 16S rRNA gene sequence analysis have become the norm to identify microbial community members in different ecosystems. The study of these sequences allows us to understand the community structure, diversity, and phylogeny of microbes in their natural environment [180,181]. In particular, quantitative PCR is a very sensitive method for the quantification of microbes from different samples [182]. In this study, we quantified the distribution of microbes harboring key functions in an active tailings pond based on the copy number of genes carrying out sulfate reduction (*dsrB*), methane production (*mcrA*) and sulfite oxidation (*sox*). Our selected ecosystem was pond 6 from Suncor Energy Inc., which was monitored over the years 2008, 2010, and 2011. The metagenome of this pond was recently obtained (unpublished data) allowing us to design primers to target the genes involved in these main microbial processes currently ongoing in tailings ponds [16,44]. This increased the accuracy of our results allowing us to establish correlation studies with the 16S rRNA gene survey and physiology measurements previously described [Chapter Four].

Initially, the primers were designed to study the actual active microbes in the pond by targeting RNA. However, failure to extract RNA from tailings prevented us from determining the actual active gene copy numbers. Hence, the results discussed here are only based on DNA approaches. However we were able to obtain information regarding the vertical spatial distribution of potential activities in the pond and compare the profiles with physiological measurments and key taxa. To our knowledge this is the first time that quantitive PCR has been used to target functional genes in tailings ponds. Therefore, the

results obtained here further broaden the understanding of the active physiology in tailings ponds.

The percentage of *dsrB*, *mcrA* and *sox* genes relative to the 16S rRNA gene in our tailings samples, confirms the importance these functions have in tailings ponds, in particular for the activities of sulfate reduction and methanogenesis [Table 6-5]. Our results showed an increase of methanogenesis from 2008 to 2010, likely due to a change in pond management by the operators. In 2008, pond 6 was actively receiving fresh tailings from the extraction plant (amended with $CaSO_4$) therefore one would expect to obtain more sulfate reduction (*dsrB* activity) compared to methanogenesis. However, in 2010 no more fresh tailings were being added to the pond, thus reducing the amount of electron acceptor available for SRB. Hence, the *mcrA* percentage was higher with respect to that of *dsrB* by 2010 [Table 6-5].These results correspond with what was observed for the 16S rRNA survey conducted on this pond for the same period (Chapter Four). The numbers obtained in 2011 were higher than expected; this could be due to the presence of several gene copy numbers in the microbial population. More experiments need to be done in order to clarify these results.

The *mcrA* gene has been used to target methanogenic Archaea in crude oil hydrocarbon-impacted environments with success, helping in the identification of certain key taxa [183] and for elucidating certain roles played by this group of microbes [184]. In addition, the specificity of the *mcrA* gene has helped to reveal more diversity in the methanogenic communities than was initially observed with the 16S rRNA in other studies [185].

Based on the 454 pyrosequencing characterization of the16S rRNA gene, pond 6 is mainly inhabited by the methanogens *Methanosaeta* and *Methanolinea* which are distributed in a heterogeneous "fluctuating" arrangement throughout all the depths studied in the pond, but with a tendency to be more abundant in the upper layers of the pond (Chapter Four). Quantitative PCR confirms the methanogenic characteristic of the pond but the correlation between these two groups of Archaea and the *mcrA* gene was not always congruent. However, for the 2008 samples the *mcrA* profiles did correlate positively with the rates of methane production activity as measured in the laboratory tests. We postulate that this could be due to the presence of a wide variety of methanogenic taxa that are distributed at discrete depths in the pond. Nine genera of methanogens were identified to the genus level, but for others the identification only covered the phylum, class, order or family level [Appendix Two:]

It is known that the *mcrA* gene is exclusively found in methanogens and in the anaerobic methane oxidizing Archaea *(*AMO*)* [186]. Therefore, another possible explanation for this lack of similarities between the 16S rRNA gene profile and the *mcrA* gene could be that our primers targeted both methanogens and ANME (anaerobic methanotrophs) in the tailings ponds. In fact, previous microbial community studies in oil sand samples using 16S rRNA gene sequencing have revealed the presence of ANME – 1a [187]. Our samples did contain Archaea sequences that are phylogenetically similar to the ANME linages such as *Methanosarcinales* and *Methanomicrobiales* [188] although in relatively low abundance. Similarly, the sulfate reducers thought to be associated with ANME (within the *Desulfosarcina*/*Desulfococcus* cluster of the *Desulfobacteraceae* [189]), were also found in our tailings 16S rRNA gene pyrosequencing characterization, in some

cases only identified to the family level [Appendix Two:]. No previous research has reported the presence of ANME in tailings ponds thus, other molecular techniques and experiments will need to be implemented in order to clarify this.

By contrast, the *dsrB* gene quantification results correlated well with the SRR activity and mirrored the profile of the dominant SRB like *Desulfocapsa sp*. [Figure 6-2, Figure 6-3, Figure 6-4(A, B, C)]. This finding supports the proposed role that members affiliated with *Desulfocapsa* sp. play in the reduction of sulfate in tailings ponds. As was explained in Chapter Four, members of this genus can serve both as sulfite disproportionating or sulfate-reducing organisms. We do not out rule the participation of other groups of SRB such as the sulfur disproportionator *Desulfobulbus* sp. [190] found in samples from 2011, or *Desulfoglaeba* sp. found in 2010 (among others), however, *Desulfocapsa* sp. is a common, abundant genus found in all years sampled, so, we can infer its importance in the sampled tailings pond. Furthermore, the mirroring of *Thiobacillus* sp. with SRR activity and *sox* gene quantification also sheds some light into the understanding of the sulfur metabolism in tailings ponds. For example, it can be hypothesized that *Thiobacillus* sp. and *Desulfocapsa* sp. may be metabolically (syntrophically) related. Syntrophic associations based on the interchange of reduced and oxidized sulfur compounds have been previously described for laboratory experiments of sulfur oxidizing *Thiobacillus thioparus* T5 and the sulfate-reducing *Desulfovibrio desulfuricans* PA2805 [191]. These interactions usually occur under oxygen-limited conditions where *Thiobacillus* sp. oxidize sulfide to elemental sulfur and thiosulfate. The resulting compounds can be used by SRB as electron acceptors while reducing hydrogen or organic compounds, thereby establishing a close relationship between the two

microbes. In addition, sulfur and thiosulfate may also be disproportionated into sulfide and sulfate, contributing to the continuity of the sulfur cycle. Other interactions like aggregations between a sulfur oxidizer and a sulfate reducer/sulfur disproportionator living in the chemocline of a meromictic lake, have also been published [192]. Based on the discussion above, the interaction between *Thiobacillus* and *Desulfocapsa* species in tailings ponds may be occurring as shown in Figure 6-5. Measurements of Fe (II) in tailings since 2010 [Appendix Twelve:] suggested that iron reduction is likely also an important process in tailings, to which *Thiobacillus* can also contribute[Figure 6-5].

In our tailings samples, *Thiobacillus* sp. mirrors the vertical distribution in the pond with that of the *sox* genes quantified, especially for 2008 and 2011 samples. Members of bacteria grouped under this genus are very diverse and have been found in different ecosystems [62]. In our study, we targeted sulfite oxidases which oxidize sulfite to sulfate [Equation 4].

Equation 4 Enzymatic reaction catalyzed by sulfite oxidases.

$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$$

The oxidation of sulfur compounds to sulfate has been well studied in *Thiobacillus* sp. in particular in the obligately chemolithoautotrophic facultatively anaerobic bacterium *Thiobacillus denitrificans* [193]. The sequence of its genome revealed more than 50 genes associated with sulfur-compound oxidation (including *sox* genes, *dsr* genes, and genes associated with the AMP-dependent oxidation of sulfite to sulfate) [193].

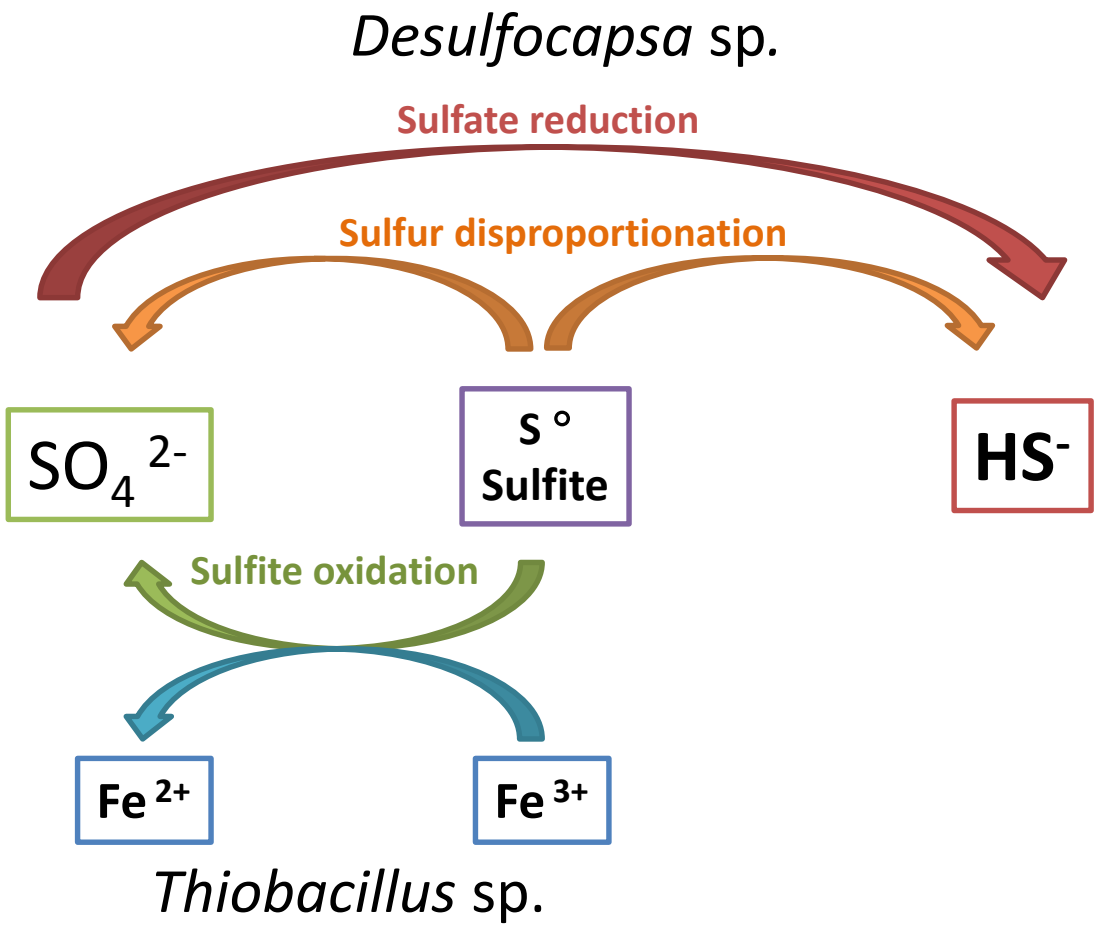


Figure 6-5 Proposed mechanism of microbial interaction between *Desulfocapsa* sp. and *Thiobacillus* sp. occuring in oil sands tailings ponds.

The sulfate yielded by this enzymatic reaction may well be the reason why sulfate is sometimes abundant at some depths in tailings ponds where all the sulfate should theoretically be consumed (e.g., in deeper layers). The oxidation of sulfur compounds coupled to iron reduction has also been well investigated. Providing there is ferric iron (Fe^{3+}) available, this mechanism could possibly be occurring. For example, the oxidation of 1 mol of elemental sulfur coupled to the reduction of 6 mol of iron Fe (III) giving 6 mols of Fe (II) with the concomitant yield of 1 mol of sulfate, has previously been described to occur in some strains of *Thiobacillus ferroxidans* [194]. In addition, the oxidation of sulfur compounds has been observed when coupled to nitrate reduction [195]. However, we do not support the latter, as no nitrate has been detected in these tailings.

In summary, the quantification of the specific genes involved in the metabolic oxido-reduction of sulfur compounds and the production of methane, has helped us corroborate the major microbial physiological processes ongoing in tailings ponds. In particular, the association between *Desulfocapsa* and *Thiobacillus* seems to be playing an important role in the sulfur metabolism and cycling in the tailings. More studies need to be carried out regarding any putative ANME community members in the tailings, thereby discerning the major CH_4 cycling involved in oil sands tailings ponds.

Chapter Seven: **The effects of substrates and alternate electron acceptors on anaerobic microbial communites in oil sands tailings ponds**

7.1 Introduction

It was discussed in Chapter One that naphtha, NA, or bitumen could potentially be carbon sources[1] in oil sands tailings ponds. Several studies regarding the use of naphtha components such as alkanes and BTEX compounds have shown that methanogenic communities in tailings ponds and other environments are able to thrive at their expense [41,47,68,69]. Biodegradation of NAs by tailing pond microbes has also been carried out using mainly commercially available NAs which can serve as surrogates to natural NAs [65] (see Chapter Five). These studies have been carried out using tailings samples obtained mainly from Syncrude operations or non-tailings sources. In contrast, the substrates used by microbial communities in other tailings ponds (e.g., managed by other oil sands operators) are poorly understood.

Therefore, using anaerobic samples from a Suncor tailings pond, we assessed the effects of naphtha, NA, or bitumen as carbon sources on the tailings microbial communities. We focussed mainly on the effects of these carbon sources under methanogenic and sulfate-reducing conditions, as these physiologies are prevalent in the Suncor tailings ponds [Chapter Four and Chapter Six]. In addition, the effects of nitrate addition on tailings communities and on sulfate reduction and methanogenesis was explained in the content of using an alternate densification reagents.

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[1] For simplicity, carbon and energy sources will be referred to as carbon sources

Understanding how hydrocarbon-degrading communities function in tailings ponds due to the presence of various carbon sources and nutrients or electron acceptors can be of significant importance to improve pond management.

7.2 Methods

7.2.1 Anaerobic enrichment to determine the effects of carbon sources for sulfate reduction and methanogenesis in tailings ponds.

Enrichments were set up under sulfate reducing and methanogenic conditions, using tailings as the microbial inoculum and NA, naphtha, or bitumen as the carbon sources. Since tailings contain enough nutrients for the indigenous population to grow and reproduce, several transfers were necessary before the effect of the various carbon sources were assessed. Three transfers were accomplished in a period of a year and a half.

Microbial inoculum:

The microbial inoculum was prepared from Suncor tailings pond 6 sampled in 2010, using the samples collected from 6 and 22 mbs. The selection of the depths was based on distinct chemical profiles exhibited [Figure 4-8]. For instance, at 6 mbs the highest concentration of sulfide was measured (0.5 mM) and at 22 mbs, sulfide concentrations were among the lowest (0.1 mM) [Figure 4-8 (B)], suggesting that sulfate reduction may be more important in incubations prepared from the 6 mbs sample than the 22 mbs sample.

The experiment started with a 5 mL pre-culture which was transferred (1 mL) after 45 days of incubation into 60 mL serum bottles containing Pfennig medium . The successive transfers (5 mL) were done at approximately every 150 days, when sulfate was totally consumed, and methane was produced in the negative controls (presumably at the expense of residual tailings substrates). Figure 7-1 shows the appearance of the bottles for each of the transfers.

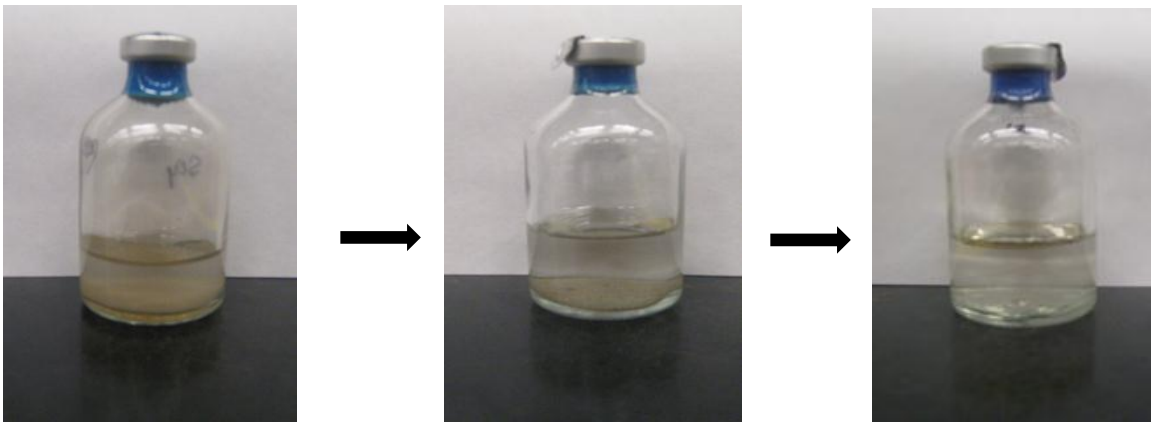
Carbon sources:

Natural NA were extracted from tailings pond 6 surface water according to a modification of the method of Holowenko *et al.*, (2001) [32] and were distributed in anaerobic serum bottles with Pfennig medium at a concentration of 20 mg $\cdot$ L^{-1} (0.3 %). Naphtha, obtained from Plant 4 at Suncor, was used at 0.5 vol %. This substrate was added together with 1 mL of heptamethylnonane to allow for slow release of naphtha into the medium and to minimize toxicity. Finally, another set of experiments with bitumen, extracted from oil sands using DCM, as the only carbon source, were prepared. Each serum bottle was prepared with 15 mL of Pfennig medium [Table 3-8] supplemented with 0.2 mL of vitamins [Table 3-10] and 5 mM of sulfate to the sulfate-reducing enrichments only.

Experiment:

Table 7-1 summarizes the experimental design for the enrichments prepared from the 6 and 22 mbs samples. For the first two transfers, the experiments had no replicates. Only for the final set of experiments, was each condition prepared in triplicate. Bottles were incubated in the dark at room temperature.

Methane formation (section 3.3.2.2) and sulfate depletion (section 3.2.3) were measured every month. Microbial community composition was analyzed for each bottle soon after the third transfer was initiated.



1st transfer 2nd transfer 3rd transfer

Figure 7-1 Appearance of enrichments after each transfer (done ~ every 150 days). Notice how residual tailings are more visible in the first two transfers, but a small amount still remains in the third transfer.

Table 7-1 Tailings enrichments amended with naphtha (0.5 vol %) or NAs (20 mg · L^{-1}) under methanogenic or sulfate-reducing conditions. Each condition was set up in triplicate in 60 mL serum bottles that contained 15 mL Pfennig salts medium. The microbial inoculum was from Suncor tailings pond 6 sampled in 2010 from 6 or 22 mbs.

6 mbs tailings enrichments	*22 mbs tailings enrichments*
Medium + sulfate + NA (6m)	Medium + sulfate + NA (22m)
Medium + NA (6m)	Medium + NA (22m)
Medium + sulfate + Naphtha (6m)	Medium + sulfate + Naphtha (22m)
Medium + Naphtha (6m)	Medium + Naphtha (22m)
Medium + sulfate (- control) (6m) (no electron donor added)	Medium + sulfate (- control) (22m) (no electron donor added)
Medium (-control) (6m) (no electron acceptor added)	Medium (-control) (22m) (no electron acceptor added)

7.2.2 Nitrate as an alternate electron acceptor in the presence of naphtha

Microbial inoculum and carbon source:

Tailings (10 g) from pond 5 sampled in 2010 (3 mbs) were used as the source of microbial inoculum for determining the effect of nitrate on tailings microbial communities. This depth showed the highest methanogenesis rate (0.4 nmol . cm^{-3} . day^{-1}) [Figure 4-15 (C)].

Naphtha (0.5 %) was used as the carbon source. It was distributed into 60 mL serum bottles that contained 10 mL of Pfennig medium (supplemented with 0.2 mL vitamines mixture [Table 3-10]) together with 1 mL of hepthamethylnonane. Sulfate and nitrate were added at 5 or 10 mM final concentrations.

Experiment:

Table 7-2 summarizes the experimental bottles prepared for this part of the study. Each experimental condition was set up in duplicate with a negative control for each condition (tailings and medium, no substrate and no electron acceptor added). Bottles were incubated in the dark at room temperature. Methane and sulfate concentrations were measured by GC or HPLC as described in sections 3.3.2.2 and 3.2.3, respectively. Sterile and non-sterile controls (tailings + naphtha, or tailings + medium) were also prepared. The experiment covered a period of 256 days after which time the microbial communities were analyzed by 454 pyrosequencing [Section 3.4.3].

Table 7-2 Tailings enrichment experiment using naphtha (0.5 %) as the carbon source with nitrate and/or sulfate as the electron acceptors added at 5 or 10 mM. The microbial inoculum was from pond 5 2010, 3 mbs tailings.

Enrichment	SO_4^{2-} *(5 mM)*	SO_4^{2-} *(10 mM)*	NO_3^- *(5 mM)*	NO_3^- *(10 mM)*
tailings+naphtha+ medium			✓	
tailings+naphtha+ medium				✓
tailings+naphtha+ medium	✓		✓	
tailings+naphtha+ medium		✓	✓	
tailings+naphtha+ medium	✓			✓

7.3 Results

7.3.1 Methane and sulfate in carbon source enrichments experiments

Figure 7-2 shows the results of the first, second, and third transfers on the carbon sources tested under methanogenic conditions. Samples from 6 mbs showed an initial preference for NA (after first transfer) [Figure 7-2 (A)] based on the amount of methane produced. The experiments enriched with tailings from 22 mbs amended with naphtha, produced the highest methane levels in the first transfer [Figure 7-3(B)]. During the first and second transfers, methane produced in the experiments with both carbon sources (NAs and naphtha) produced relatively low methane (~ 50 µmol), however, after the third transfer, the experiments with tailings from 22 mbs amended with naphtha, peaked to around 120 µmol of methane [Figure 7-2(B)]. After the third transfer, experiments with 6 mbs tailings and naphtha showed higher methane production in comparison with experiments with NAs for the same depth of tailings. However, methane production for this depth was always lower than for the experiments with tailings from 22 mbs amended with naphtha [Figure 7-2(B)]. The values of methane obtained from NAs added as a carbon source at both depths were very similar to that of the negative control, where only the bacterial inoculum (tailings) and medium were incubated. This suggests that methane is coming from any background substrates present in the residual tailings rather than from NA metabolism. Although less tailings was present in the serum bottles after the third transfer, a small amount still remained [Figure 7-1]. Bitumen did not serve as carbon source under methanogenic or sulfate-reducing conditions, as no methane was produced or sulfate reduced from any of the experiments. For this reason, no sucessive

transfers were carried out for this substrate [Figure 7-2]. Under sulfate-reducing conditions, similar results were obtained, in which sulfate was depleted in the first and second transfers in the NA- and naphtha-amended bottles relative to controls (data not shown).

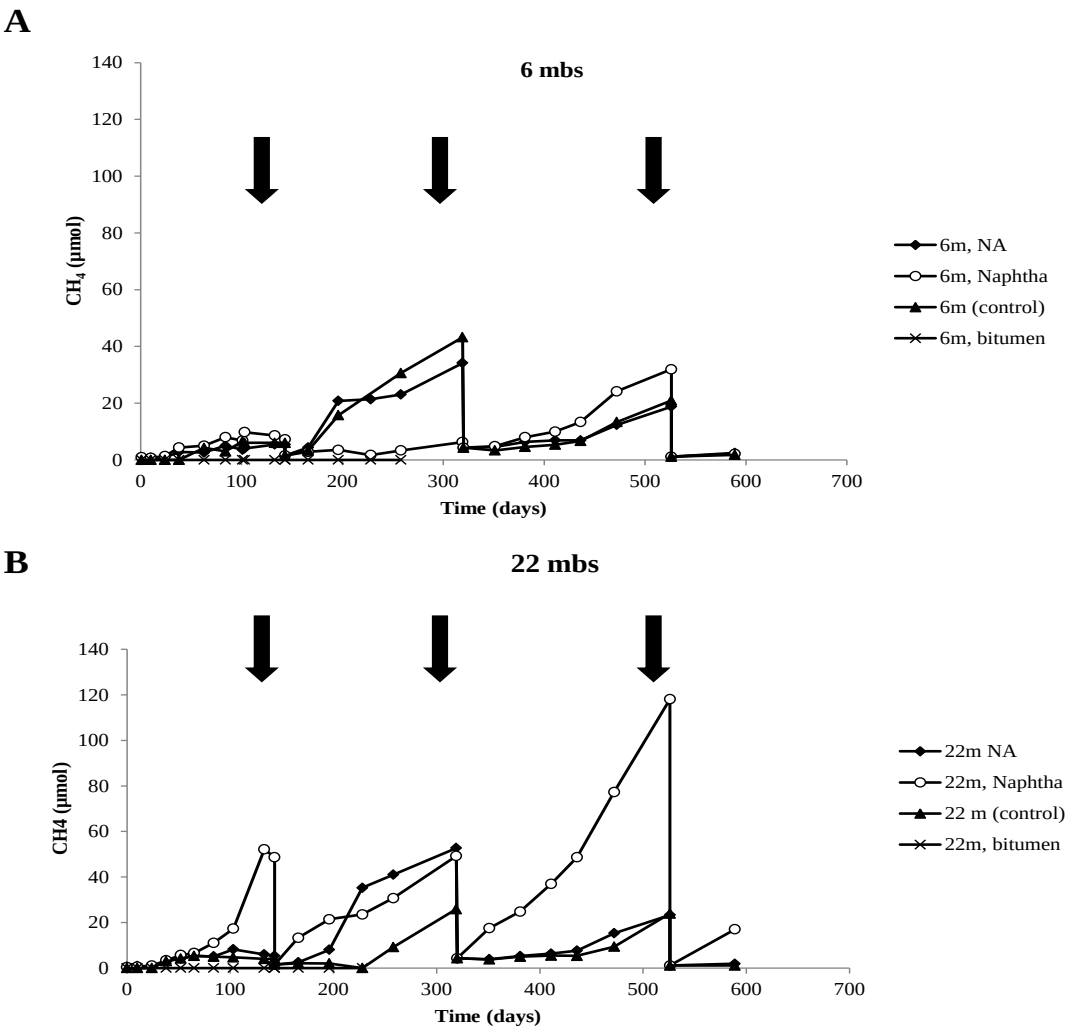


Figure 7-2 Monitoring of methane production by enrichments amended with naphtha or natural NAs to enrichments with tailings from 6 and 22 mbs from pond 6 2010. The arrows indicate each transfer.

After 188 days of incubation following the third transfer, methane production and sulfate depletion were observed when either NA or naphtha substrates were present [Figure 7-3]. However, enrichments amended with naphtha showed more sulfate reduction and methane production relative to the sulfate-free controls, especially when the 22 mbs sample was used as the microbial inoculum [Figure 7-3(C, D)]. In these enrichments, sulfate depletion started after 33 days of incubation and methane production started to increase soon after 97 days [Figure 7-3 (C, D)]. With the 6 mbs sample, no additional depletion of sulfate or production of methane was observed after 100 days of incubation relative to the substrate-free controls [Figure 7-3 (A, B)].

The highest methane production was approximately 32 µmol and it was observed in the samples collected from 22 mbs, while in the 6 mbs enrichments, only 3 µmol of methane was produced after 188 days. No difference in methane production or sulfate reduction in the naphtha or natural NA was observed when tailings originating from the 6 mbs was used as the microbial inoculum [Figure 7-3 (A, B)].

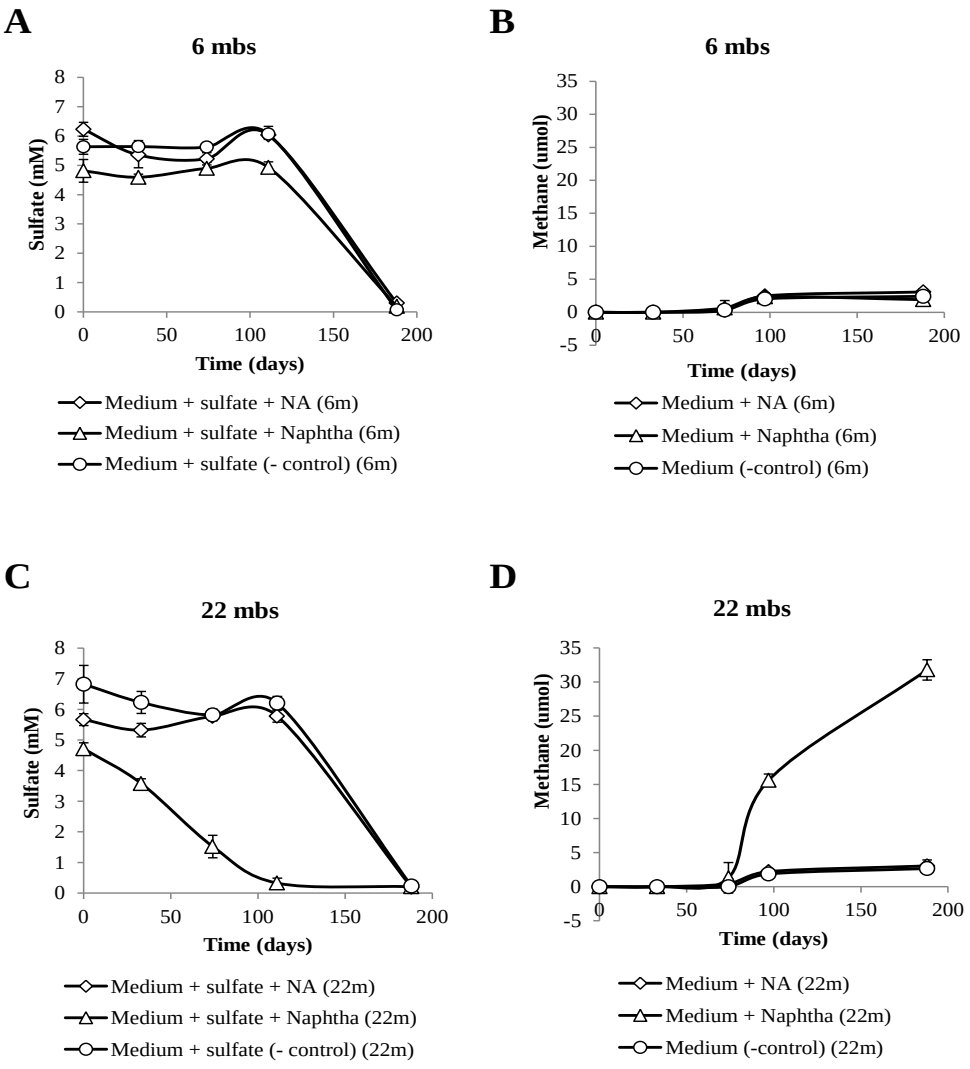


Figure 7-3 Methane production and sulfate depletion observed after the third transfer of tailings enrichments with natural NA or naphtha as carbon sources using samples collected from 6 and 22 mbs of Suncor Pond 5. (A) Sulfate depletion for enrichments with tailings from 6 mbs; (B) methane production from enrichments with tailings from 6 mbs; (C) sulfate depletion from enrichments with tailings from 22 mbs; (D) methane production from enrichments with tailings collected from 22 mbs. The error bars represent the standard error of 3 replicates.

7.3.2 Microbial community when different carbon sources are available in tailings

The microbial community profiles were examined following sulfate – amended or sulfate - free incubations enriched on NA or naphtha from the 6 and 22 mbs microbial communities following the third transfer. For the 6 mbs-derived enrichments, the identified methanogens grouped under the genera *Methanospirillum*, *Methanofollis*, and *Methanobacterium* were enriched, particularly when NA was used as the carbon source. *Methanospirillum* was the most abundant genus reaching a maximum of 44 % of the pyrosequencing OTUs when NA and sulfate were in the medium. When no sulfate but NA were present (e.g. under methanogenic conditions), an abundance of only 25 % of this methanogen was found [Figure 7-4] (A). The addition of naphtha as the sole source of carbon enriched species related to *Desulfobacter* under sulfate reducing conditions, but *Thauera sp.* were most abundant when sulfate was absent. Naphtha enrichments from the 6 mbs samples also selected for the methanogens mentioned above but only at an abundance of 4 % of the pyrosequencing OTUs [Figure 7-4 (A)]. Other genera refers to approximately 40 different genera with abundance lower than 3% [Figure 7-4].

Enrichments prepared with tailings from 22 mbs showed a greater diversity than those with 6 mbs. When NA and sulfate were present, organisms affiliating with *Acidaminobacter* and *Sediminibacterium* were dominant, comprising 13% and 8% of pyrosequencing OTUs, respectively. For this enrichment, methanogens grouped under the genera *Methanospirillum, Methanobacterium,* and *Methanosaeta* at an abundance of only 3 to 5 % of the pyrosequencing OTUs identified at the genus level. However, when no sulfate was available, and NA was the only carbon source, 5 different methanogenic

genera (*Methanospirillum, Methanofollis, Methanobacterium, Methanosaeta, Methanolinea),* were prominent along with bacteria grouped under *Thauera* and *Acidovorax.*

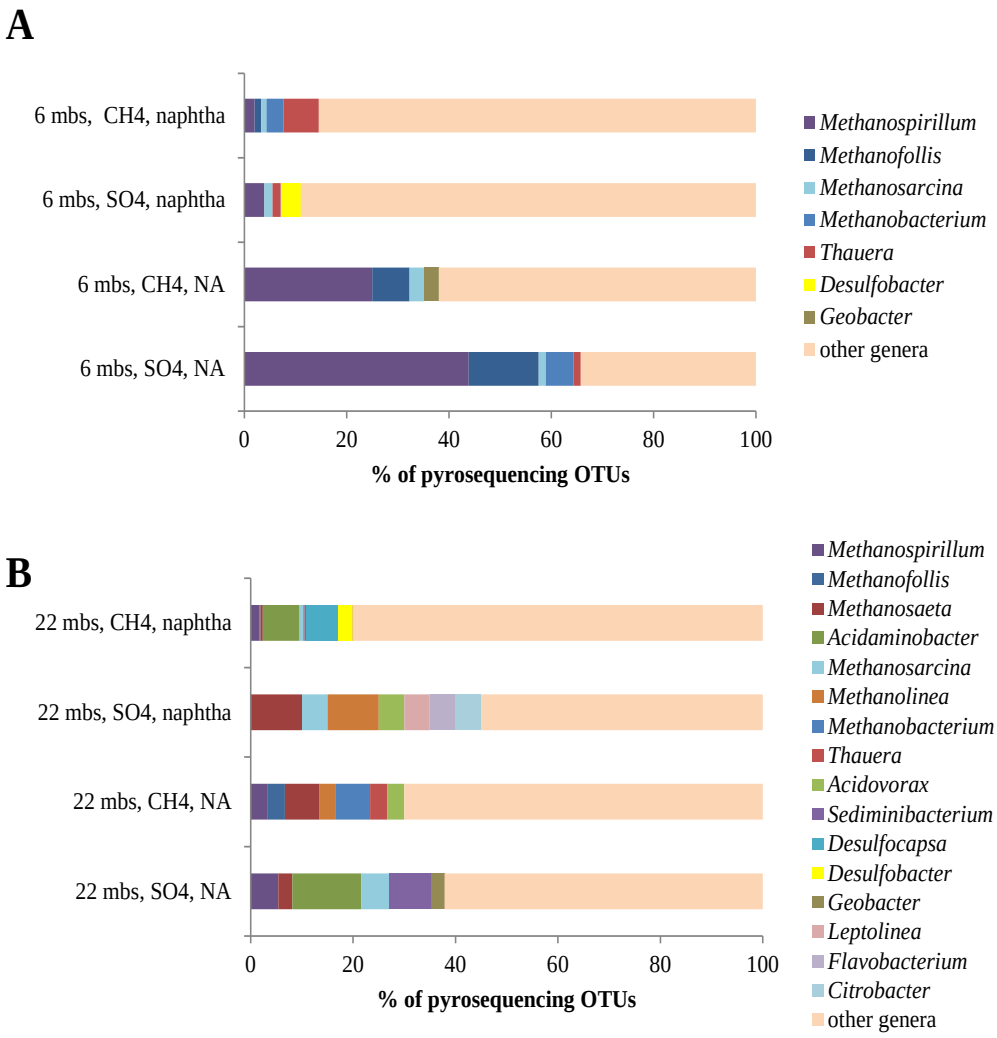


Figure 7-4 Microbial community analysis of tailings enrichments from pond 5 (2010) samples identified at the genus level after the third tranfer of NA and naphtha-amended enrichments. Naphtha and NA were used as sole carbon sources under methanogenic or sulfate reducing conditions. (A) Enrichments prepared with tailings from 6 mbs, (B) enrichments prepared with tailings from 22 mbs.

For the naphtha- and sulfate-amended enrichments prepared with tailings from 22 mbs, species belonging to *Leptolinea*, *Flavobacterium*, *Acidovorax* and *Citrobacter* were abundant. Also, methanogens affiliating with *Methanosarcina*, *Methanosaeta*, and *Methanolinea* were relatively abundant. For this same depth (22 mbs), under methanogenic conditions, different methanogens were found with the exception of *Methanosarcina*. They included *Methanospirillum*, *Methanofollis*, and *Methanobacterium* [Figure 7-4(B)].

In general, both carbon sources enriched for methanogenic Archaea and sulfate reducers among other genera including *Thauera* and *Geobacter*. However, in the 22 mbs enrichments, a more diverse population was present based on 454 pyrosequencing and identifications at the genus level. For example, members of the genera *Flavobacterium*, *Acidovorax*, *Acidaminobacter*, *Sediminibacterium*, and *Citrobacter* were in the 22 mbs enrichments but not in the 6 mbs enrichments. Naphtha as a carbon source enriched both methanogens and SRB in the presence or absence of sulfate.

7.3.3 Methane inhibition by nitrate

In addition to examining the potential for a variety of substrates to drive sulfate reduction and methanogenesis, the effect of nitrate addition as an alternate electron acceptor was also considered. Specifically, the effect of nitrate addition on methane production and sulfate reduction was assessed. In these experiments, naphtha was added as the carbon source because this carbon source appeared to be driving methanogenesis and sulfate reduction in our previous experiments [Figure 7-3(C, D)].

The addition of naphtha improved the microbial activity in all the conditions tested as the consumption of sulfate and nitrate was faster when naphtha was available [Figure 7-5 and Figure 7-6]. Little difference in the methane inhibition was observed between the control with naphtha and the experiment with the different nitrate and sulfate additions. However, methane was significantly inhibited when compared to the controls that had none of these electron acceptors [Figure 7-5 and Figure 7-6].

In all the experiments, a lag phase of approximately 100 days was observed. After this time, the methane production sharply increased in the experiments with no electron acceptors [Figure 7-5 and Figure 7-6]. For experiments with only nitrate added, after 100 days of incubation, the nitrate was completely consumed in the 5 mM nitrate enrichments or half way consumed in the 10 mM nitrate enrichments [Figure 7-5]. However, for these experiments, the nitrite concentrations remained relatively constant for the same period of time and the sulfate concentrations were also relatively constant [Figure 7-5].

In the enrichments where both sulfate and nitrate were added, nitrate was reduced faster than the sulfate, specifically in the experiments amended with naphtha [Figure 7-6]. Sulfate reduction started to occur when nitrate had been completely depleted. Interestingly, for these enrichments the concentration of nitrite increased 3 fold after 100 days and then remained constant. However, the nitrite concentration did not reach stoichiometrically-expected amounts based on initial nitrate concentrations added [Figure 7-6]. The values of nitrate shown in the graphs are always a little bit above the initial concentration added presumably due to interference in the HPLC measurements.

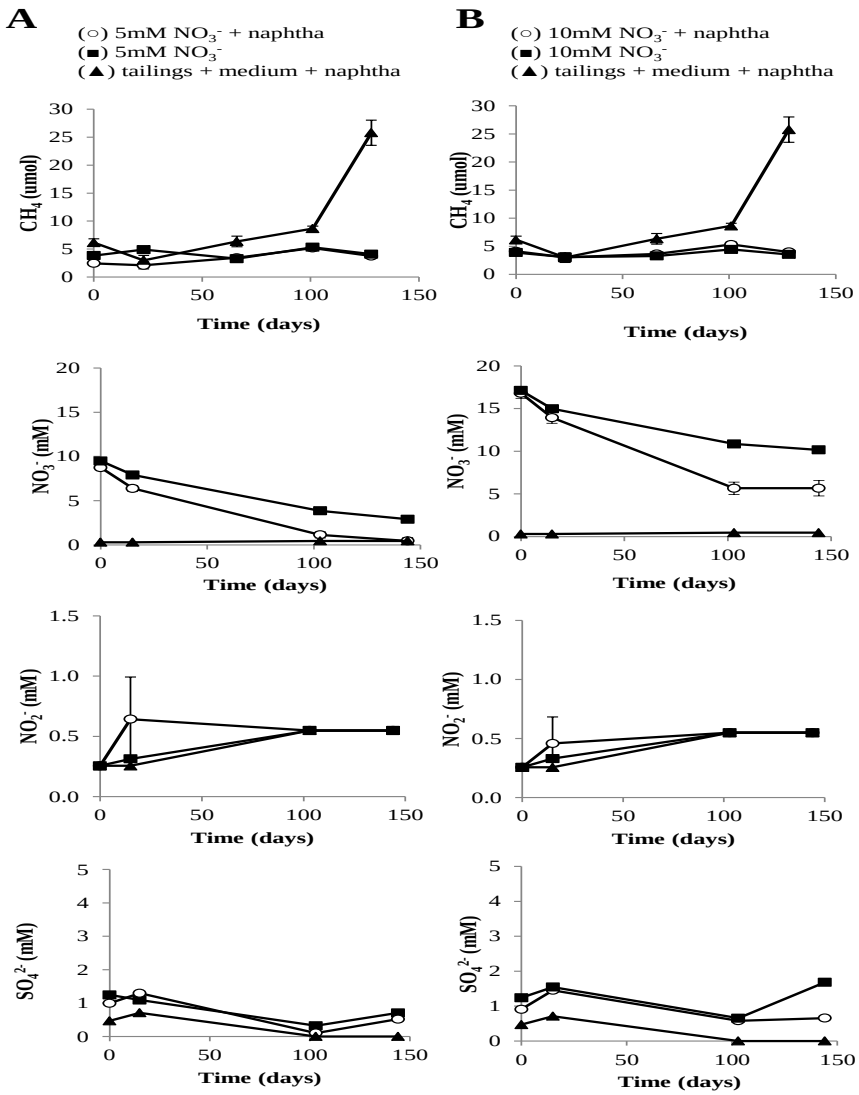


Figure 7-5 Enrichments with naphtha as the electron donor and nitrate or sulfate as the electron acceptor. Methane, sulfate, nitrate, and nitrite levels in enrichments containing (A) 5 mM nitrate, or (B) 10 mM nitrate. Error bars where visible, represent the standard error of two replicates.

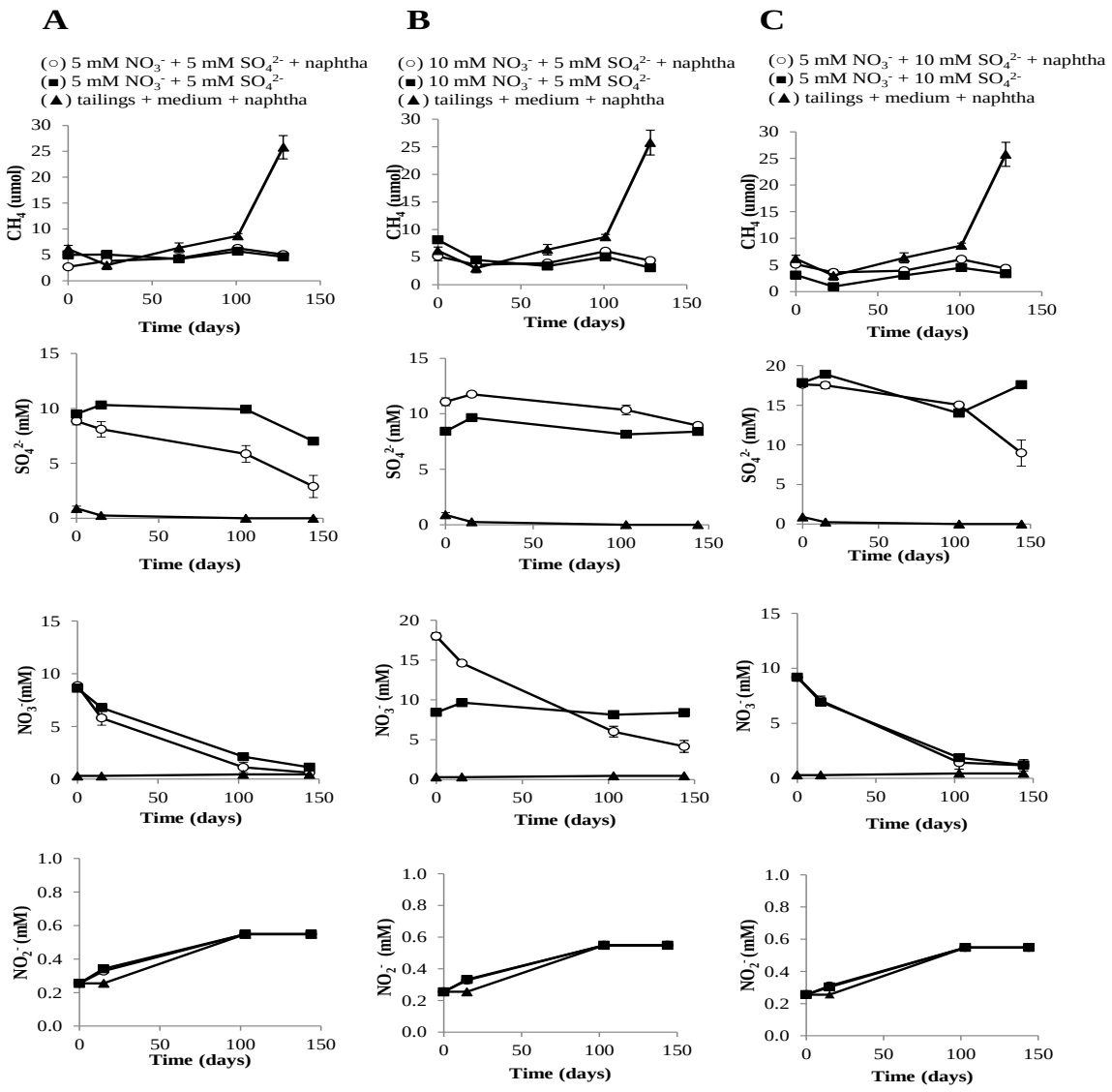


Figure 7-6 Enrichments with naphtha as the electron donor and nitrate and sulfate as the electron acceptor. Methane, sulfate and nitrate measurements in incubations containing (A) 5 mM nitrate, 5 mM sulfate, (B) 10 mM nitrate, 5 mM sulfate, (C) 5 mM nitrate, 10 mM sulfate. Error bars where visible, represent the standard error of two replicates.

7.3.4 Microbial community composition when nitrate is available

A genus-level survey of the microbial composition present in some of the nitrate-amended enrichments is shown in Figure 7-7. In general, the addition of naphtha together with the salts present in the minimal medium, enriched the microbial population originally present at the beginning of the experiment (tailings, medium, t=0).

The most abundant sequences in all the experiments belonged to the genus *Thiobacillus* with an average abundance of 12% of the pyrosequencing OTUs. The most prominent methanogens affiliated with *Methanosaeta* and *Methanolinea*, comprising on average 7.4 and 2.4 % of the pyrosequencing OTUs, respectively. Both methanogenic activity was greatly inhibited in enrichments with 5 mM NO_3^- and 10 mM SO_4^{2-}, and in enrichments where 10 mM nitrate and naphtha were available [Figure 7-7]. Regarding the sequences affiliating with SRB, the most abundant were *Desulfuromonas* sp. and *Desulfocapsa* sp. with 3.5 and 1.2 average % of pyrosequencing OTUs, respectively. The latter was more enriched (5 % of pyrosequencing OTUs) in the 5 mM NO_3^- + 10 mM SO_4^{2-} + naphtha enrichments. Known syntrophic or hydrocarbon-degrading bacteria, such as *Smithella* and *Brachymonas* were also detected in all the enrichments [Figure 7-7]. In particular, *Smithella* sp. was markedly abundant in the experiment where no alternate electron acceptor was supplied, comprising 12.7 % of the pyrosequencing OTUs. Other genera represents approximately 130 different genera with abundance lower than 3 % [Figure 7-7].

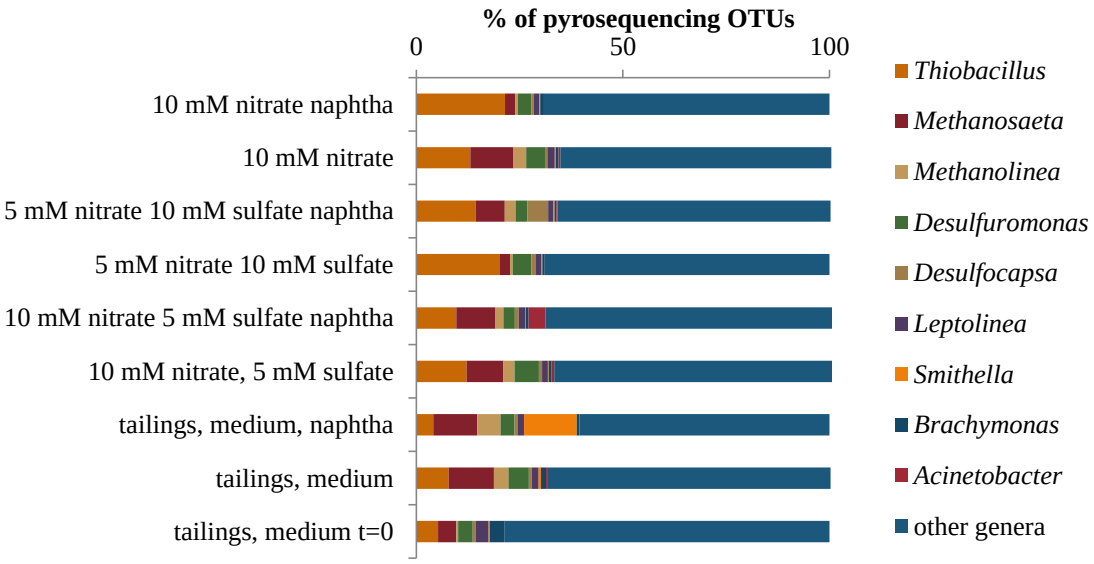


Figure 7-7 Percentage of pyrosequencing OTUs present in tailings enrichments with no added electron acceptor or with nitrate and/or sulfate added as electron acceptors. Other genera represent % of pyrosequencing OTUs < 3 % of the total community identified at the genus level.

7.4 Discussion

7.4.1 Naphtha vs. natural NAs vs. bitumen as electron donors for anaerobes in tailings ponds

The presence of less than 1% of naphtha, 40 to 70 mg $\cdot L^{-1}$ of NAs, and ~ 3% of residual bitumen in tailings ponds suggests that these compounds are the most likely available carbon and energy sources for the microbial population in the ponds. Naphtha has previously proved to support methanogenesis [41,47,68,69] and NAs have shown to be biodegradable aerobically depending on the chemical properties of the acids [34,64-67,145,149,151,166]. In the present study, bitumen was initially set up as a carbon source but since no microbial growth was detected during the first incubation period [Figure 7-2], the initial incubations were not successively transferred. The current study thus focussed on investigating the preference for natural NAs and/or naphtha in enrichments prepared from two distinct depths of tailings samples from Suncor Energy Inc. (Pond 6 2010 samples).

The microbial community composition enriched with NA or naphtha varied likely due to the presence of different substrates. The 454 pyrosequencing of the enrichments prepared with these two carbon sources gave rise to species related to the spiral shaped $H_2 - CO_2$ and formate utilizing *Methanospirillum* [196] as the most abundant taxon [Figure 7-4], in particular for tailings collected from 6 mbs with NAs as the sole source of carbon. However, the 454 pyrosequencing of the native microbial composition of pond 6 2010 at 6 mbs and 22 mbs (e.g. unenriched), did not reveal the presence of the genus

Methanospirillum in any of the depths sampled (3 – 22 mbs) [Chapter Four:]. This confirms that current technology to detect or quantify microorganisms from the environment has its limitations and we can mainly detect what we select for in the first place. Thus, we can infer that *Methanospirillum* sp. was always present in tailings but at a very low abundance, not detected by our DNA extraction and amplification protocols. However, when macro- and micronutrients were supplied (by the basal salt medium components), members of this genus were able to proliferate.

In all of the naphtha or NA-amended enrichments, the methanogenic members were more abundant than the SRB, even in the bottles amended with SO_4^{2-}. Considering that DNA was extracted soon after the third transfer had started, this result was somewhat expected. Since each successive transfer was done when the sulfate from the previous culture was depleted, and when the un-amended controls had started to produce methane, selection for the methanogenic population was probable. Interestingly, in all the conditions, at least one known sulfate reducer (*Desulfobacter* and/or *Desulfocapsa*), a hydrocarbon degrader (possibly *Thauera*), and several methanogens (*Methanospirillum, Methanofollis, Methanosarcina, Methanobacterium, Methanolinea, Methanosaeta*) were present. This is similar to a result published by Ficker *et al* (1999) who examined microbial community composition in an anaerobic toluene-degradng culture [197]. In a 10-year experiment, they observed that in a methanogenic consortium enriched with material from a creosote-contaminated aquifer and maintained with toluene as the sole source of carbon, two methanogenic Archaea (*Methanosaeta* and *Methanospirillum*) together with a sulfate reducer (*Desulfotomaculum*) and a bacterium not related to any previously

described genus were associated with the degradation of toluene [197]. Similarly, Ulrich and Edwards [198] obtained a mixed community of sulfate reducers, methanogens and syntrophs in an anaerobic culture amended with benzene as the source of carbon. They suggested that one of the bacteria may be responsible for the toluene or benzene attack, generating partially oxidized products such as fatty acids or alcohols that become available for the other bacteria (presumably the SRB acting as a fermenter) that would further degrade these low molecular weight compounds into acetate, CO_2 and H_2. Lastly, methanogens would complete the degradation pathway to CO_2 and CH_4 [197,198]. From our results, we can speculate that *Thauera* sp. may be an important primary hydrocarbon degrader while *Desulfobacter* sp. and/or *Desulfocapsa* sp. are acting as syntrophs providing the small molecular weight compounds for the hydrogenoetrophic (*Methanospirillum*) or acetoclastic methanogens (*Methanosaeta*). *Thauera* sp. are well known for their capability of growing with toluene as the sole source of carbon under nitrate-reducing conditions. Some members of this genus carry out the fumarate addition reaction, a common mechanism in anaerobic degradation [199]. It is not clear why an organism known mainly for nitrate reduction would become enriched in a methanogenic enrichment from tailings ponds. However, *Thauera* species have previously been reported to be present in oil sands tailings ponds [38,40], in industrial wastewater treatment plants [200], and in an Alberta oilfield where methanogens also dominate [201].

The differences in the microbial community composition obtained in our cultures enriched from 6 mbs and 22 mbs zones, confirms the microbial heterogeneity of the tailings ponds as well as the metabolic variety observed in these complex ecosystems.

Interestingly, the samples collected from the deeper depth were more diverse. At 22 mbs, we identified many putative naphtha degraders such as *Flavobacterium* sp. [163,202], *Acidovorax* sp. [130,131], *Acidaminobacter* sp. [203], and *Citrobacter* sp. [204] that were not detected at 6 mbs. These bacteria may well be associated in syntrophic interactions with the methanogens that were also prevalent. Among them, the H_2- and CO_2 -utilizing *Methanospirillum* sp. and *Methanofollis* sp. were the most dominant in all the conditions. Previous studies have detected these Archaea*l* groups in hydrocarbon-associated environments [140,205]. Organisms affiliated with *Methanosaeta*, *Methanolinea*, and *Methanobacterium* were also naturally found in the tailings ponds [Chapter Four], and have been previously enriched in laboratory incubations containing hydrocarbons [39,69]. Other bacteria like *Sediminibacterium* sp. (previously detected by molecular techniques from a uranium mine [206]) may also be contributing to the degradation of hydrocarbon substrates however not much literature is available regarding this genus.

Overall, under enrichment conditions, there seems to be a preference for naphtha over NA by anaerobic tailings microbial populations as a carbon source, both under methanogenic and sulfate reducing conditions [Figure 7-3]. Initially, enrichments prepared from 6 mbs samples appeared to be less inhibited by NA than by naphtha (based on methane production, sulfate reduction) but after the third transfer naphtha was favoured, as enhanced methane production and sulfate consumption was observed relative to the controls. In the NA-amended enrichments, methane production and sulfate reduction were not enhanced relative to the controls [Figure 7-2]. It is possible that in the first transfers, the microbial population was living more at the expense of residual

hydrocarbon compounds attached to the clay particles. As the tailings were diluted with the successive transfers, the substrates were no longer available hence the microbial community was potentially limited by carbon sources and not able to utilize the supplied natural NAs. Even under aerobic conditions, degradation of natural NAs has been shown to be more difficult than model and commercially available NAs [207] due to their complex chemical structure (branched alkyl chains), the range number of compounds present in naturally occuring samples[83,149], as well as their toxicity and surfactant properties [36]. However, Herman *et al*. [65,67,146] have demonstrated that microorganims from oil sands tailings have the potential to degrade both alkyl side chain and carboxylated cycloalkane ring components of NAs but the rate at which the degradation occurs seems to be affected by the limitation of nitrogen and phosphorous and such activity is under aerobic conditions. It is most likely that more incubation time is needed before we see a marked depletion of these compounds under energetically less favored anaerobic conditions.

On the other hand, biodegradation studies with naphtha [47], typically comprising a mixture of short chain alkanes [41], long chain alkanes from bitumen components [68], and monoaromatic compounds have previously been conducted under methanogenic conditions with endogenous microbial populations from Syncrude Canada Ltd. tailings ponds. The results showed that the methanogenic communities are capable of degrading these compounds in the order of $C_{10} > C_8 > C_7 > C_6$ [47]. Similarly, longer chain alkanes (C_{14}, C_{16}, and C_{18}) were also completely degraded although longer incubation times were needed (~440 days) with longer adaptation phase (180 – 280 days) before any methane production occurred compared to the degradation of short chain alkanes (~ 10 days) [68].

BTEX compounds were also found to drive methane production in tailings enrichments [47]. Our results confirm that naphtha components are serving as key carbon substrates in Suncor tailings ponds. Although naphtha compounds were not measured, our enrichment studies showed significantly enhanced levels of methane production and sulfate reduction when whole naphtha was supplied relative to controls, suggesting that the degradation of naphtha was driving these processes. Figure 7-8 shows an overview of which species were enhanced when naphtha was available in comparison to species found naturally in tailings pond 6.

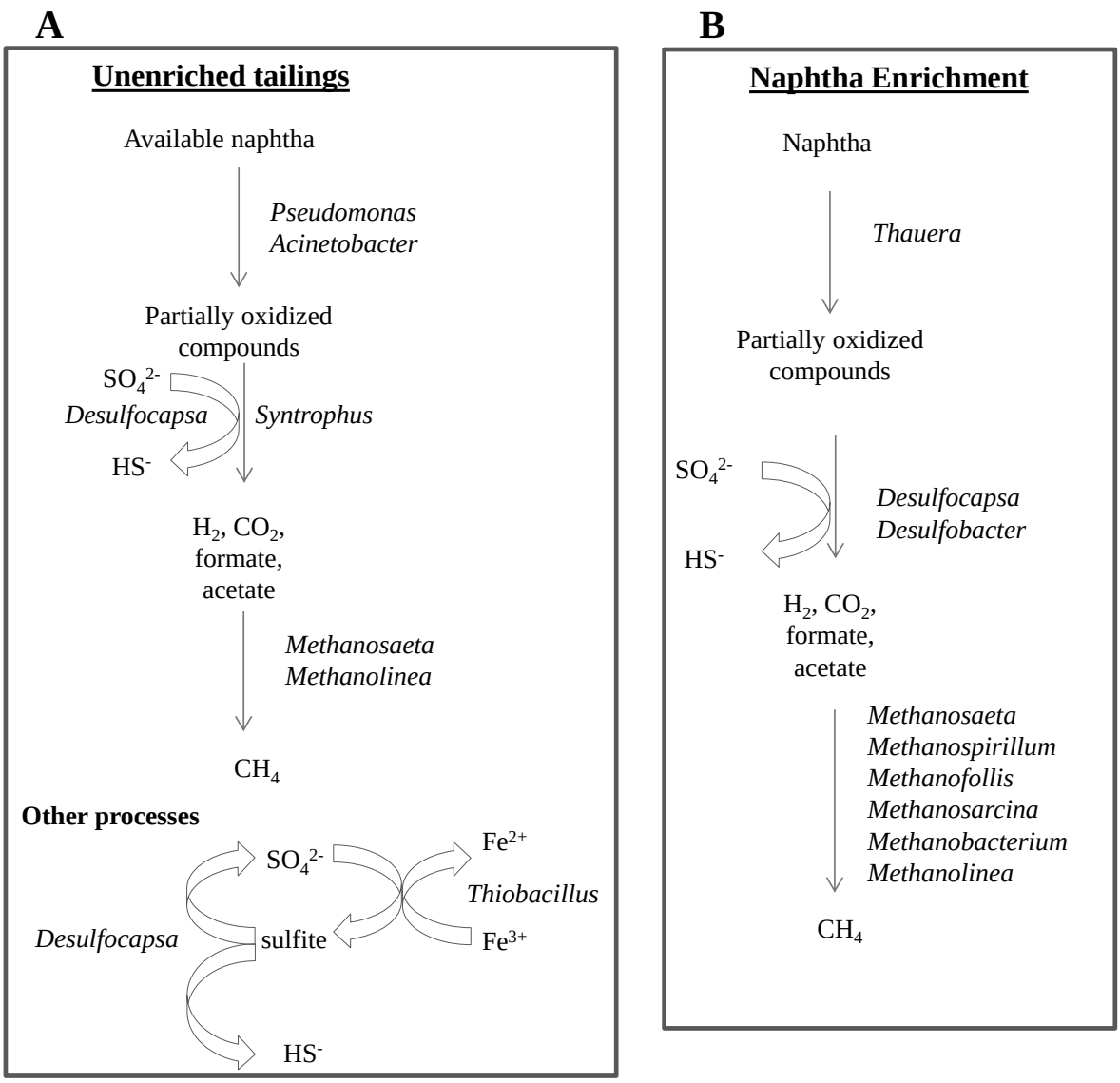


Figure 7-8 Microbial processess in Suncor oil sands tailings pond 6 2010. (A) unamended tailings, (B) naphtha-amended tailings. The microbial genera were identified using 454 pyrosequencing and only the most abundant taxa are presented.

7.4.2 Nitrate as an alternate electron acceptor in tailings ponds

The addition of gypsum to tailings ponds to enhance solids sedimentation (e.g. in CT operations) has the drawback of supplying sulfate to SRB with the concomitant risk of producing hydrogen sulfide. Under typical tailings conditions, only around 3 % of the total sulfide is expected to escape to the surface as H_2S gas, due to the alkalinity of the tailings [16]. At pH 7 – 8, most of the sulfide is present as HS^-, which has a tendency to precipitate as iron and other metal sulfides [16]. Substituting gypsum with calcium nitrate to enhance tailings sedimentation rates, and to avoid the production of sulfide, could be considered by the oil sands operators as an alternative pond management practice if sulfide emissions become an issue in the area.

Nitrate reduction produces a higher energy ($\Delta G°'$) than sulfate reduction as its reduction potential is more positive. Therefore, in anaerobic systems bacteria can gain more energy in transferring electrons when nitrate is available. Thus, as the reduction of NO_3^-/ NO_2^- has a $E'_0 = + 0.4$ volts whereas SO_4^{2-}/ HS^- only has a $E'_0 = – 0.2$. Volts. If $\Delta G°' = – nF\Delta E_0'$, nitrate reduction would yield more energy to the bacteria than sulfate reduction [54]. Further, nitrate reduction would be expected to inhibit methanogenesis.

In our experiments, the addition of nitrate at 5 or 10 mM together with sulfate at similar concentrations was tested. The rationale behind this experimental design was to determine whether there would be a shift in the inhibition of methane, currently occuring by SRB when sulfate is available, to heterotrophic nitrate-reducing bacteria (hNRB) if nitrate were to be added to tailings [Figure 7-9].

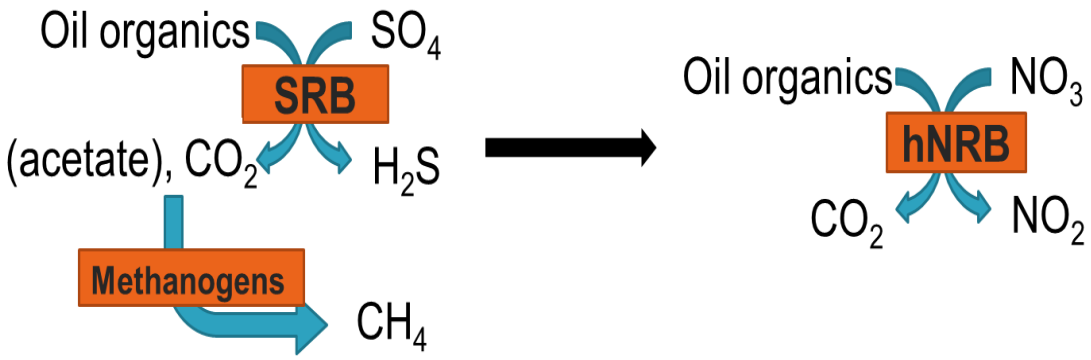


Figure 7-9 Schematic representation of the displacement of SRB by hNRB when nitrate is available in tailings ponds.

As was expected, methane production was inhibited by the addition of nitrate and/or sulfate [Figure 7-5 and Figure 7-6] to enrichments. The inhibition correlated with a decrease in the relative abundance of syntroph *Smithella*, and/or a decrease in the abundance of the methanogens *Methanosaeta* and *Methanolinea;* especially when 10 mM sulfate and/or 10 mM nitrate were added to the medium [Figure 7-7]. It is well known that methanogens need small molecular weight compounds in order to carry out their metabolism (e.g. acetate, formate, H_2/CO_2) and that these compounds are supplied by the syntrophs [116]. The abundance of *Smithella* sp. in our cultures indicates that these bacteria may be involved in the degradation of hydrocarbon (naphtha), producing substrates for the methanogens. Siddique *et al*. [69] also detected *Smithella* sp. in association with *Syntrophus* sp. in methanogenic cultures amended with naphtha and BTEX. They attributed the presence of this association to the initial anaerobic activation of alkanes and/or subsequent beta-oxidation of their metabolites to acetate [69]. Similarly, *Smithella* was among some of the dominant species in an oil reservoir treated with nitrate to control souring [201], and in an alkane-degrading methanogenic culture enriched from river sediments [106]. In our tailings enrichment cultures, the abundance of this genus in the control (tailings + naphtha + medium), together with *Syntrophus* sp. and *Leptolinea* sp. (~2.0 average % of pyrosequencing OTUs) and the methanogens, suggests the interconnection among them when no sufficient electron acceptor is around. However, the addition of nitrate and sulfate causes shifts in the community, inhibiting the methane/syntrophic association (e.g. genera associated with these activities decreased in abundance) while enriching for *Thiobacillus* sp. [Figure 7-7].

In Chapter Six, the metabolic versatility of *Thiobacillus* sp. was discussed. In particular, the denitrifying chemolithotroph *Thiobacillus denitrificans*, a bacterium capable of reducing nitrate with ferrous iron [193,208] and FeS [209] as electron donors. Equation 5 describes the oxidation of pyrite when nitrate is available, completely reducing nitrate to N_2 gas (a) or incomplete reduction to nitrite (NO_2^-) (b) [195].

Equation 5 Pyrite oxidation under nitrate reduction conditions

$$FeS_{2(s)} + 3NO_{3\,(aq)}^{-} + 2H_2O \rightarrow Fe(OH)_{3(s)} + 2SO_{4\,(aq)}^{2-} + 1.5N_{2\,(g)} + H^+ \qquad \Delta G^{\circ\prime} = -2439 \text{ kJ/mol} \qquad (a)$$

$$FeS_{2(s)} + 7.5NO_{3\,(aq)}^{-} + 3.5H_2O \rightarrow Fe(OH)_{3(s)} + 2\,SO_{4\,(aq)}^{2-} + 7.5NO_{2\,(aq)}^{-} + 4H^+ \qquad \Delta G^{\circ\prime} = -1954 \text{ kJ/mol} \qquad (b)$$

Thiobacillus, originally present in the tailings samples at time zero [Figure 7-7], was enriched in all the experiments where either nitrate or sulfate was added, independently of the presence of naphtha. This suggests that the predominance of this microorganism in tailings (e.g. also observed in pond 5 and 6 samples, Chapter Four) does not rely on naphtha as electron donor but instead it is likely using FeS_2 (Fe(II) or reduced S compounds) in tailings as the electron donor (refer to Appendix Twelve: for Fe(II) values). Since no difference was seen in the % abundance of *Thiobacillus* when only nitrate or nitrate/sulfate was added, we hypothesize that members of this genus are either using reduced nitrate compounds as electron acceptor or sulfur compounds as electron donors. Based on the stoichiometry of the reduction of nitrate to nitrite, and the results obtained, we can infer that the added nitrate was either assimilated or completely reduced to NO, N_2O or N_2 gas (denitrification) by *Thiobacillus*. Since the nitrite concenrations did not vary during the whole incubation time in enrichments amended

with nitrate, we can infer that as nitrite was formed, it was rapidly reduced by *Thiobacillus* [Figure 7-5]. In contrast, in enrichments amended with both sulfate and nitrate, nitrite concentrations did increase during the incubation time [Figure 7-6]. When sulfate is available, SRB can reduce these compounds to sulfide which can then be used as electron donors by *Thiobacillus* while reducing nitrate [210]. Therefore, microbes present in these enrichments could potentially use naphtha or reduced sulfur compounds as electron donors while reducing nitrate, thus allowing for the accumulation of nitrite [Figure 7-6]. Figure 7-10 shows an overview of the proposed metabolic processes in these enrichments.

Either way, the reduction of nitrate can possibly lead to the production of NO and N_2O. These compounds can have detrimental consequences to the environment as their accumulation can lead to ozone layer deterioration and the production of acid rain (as nitric acid) [54].

In summary, the addition of nitrate to tailings ponds could be used as an alternative to inhibit methane production but the denitrification products could represent a higher environmental risk than hydrogen sulfide produced from the reduction of sulfate. Future studies should examine the production of these potentially harmful byproducts.

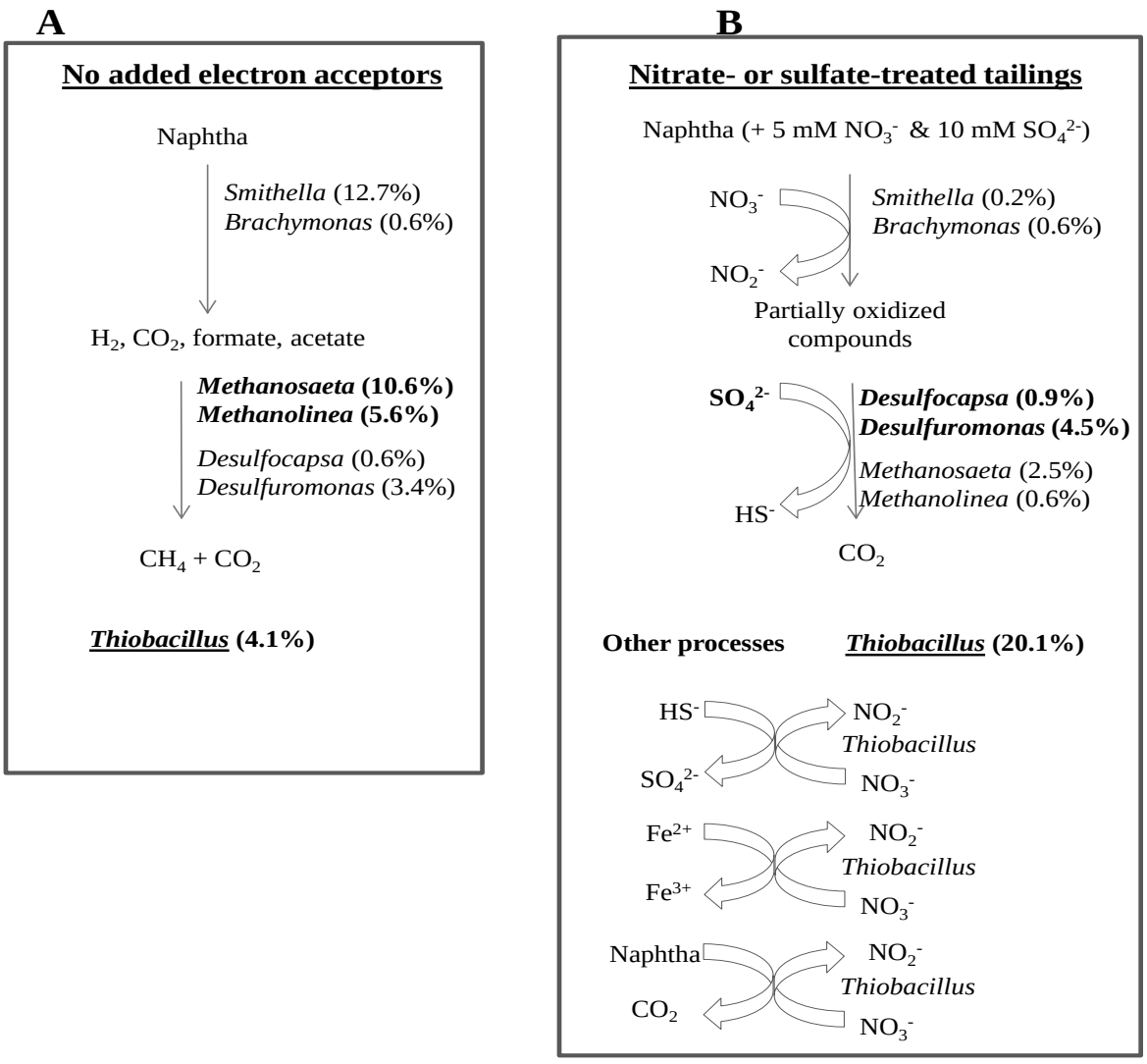


Figure 7-10 Proposed model of microbial community shifts when nitrate and/or sulfate is added as alternate electron acceptors to tailings (pond 5 2010) amended with naphtha. Left panel, no added electron acceptors showing increased average abundance of *Smithella* and methanogens and lower abundance of *Thiobacillus* and SRB. Right panel, when nitrate and/or sulfate added, lower average abundance of *Smithella* and methanogens, and increased abundance of *Thiobacillus* and SRB.

Chapter Eight: **Conclusions**

At the start of this thesis work, it was hypothesized that changes to tailings pond management operations would result in a shift in the pond microbial communities and activities based on changes in selective pressures to which the microbial communities were exposed. For example, it was hypothesized that the addition of calcium sulfate (gypsum) inhibits methanogenesis, and that as a pond ages, methanogens will dominate the microbial community due to the lack of alternate electron acceptors, hence increasing the probability of producing methane emissions. It was also hypothesized that pond closure (e.g. a scenario where no new tailings or treatments are added to a tailings pond) can potentially shift the microbial community composition and activities, but the outcome was not known (e.g. does pond closure lead to positive or negative effects?). Thus, gaining information about the key microbial players and activities that occur in tailings ponds subject to different management strategies can help operators predict whether certain operations will lead to desired (e.g. biodegradation/bioremediation of tailings chemicals) or undesired effects (e.g. gas emissions).

To address the hypothesis, the microbial communities and physiologies in oil sands tailings ponds were determined using samples collected from two tailings ponds being operated using different management approaches. These parameters were monitored in an active pond that was treated with gypsum (Suncor pond 6) and in another tailings pond (also treated with gypsum) before and after closure (designated as an inactive pond, Suncor pond 5). In order to thoroughly investigate microbial communities and activities over time, depth-dependent sets of samples were collected and analyzed from the years

2008 to 2011. A variety of chemical analyses, microbial activity assays, microbial community composition analyses, functional gene studies, and substrate determinations were performed. The anaerobic microbial community composition together with their main metabolic activities (sulfate reduction and methanogenesis rates), and the quantification of the gene copy number for the genes involved in these reactions were evaluated and compared. In order to determine the preferred substrate for anaerobic tailings ponds communities, enrichments were established with different substrates (bitumen, naphtha, or NAs) and methane production and sulfate reduction were monitored along with an examination of the microbes presumably involved in consuming the substrates. The microbial community composition and activities at the pond surface were also assessed to examine the potential for aerobic surface microbes to use gases that can potentially be emitted (e.g. H_2S) and pure cultures were isolated and studied for their ability to degrade NAs which are a source of known toxicity in tailings ponds.

The microbial community analysis in the active pond (pond 6) showed that the main groups of organisms inhabiting the anaerobic depths comprise, in order of abundance, the phyla Proteobacteria, Euryarchaeota, Chloroflexi, and Firmicutes, with the first two phyla dominating (each comprising around 30 % of the average of pyrosequencing OTUs). It was observed that these proportions change depending on the management of the pond. For example, for the inactive pond (pond 5) prior to closure, Proteobacteria comprised approximately 70% of the total OTUs while Euryarchaeota comprised ~26 % of the average of pyrosequencing OTUs. However, after a year of no tailings inputs, Proteobacteria abundance increased slightly to 77%, while Euryarchaeota

abundance decreased by approximately half (down to 12% of the total average pyrosequencing OTUs). Similarly, at the genus level, the active pond (pond 6) harboured methanogens as the major microbial groups whereas for the inactive pond (pond 5, after a year following closure) a shift from a primarily methanogenic community to one more dominated by putative hydrocarbon degraders was observed.

The 454 pyrosequencing of the 16S rRNA genes and the qPCR analysis of specific functional genes indicated that the methanogens most commonly found in these tailings ponds were *Methanosaeta* sp. and *Methanolinea* sp. In the active pond (pond 6), the abundance of these methanogens closely paralleled that of *Syntrophus* sp. that were also identified by 16S rRNA gene analysis, suggesting that syntrophic reactions are prevalent in the tailings ponds, likely functioning to degrade carbon substrates to methane. This observation supports previous findings in a Syncrude tailings pond and in other hydrocarbon-degrading environments [68,69]. Microbial community analysis also showed the dominance of 16S rRNA gene sequences associated with *Desulfocapsa* sp. and *Thiobacillus* sp., organisms that are well known to be involved in sulfur metabolism. *Thiobacillus* sp. are metabolically versatile in that they can be involved in reactions involving sulfite oxidation, sulfate reduction, iron oxidation, iron reduction, and nitrate reduction. Based on the finding of these two organisms in parallel, it is speculated that a close commensalistic interaction is taking place between these two groups of bacteria. Other organisms revealed by 16S rRNA gene sequencing that may be possibly playing important roles in the biodegradation of hydrocarbon-like compounds in tailings ponds

include members of the genera *Acinetobacter* and *Pseudomonas* that were found in all tailings samples.

The qPCR analysis targeting the *dsr*B gene correlated well as a function of depth with the sulfate reduction rates and 16S rRNA gene sequencing results revealing the abundance of SRB like *Desulfocapsa*. Interestingly, the *sox* genes also correlated positively as a function of depth with the sulfate reduction rate and with the abundance of *Thiobacillus*. However, the *sox* genes were not detected near the pond surface, where aerobic sulfide oxidation is expected to occur and where laboratory experiments showed slightly higher rates of sulfide oxidation in live tailings tests (e.g. biotic tests) compared to sterile tailings tests (e.g. abiotic tests). These data suggest that abiotic sulfide oxidation is more prevalent than biological sulfide oxidation near the pond surface to help prevent sulfide emissions from the pond. The qPCR data for methanogens (e.g. quantifying the *mcrA* gene) did not correlate as well with the methanogenic Archaea obtained by 454 pyrosequencing of the 16S rRNA gene. It was speculated that this may be related to the presence of *mcrA* that can also be found in anaerobic methane oxidizers which may be present in tailings based on other studies [187]. More studies regarding this idea need to be carried out.

The surface water contained a completely different microbial community composition in comparison to the anaerobic layers which was not unexpected given the availability of oxygen as an electron acceptor at the surface, but not at depth. The tailings pond microorganisms found at the pond surface are dominated by genera that have been previously found to degrade hydrocarbons or that are metabolically associated with

algae. In a small proportion, sulfide oxidizers can also be found. Three isolates were obtained from tailings pond surface water that are able to effectively degrade model NA compounds, which shows that organisms at the tailings ponds surface may be able to metabolize some carbon substrates added to the ponds following bitumen extraction. These isolates can now be used as model organisms to study the biodegradation of NA in more depth (such as for determining key enzymes and genes involved in the NA metabolic pathways). Among the isolates obtained, some have been reported as aerobic sulfide oxidizers (*Xanthobacter* sp.), although this process does not appear to be operating in the ponds based on the studies conducted here. As mentioned earlier, if sulfide reaches the surface due to sulfate reduction occurring at depth, it will most likely be chemically converted to a more oxidized form.

The preference in substrate utilization by the anaerobic microbial populations in tailings ponds seem to be more directed towards naphtha than to NAs as more microbial activity was observed in cultures amended with naphtha than with NAs. Bitumen did not serve as a carbon source under the anaerobic conditions tested. A common pattern of a sulfate reducer, a methanogen, and a hydrocarbon degrader was seen in all the enrichments confirming the cooperation among the species to degrade complex organic compounds.

The effectiveness of nitrate as an alternate electron acceptor to inhibit methanogenesis if sulfide emissions are ever an issue was also shown. However the fate of the final products of denitrification is not known and could be deleterious to the environment if nitrous oxides are produced. Interestingly the genus most enriched under

nitrate-amended conditions was *Thiobacillus*, which suggests the importance of this group of bacteria in tailings ponds, specifically due to their versatile metabolism associated with sulfur and iron. Through these studies, this genus has emerged as a major microbial player in oil sands tailings ponds that can play an important role in many metabolic processes, thus further studies targeting these bacteria is warranted.

Comparing the two ponds studied, we can conclude that for the active pond, microbial activity was found throughout the depths studied (first 22 mbs), whereas for the inactive pond, most of the microbial activity was concentrated closer to the surface. However, a significant reduction of microbial activity was observed after pond 5 was dewatered and closed. Sulfate reduction activity in the active pond (pond 6) was also decreased due to a reduction of tailings inputs. The phylogenetic relationships between all of the samples taken from both ponds under study showed that each pond, and each sampling time are not closely related to each other, supporting the hypothesis that changes in pond management can dramatically alter the community structure and thus key activities in tailings ponds. However, the results herein obtained cannot yet be generalized to other tailings ponds until further studies are conducted with other tailings ponds (e.g. microbial monitoring studies of another pond undergoing closure) showing similar shifts in microbial community composition and activities.

In summary, tailings ponds harbours a wide variety of microorganisms that likely interact to carry out a variety of processes including sulfur metabolism, methane production, and biodegradation both aerobically and anaerobically. Other processes like nitrate and iron reduction can also occur as the tailings contain the microbes with genetic

capabilities to support these metabolic processes. The abundance and spatial distribution of microbial communities is closely related to the substrate availability, which can vary depending on the implemented pond management strategy. Therefore, the microbial study of oil sands tailings ponds should not be underestimated and should be followed carefully by operators in the oil sands region as part of the pond monitoring routine, both to prevent negative environmental damage and/or to predict the outcome of a management scheme.

References

1. Alberta Go. 2011 July 17, 2012. Responsible Actions: A Plan for Alberta's Oil Sands. Annual Progress Report 2011. Government of Alberta <http://www.energy.alberta.ca/pdf/OSSResponsibleActionsProgressReport2011.pdf>. Accessed 2012 July 17, 2012.
2. Kasperski KL, Mikula RJ. Waste Streams of Mined Oil Sands: Characteristics and Remediation. Elements 2011;7(6):387-392.
3. Chalaturnyk R, Scott J, Ozum B. Management of oil sands tailings. Petroleum Science and Technology 2002:1025-1046.
4. Goverment A. 2011 Alberta´s Oil Sands. Facts about Tailings Management. <http://www.oilsands.alberta.ca/tailings.html>.
5. Hooshiar A, Uhlik P, Ivey DG, Liu Q, Etsell TH. Clay minerals in nonaqueous extraction of bitumen from Alberta oil sands Part 2. Characterization of clay minerals. Fuel Processing Technology 2012;96:183-194.
6. Kaminsky HAW, Etsell TH, Ivey DG, Omotoso O. Distribution of clay minerals in the process streams produced by the extraction of bitumen from athabasca oil sands. Canadian Journal of Chemical Engineering 2009;87(1):85-93.
7. Tenenebaum DJ. Oils Sands Development: A Health Risk Worth Taking? Environmental Health Perspectives 2009;117(4):A150-A156.
8. Gray GR, Luhning R. Bitumen. The Canadian Encyclopedia. Volume 20102010.
9. Clark KA, Pasternack DS. Hot water separation of bitumen from Alberta bituminous sand. Industrial and Engineering Chemistry 1932;24:1410-1416.
10. Kleindienst M. 2005 The Athabasca oil sands in north-west Canada, a short geological overview. <http://www.geo.tu-freiberg.de/oberseminar/os05_06/maria_kleindienst.pdf>. Accessed 2012.
11. Masliyah J, Zhou ZJ, Xu ZH, Czarnecki J, Hamza H. Understanding water-based bitumen extraction from athabasca oil sands. Canadian Journal of Chemical Engineering 2004;82(4):628-654.
12. Li C. Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification: University of Alberta; 2010. 193 p.
13. Chalaturnyk R, Scott J, Ozum B. Oil sands tailings production for environmental disposal. In: Wang YH, Ge SR, Guo GL, editors. Mining Science and Technology. Mining Science and Technology2004. p 653-658.
14. Resources ACo. 2004 July 18, 2012. Oil sands technology roadmap: unlocking the potential. <http://www.acr-alberta.com/OSTR_report.pdf>. Accessed 2012 July 18, 2012.
15. Bordenave S, Kostenko V, Dutkoski M, Grigoryan A, Martinuzzi RJ, Voordouw G. Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings. Chemosphere 2010;81(5):663-668.
16. Salloum MJ, Dudas MJ, Fedorak PM. Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Management and Research 2002;20(2):162-171.

17. Proskin S, Sego D, Alostaz M. Oil Sands MFT Properties and Freeze-Thaw Effects. Journal of Cold Regions Engineering 2012;26(2):29-54.
18. Kasperski KL. A review of properties and treatment of oil sands tailings. AOSTRA Journal of Research 1992;8:11-53.
19. Allen EW. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science 2008;7(2):123-138.
20. Adeyinka OB, Samiei S, Xu ZH, Masliyah JH. Effect of particle size on the rheology of athabasca clay suspensions. Canadian Journal of Chemical Engineering 2009;87(3):422-434.
21. Mercier PHJ, Ng S, Moran K, Sparks BD, Kingston D, Kotlyar LS, Kung J, Woods J, Patarachao B, McCracken T. Colloidal Clay Gelation: Relevance to Current Oil Sands Operations. Petroleum Science and Technology 2012;30(9):915-923.
22. Kotlyar LS, Sparks BD, Schutte R. Role of bicarbonate ions in the stability of ultra-fines suspensions. Journal of Environmental Science and Health Part a-Environmental Science and Engineering & Toxic and Hazardous Substance Control 1995;30(6):1277-1288.
23. Majid A, Sparks BD, Ripmeester JA. Isolation and characterization of humic matter from syncrude sludge pond tailings. Fuel 1992;71(2):165-168.
24. Powte C, Biggar, KW, Silva, MJ, McKenna, GT, Scordo, EB. Review of Oil Sands Tailings Technology. Network OSRaI, editor. Edmonton, Alberta, Canada: Tylor and Francis group; 2011. 381–391 p.
25. Beier N, Sego D. The Oil Sands Tailings Research Facility. Geotechnical News 2008:72-77.
26. Hocking MB, Lee GW. Effect of chemical agents on settling rates of sludges from effluent of hot-water extraction of athabasca oil sands. Fuel 1977;56(3):325-333.
27. Long J, Li H, Xu Z, Masliyah JH. Role of colloidal interactions in oil sand tailings treatment. Aiche Journal 2006;52(1):371-383.
28. Matthews JG, Shaw WH, MacKinnon MD, Cuddy RG. Development of composite tailings technology at Syncrude Canada. In: Singhal RK, Mehrotra AK, editors. Environmental Issues and Management of Waste in Energy and Mineral Production2000. p 455-463.
29. Clemente JS, Fedorak PM. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 2005;60(5):585-600.
30. Headley JV, Peru KM, Barrow MP. Mass Spectrometric Characterization of Naphthenic Acids in Environmental Samples: A Review. Mass Spectrometry Reviews 2009;28(1):121-134.
31. Quagraine EK, Peterson HG, Headley JV. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 2005;40(3):685-722.

32. Holowenko FM, MacKinnon MD, Fedorak PM. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research 2002;36(11):2843-2855.
33. Headley JV, McMartin DW. A review of the occurrence and fate of naphthenic acids in aquatic environments. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 2004;39(8):1989-2010.
34. Clemente JS, MacKinnon MD, Fedorak PM. Aerobic biodegradation of two commercial naphthenic acids preparations. Environmental Science & Technology 2004;38(4):1009-1016.
35. Rogers VV, Wickstrom M, Liber K, MacKinnon MD. Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings. Toxicological Sciences 2002;66(2):347-355.
36. Frank RA, Kavanagh R, Burnison BK, Arsenault G, Headley JV, Peru KM, Van Der Kraak G, Solomon KR. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 2008;72(9):1309-1314.
37. Foght JM, Fedorak PM, Westlake DWS, Boerger H. Microbial Content and Metabolic Activities in the Syncrude Tailings Pond. AOSTRA J. of Research 1984;1:139 – 146.
38. Golby S, Ceri H, Gieg L, Chatterjee I, Marques L, Turner R. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. Fems Microbiology Ecology 2012;79(1):240-250.
39. Penner TJ. Analysis of Methanogenic Microbial Communities from OIl Sands Processing Tailings. Edmonton: University of Alberta; 2006.
40. Penner TJ, Foght JM. Mature fine tailings from oil sands processing harbour diverse methanogenic communities. Canadian Journal of Microbiology 2010;56(6):459-470.
41. Siddique T, Fedorak PM, Foght JM. Biodegradation of short-chain n-alkanes in oil sands tailings under methanogenic conditions. Environmental Science & Technology 2006;40(17):5459-5464.
42. Siddique T, Gupta R, Fedorak PM, MacKinnon MD, Foght JM. A first approximation kinetic model to predict methane generation from an oil sands tailings settling basin. Chemosphere 2008;72(10):1573-1580.
43. Haveroen ME, MacKinnon MD, Fedorak PM. Polyacrylamide added as a nitrogen source stimulates methanogenesis in consortia from various wastewaters. Water Research 2005;39(14):3333-3341.
44. Fedorak PM, Coy DL, Salloum MJ, Dudas MJ. Methanogenic potential of tailings samples from oil sands extraction plants. Canadian Journal of Microbiology 2002;48(1):21-33.
45. Fedorak PM, Coy DL, Dudas MJ, Simpson MJ, Renneberg AJ, MacKinnon MD. Microbially-mediated fugitive gas production from oil sands tailings and increased tailings densification rates. Journal of Environmental Engineering and Science 2003;2(3):199-211.

46. Holowenko FM, MacKinnon MD, Fedorak PM. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Canadian Journal of Microbiology 2000;46(10):927-937.
47. Siddique T, Fedorak PM, McKinnon MD, Foght JM. Metabolism of BTEX and naphtha compounds to methane in oil sands tailings. Environmental Science and Technology 2007;41(7):2350-2356.
48. Rogers VV, Liber K, MacKinnon MD. Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. Chemosphere 2002;48(5):519-527.
49. Development. AGAEaSR. 2012 July 19, 2012. Alberta's oil sand. Clean energy future. Tailings ponds. Accessed 2012 July 19, 2012.
50. McFadyen D. Directive 074: Tailings Performance Criteria and Requirements for Oil Sands Mining Schemes. In: (ERCB) ERCB, editor. Calgary, Alberta, Canada2009. p 14.
51. Inc. SE. 2012 Tailings management. <http://www.suncor.com/en/responsible/3229.aspx>.
52. Wells P, Caldwell, J, Fournier, J. Suncor Pond 5 coke cap: the story of its conception, testing, and advance to full-scale construction. Infomine. LibraryMine2010.
53. Yergeau E, Lawrence JR, Sanschagrin S, Waiser MJ, Korber DR, Greer CW. Next-generation sequencing of microbial communities in the athabasca river and its tributaries in relation to oil sands mining activities. Applied and environmental microbiology 2012;78(21).
54. Madigan MT, Martinko JM, Dunlap PV, Clark DP. Brock Biology of Microorganisms. Carlson LBaG, editor: Pearson Education Inc.; 2009. 1061 p.
55. Ramos-Padron E, Bordenave S, Lin SP, Bhaskar IM, Dong XL, Sensen CW, Fournier J, Voordouw G, Gieg LM. Carbon and Sulfur Cycling by Microbial Communities in a Gypsum-Treated Oil Sands Tailings Pond. Environmental Science & Technology 2011;45(2):439-446.
56. Stams AJM. METABOLIC INTERACTIONS BETWEEN ANAEROBIC-BACTERIA IN METHANOGENIC ENVIRONMENTS. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 1994;66(1-3):271-294.
57. Grigoryan A, Voordouw G. Microbiology to help solve our energy needs - Methanogenesis from oil and the impact of nitrate on the oil-field sulfur cycle. In: Wiegel J, Maier RJ, Adams MWW, editors. Incredible Anaerobes: from Physiology to Genomics to Fuels. Volume 1125, Annals of the New York Academy of Sciences2008. p 345-352.
58. Hedrich S, Schloemann M, Johnson DB. The iron-oxidizing proteobacteria. Microbiology-Sgm 2011;157.
59. Jorgensen CJ, Jacobsen OS, Elberling B, Aamand J. Microbial Oxidation of Pyrite Coupled to Nitrate Reduction in Anoxic Groundwater Sediment. Environmental Science & Technology 2009;43(13):4851-4857.

60. Leifer I, Patro RK. The bubble mechanism for methane transport from the shallow sea bed to the surface: A review and sensitivity study. Continental Shelf Research 2002;22(16):2409-2428.
61. Kelly DP, Shergill JK, Lu WP, Wood AP. Oxidative metabolism of inorganic sulfur compounds by bacteria. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 1997;71(1-2):95-107.
62. Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J. Oxidation of reduced inorganic sulfur compounds by bacteria: Emergence of a common mechanism? Applied and Environmental Microbiology 2001;67(7):2883-2894.
63. Fuchs T, Huber H, Burggraf S, Stetter KO. 16S rDNA-based phylogeny of the archaeal order Sulfolobales and reclassification of *Desulfurolobus ambivalens* as Acidianus ambivalens comb nov. Systematic and Applied Microbiology 1996;19(1):56-60.
64. Del Rio LF, Hadwin AKM, Pinto LJ, MacKinnon MD, Moore MM. Degradation of naphthenic acids by sediment micro-organisms. Journal of Applied Microbiology 2006;101(5):1049-1061.
65. Herman DC, Fedorak PM, Costerton JW. Biodegradation of cycloalkane carboxylic-acids in oil sands tailings. Canadian Journal of Microbiology 1993;39(6):576-580.
66. Lai J, Pinto L, Kiehlmann E, BendellYoung L, Moore M. Factors that affect the degradation of naphthenic acids in oil sands wastewater by indigenous microbial communities. Environmental Toxicology and Chemistry 1996;15(9):1482-1491.
67. Herman DC, Fedorak PM, Mackinnon MD, Costerton JW. Biodegradation of naphthenic acids by microbial-populations indigenous to oil sands tailings. Canadian Journal of Microbiology 1994;40(6):467-477.
68. Siddique T, Penner T, Semple K, Foght JM. Anaerobic Biodegradation of Longer-Chain n-Alkanes Coupled to Methane Production in Oil Sands Tailings. Environmental Science & Technology 2011;45(13):5892-5899.
69. Siddique T, Penner T, Klassen J, Nesbo C, Foght JM. Microbial communities involved in methane production from hydrocarbons in oil sands tailings. Environmental science & technology 2012;46(17):9802–9810.
70. Widdel F, Rabus R. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Current Opinion in Biotechnology 2001;12(3):259-276.
71. Zengler K, Richnow HH, Rossello-Mora R, Michaelis W, Widdel F. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 1999;401(6750):266-269.
72. Wyndham R, Costerton J. *In vitro* microbial-degradation of bituminous hydrocarbons and *in situ* colonization of bitumen surfaces within the Athabasca oil sands deposit. Applied and Environmental Microbiology 1981;41(3):791-800.
73. Frank RA, Fischer K, Kavanagh R, Burnison BK, Arsenault G, Headley JV, Peru KM, Van der Kraak G, Solomon KR. Effect of Carboxylic Acid Content on the Acute Toxicity of Oil Sands Naphthenic Acids. Environmental Science & Technology 2009;43(2):266-271.

74. Videla PP, Farwell AJ, Butler BJ, Dixon DG. Examining the Microbial Degradation of Naphthenic Acids Using Stable Isotope Analysis of Carbon and Nitrogen. Water Air and Soil Pollution 2009;197(1-4):107-119.
75. Blakley ER. Microbial degradation of cyclohexanecarboxylic acid - pathway involving aromatization to form para-hydroxybenzoic acid. Canadian Journal of Microbiology 1974;20(10).
76. Blakley ER, Papish B. The metabolism of cyclohexanecarboxylic acid and 3-cyclohexenecarboxylic acid by pseudomonas-putida. Canadian Journal of Microbiology 1982;28(12).
77. Rho EM, Evans WC. Aerobic metabolism of cyclohexanecarboxylic acid by acinetobacter-anitratum. Biochemical Journal 1975;148(1).
78. Rontani JF, Bonin P. Utilization of normal-alkyl-substituted cyclohexanes by a marine alcaligenes. Chemosphere 1992;24(10):1441-1446.
79. Han XM, MacKinnon MD, Martin JW. Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 2009;76(1):63-70.
80. Quagraine EK, Headley JV, Peterson HG. Is biodegradation of bitumen a source of recalcitrant naphthenic acid mixtures in oil sands tailing pond waters? Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 2005;40(3):671-684.
81. Whitby C, Laskin A, Sariaslani S, Gadd G. Microbial Naphthenic Acid Degradation. Advances in Applied Microbiology, Vol 70 2010;70:93-125.
82. Kannel PR, Gan TY. Naphthenic acids degradation and toxicity mitigation in tailings wastewater systems and aquatic environments: A review. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 2012;47(1):1-21.
83. Smith BE, Lewis CA, Belt ST, Whitby C, Rowland SJ. Effects of Alkyl Chain Branching on the Biotransformation of Naphthenic Acids. Environmental Science & Technology 2008;42(24):9323-9328.
84. Holowenko FM, Mackinnon MD, Fedorak PM. Naphthenic acids and surrogate naphthenic acids in methanogenic microcosms. Water Research 2001;35(11):2595-2606.
85. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiological Reviews 1995;59(1):143-169.
86. Foght J. Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects. Journal of Molecular Microbiology and Biotechnology 2008;15(2-3):93-120.
87. Kirk JL, Beaudette LA, Hart M, Moutoglis P, Khironomos JN, Lee H, Trevors JT. Methods of studying soil microbial diversity. Journal of Microbiological Methods 2004;58(2):169-188.
88. More MI, Herrick JB, Silva MC, Ghiorse WC, Madsen EL. QUANTITATIVE CELL-LYSIS OF INDIGENOUS MICROORGANISMS AND RAPID EXTRACTION OF MICROBIAL DNA FROM SEDIMENT. Applied and Environmental Microbiology 1994;60(5).

89. Tebbe CC, Vahjen W. Interference of humic acids and dna extracted directly from soil in detection and transformation of recombinant-dna from bacteria and a yeast. Applied and Environmental Microbiology 1993;59(8):2657-2665.
90. Cline JD. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnology and Oceanography 1969;14(3):454-&.
91. Cordruwisch R. A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. Journal of Microbiological Methods 1985;4(1):33-36.
92. Lambo AJ, Noke K, Larter SR, Voordouw G. Competitive, Microbially-Mediated Reduction of Nitrate with Sulfide and Aromatic Oil Components in a Low-Temperature, Western Canadian Oil Reservoir. Environmental Science & Technology 2008;42(23):8941-8946.
93. Standard A. D2216-05 Standard test methods for laboratory determination of water (moisture) content of soil and rock by mass. ASTM International; 2005.
94. G.A. U, Krumholz LR, Suflita JM. A Rapid and Simple Method for Estimating Sulfate Reduction Activity and Quantifying Inorganic Sulfides. Applied and Environmental Microbiology 1997;63(4):1672 – 1630.
95. Kaster KM, Voordouw G. Effect of nitrite on a thermophilic, methanogenic consortium from an oil storage tank. Applied Microbiology and Biotechnology 2006;72(6):1308-1315.
96. Takada-Hoshino Y, Matsumoto N. An improved DNA extraction method using skim milk for soils that strongly adsorb DNA. Microbes Environ 2004;19(1):13-19.
97. Park HS, Chatterjee I, Dong XL, Wang SH, Sensen CW, Caffrey SM, Jack TR, Boivin J, Voordouw G. Effect of Sodium Bisulfite Injection on the Microbial Community Composition in a Brackish-Water-Transporting Pipeline. Applied and Environmental Microbiology 2011;77(19):6908-6917.
98. Shannon CE. A mathematical theory of communication. Bell System Technical Journal 1948;27(3).
99. Simpson EH. Measurement of diversity. Nature 1949;163(4148).
100. Hill TCJ, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. Fems Microbiology Ecology 2003;43(1).
101. Gotelli NJ, Colwell RK. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 2001;4(4).
102. Pfennig N, Biebl H. Desulfuromonas-acetoxidans gen-nov and sp-nov - new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Archives of Microbiology 1976;110(1).
103. Engelbrektson A, Kunin V, Wrighton KC, Zvenigorodsky N, Chen F, Ochman H, Hugenholtz P. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. Isme Journal 2010;4(5).
104. Parkes RJ, Brock F, Banning N, Hornibrook ERC, Roussel EG, Weightman AJ, Fry JC. Changes in methanogenic substrate utilization and communities with depth in a salt-marsh, creek sediment in southern England. Estuarine Coastal and Shelf Science 2012;96:170-178.

105. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and Molecular Biology Reviews 1997;61(2):262-&.
106. Gray ND, Sherry A, Grant RJ, Rowan AK, Hubert CRJ, Callbeck CM, Aitken CM, Jones DM, Adams JJ, Larter SR and others. The quantitative significance of Syntrophaceae and syntrophic partnerships in methanogenic degradation of crude oil alkanes. Environmental Microbiology 2011;13(11).
107. Patel GB, Sprott GD. Methanosaeta-concilii gen-nov, sp-nov (methanothrix-concilii) and methanosaeta-thermoacetophila nom-rev, comb-nov. International Journal of Systematic Bacteriology 1990;40(1):79-82.
108. Whitman WB, Bowen TL, Boone DR. The Methanogenic Bacteria. Prokaryotes: A Handbook on the Biology of Bacteria, Vol 3, Third Edition: Archaea. Bacteria: firmicutes, actinomycetes 2006.
109. Carbonero F, Oakley BB, Hawkins RJ, Purdy KJ. Genotypic Distribution of a Specialist Model Microorganism, Methanosaeta, along an Estuarine Gradient: Does Metabolic Restriction Limit Niche Differentiation Potential? Microbial Ecology 2012;63(4):856-864.
110. Purdy KJ, Nedwell DB, Embley TM. Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting Antarctic sediments. Applied and Environmental Microbiology 2003;69(6):3181-3191.
111. Berdugo-Clavijo C, Dong XL, Soh J, Sensen CW, Gieg LM. Methanogenic biodegradation of two-ringed polycyclic aromatic hydrocarbons. Fems Microbiology Ecology 2012;81(1):124-133.
112. Sanz JL, Rodriguez N, Diaz EE, Amils R. Methanogenesis in the sediments of Rio Tinto, an extreme acidic river. Environmental Microbiology 2011;13(8):2336-2341.
113. Elferink S, Vorstman WJC, Sopjes A, Stams AJM. Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater. Fems Microbiology Ecology 1998;27(2):185-194.
114. Popp N, Schloemann M, Mau M. Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiology-SGM 2006;152(11):3291-3304.
115. Imachi H, Sakai S, Sekiguchi Y, Hanada S, Kamagata Y, Ohashi A, Harada H. Methanolinea tarda gen. nov., sp nov., a methane-producing archaeon isolated from a methanogenic digester sludge. International Journal of Systematic and Evolutionary Microbiology 2008;58:294-301.
116. McInerney MJ, Struchtemeyer CG, Sieber J, Mouttaki H, Stams AJM, Schink B, Rohlin L, Gunsalus RP. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Incredible Anaerobes: from Physiology to Genomics to Fuels 2008;1125:58-72.
117. Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Aitken CM, Bennett B, Huang H, Brown A, Bowler BFJ and others. Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 2008;451(7175):176-U6.

118. Finster K. Microbiological disproportionation of inorganic sulfur compounds. Journal of Sulfur Chemistry 2008;29(3-4).
119. Frederiksen TM, Finster K. The transformation of inorganic sulfur compounds and the assimilation of organic and inorganic carbon by the sulfur disproportionating bacterium Desulfocapsa sulfoexigens. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 2004;85(2).
120. De Vrieze J, Hennebel T, Boon N, Verstraete W. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. Bioresource Technology 2012;112:1-9.
121. Gagne F, Andre C, Douville M, Talbot A, Parrott J, McMaster M, Hewitt M. An examination of the toxic properties of water extracts in the vicinity of an oil sand extraction site. Journal of Environmental Monitoring 2011;13(11):3075-3086.
122. Pal A, Paul AK. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian Journal of Microbiology 2008;48(1):49-64.
123. Harner NK, Richardson TL, Thompson KA, Best RJ, Best AS, Trevors JT. Microbial processes in the Athabasca Oil Sands and their potential applications in microbial enhanced oil recovery. Journal of Industrial Microbiology & Biotechnology 2011;38(11):1761-1775.
124. Shabtai Y. Isolation and characterization of a lipolytic bacterium capable of growing in a low-water-content oil-water emulsion. Applied and Environmental Microbiology 1991;57(6):1740-1745.
125. Shabtai Y, Dayamishne N. Production, purification, and properties of a lipase from a bacterium (pseudomonas-aeruginosa ys-7) capable of growing in water-restricted environments. Applied and Environmental Microbiology 1992;58(1):174-180.
126. Morozova D, Wagner D. Stress response of methanogenic archaea from Siberian permafrost compared with methanogens from nonpermafrost habitats. Fems Microbiology Ecology 2007;61(1):16-25.
127. Liu CT, Miyaki T, Aono T, Oyaizu H. Evaluation of methanogenic strains and their ability to endure aeration and water stress. Current Microbiology 2008;56(3):214-218.
128. Mulligan CN, Yong RN, Gibbs BF. Surfactant-enhanced remediation of contaminated soil: a review. Engineering Geology 2001;60(1-4):371-380.
129. Aburto A, Fahy A, Coulon F, Lethbridge G, Timmis KN, Ball AS, McGenity TJ. Mixed aerobic and anaerobic microbial communities in benzene-contaminated groundwater. Journal of Applied Microbiology 2009;106(1):317-328.
130. Singleton DR, Ramirez LG. Isolation of a Phenanthrene. Degrading Acidovorax Strain and Identification of Associated Genetic Elements. Abstracts of the General Meeting of the American Society for Microbiology 2008;108.
131. Singleton DR, Ramirez LG, Aitken MD. Characterization of a Polycyclic Aromatic Hydrocarbon Degradation Gene Cluster in a Phenanthrene-Degrading Acidovorax Strain. Applied and Environmental Microbiology 2009;75(9):2613-2620.

132. Singleton DR, Richardson SD, Aitken MD. Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. Biodegradation 2011;22(6):1061-1073.
133. Eriksson M, Sodersten E, Yu ZT, Dalhammar G, Mohn WW. Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. Applied and Environmental Microbiology 2003;69(1):275-284.
134. Zhang CL, Bennett GN. Biodegradation of xenobiotics by anaerobic bacteria. Applied Microbiology and Biotechnology 2005;67(5):600-618.
135. Miot J, Benzerara K, Morin G, Kappler A, Bernard S, Obst M, Ferard C, Skouri-Panet F, Guigner J-M, Posth N and others. Iron biomineralization by anaerobic neutrophilic iron-oxidizing bacteria. Geochimica et Cosmochimica Acta 2009;73(3):696-711.
136. Biegert T, Fuchs G, Heider F. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium Thauera aromatica is initiated by formation of benzylsuccinate from toluene and fumarate. European Journal of Biochemistry 1996;238(3):661-668.
137. Andersson S, Dalhammar G, Rajarao GK. Influence of microbial interactions and EPS/polysaccharide composition on nutrient removal activity in biofilms formed by strains found in wastewater treatment systems. Microbiological Research 2011;166(6):449-457.
138. Castro-Gutierrez VM, Rodriguez-Rodriguez CE, Vargas-Azofeifa I. Hydrocarbon Degrading Microflora in a Tropical fuel-Contaminated Aquifer: Assessing the Feasibility of PAH Bioremediation. International Journal of Environmental Research 2012;6(1):345-352.
139. Lors C, Ryngaert A, Perie F, Diels L, Damidot D. Evolution of bacterial community during bioremediation of PAHs in a coal tar contaminated soil. Chemosphere 2010;81(10):1263-1271.
140. Zhao L-x, Gao P-k, Cao M-n, Gao M-l, Li G-q, Zhu X-d, Ma T. Research on Population Structure and Distribution Characteristic of Indigenous Microorganism in Post-polymer-Flooding Oil Reservoir. Huanjing Kexue 2012;33(2):625-632.
141. Lindsay MBJ, Wakeman KD, Rowe OF, Grail BM, Ptacek CJ, Blowes DW, Johnson DB. Microbiology and Geochemistry of Mine Tailings Amended with Organic Carbon for Passive Treatment of Pore Water. Geomicrobiology Journal 2011;28(3):229-241.
142. de Jong GAH, Tang JA, Bos P, de Vries S, Kuenen JG. Purification and characterization of a sulfite : cytochrome c oxidoreductase from Thiobacillus acidophilus. Journal of Molecular Catalysis B-Enzymatic 2000;8(1-3):61-67.
143. Coates JD, Lonergan DJ, Philips EJP, Jenter H, Lovley DR. Desulfuromonas palmitatis sp nov, a marine dissimilatory Fe III reducer that can oxidize long-chain fatty acids. Archives of Microbiology 1995;164(6):406-413.
144. Finster K, Coates JD, Liesack W, Pfennig N. Desulfuromonas thiophila sp. nov, a new obligately sulfur-reducing bacterium from anoxic freshwater sediment. International Journal of Systematic Bacteriology 1997;47(3):754-758.

145. Whitby C, Johnson R, Smith B, Robinson K, Rowland S. Biodegradation of naphthenic acids. Geochimica Et Cosmochimica Acta 2009;73(13):A1433-A1433.
146. Herman DC, Fedorak PM, Mackinnon MD, Costerton JW. Biodegradation of naphthenic acids and the reduction of acute toxicity of oil sands tailings. Hydrocarbon Bioremediation 1994:274-277.
147. Antic MP, Jovancicevic BS, Ilic M, Vrvic MM, Schwarzbauer J. Petroleum pollutant degradation by surface water microorganisms. Environmental Science and Pollution Research 2006;13(5):320-327.
148. Biryukova OV, Fedorak PM, Quideau SA. Biodegradation of naphthenic acids by rhizosphere microorganisms. Chemosphere 2007;67(10):2058-2064.
149. Han XM, Scott AC, Fedorak PM, Bataineh M, Martin JW. Influence of molecular structure on the biodegradability of naphthenic acids. Environmental Science & Technology 2008;42(4):1290-1295.
150. Johnson R, Smith B, Sutton P, McGenity T, Rowland S, Whitby C. Microbial biodegradation of aromatic alkanoic naphthenic acids is affected by the degree of alkyl side chain branching. Isme Journal 2011;5(3):486-496.
151. Johnson RJ, West CE, Swaih AM, Folwell BD, Smith BE, Rowland SJ, Whitby C. Aerobic biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two different pathways by a new isolate of Mycobacterium. Environmental Microbiology 2012;14(4):872-882.
152. DJ L. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics: John Wiley and Sons. Chichester, UK.; 1991. p 115-175.
153. Ougham HJ, Trudgill PW. Metabolism of cyclohexane acetic-acid and cyclohexane butyric-acid by arthrobacter sp strain ca1. Journal of Bacteriology 1982;150(3):1172-1182.
154. Ercole C, Sulpizii MP, Veglio F, Bartolini S, Toro L, Lepidi A. Desulfurization of model aromatic molecules by Pedomicrobium sp. Fuel Processing Technology 1997;52(1-3):191-197.
155. Padden AN, Kelly DP, Wood AP. Chemolithoautotrophy and mixotrophy in the thiophene-2-carboxylic acid-utilizing Xanthobacter tagetidis. Archives of Microbiology 1998;169(3):249-256.
156. Maciel BM, Santos ACF, Dias JCT, Vidal RO, Dias RJC, Gross E, Cascardo JCM, Rezende RP. Simple DNA extraction protocol for a 16S rDNA study of bacterial diversity in tropical landfarm soil used for bioremediation of oil waste. Genetics and Molecular Research 2009;8(1):375-388.
157. Hube AE, Heyduck-Soeller B, Fischer U. Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture. Systematic and Applied Microbiology 2009;32(4):256-265.
158. Headley J, Du J, Peru K, Gurprasad N, McMartin D. Evaluation of algal phytodegradation of petroleum naphthenic acids. Journal of Environmental Science and Health Part a-Toxic/hazardous Substances & Environmental Engineering 2008;43(3):227-232.

159. Quesnel DM, Bhaskar IM, Gieg LM, Chua G. Naphthenic acid biodegradation by the unicellular alga Dunaliella tertiolecta. Chemosphere 2011;84(4):504-511.
160. Kryachko Y, Dong X, Sensen CW, Voordouw G. Compositions of microbial communities associated with oil and water in a mesothermic oil field. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 2012;101(3):493-506.
161. Chang Y-T, Lee J-F, Chao H-P. Variability of communities and physiological characteristics between free-living bacteria and attached bacteria during the PAH biodegradation in a soil/water system. European Journal of Soil Biology 2007;43(5-6):283-296.
162. Tumaikina YA, Turkovskaya OV, Ignatov VV. Degradation of hydrocarbons and their derivatives by a microbial association on the base of Canadian pondweed. Applied Biochemistry and Microbiology 2008;44(4):382-388.
163. Kao CM, Chien HY, Surampalli RY, Chien CC, Chen CY. Assessing of Natural Attenuation and Intrinsic Bioremediation Rates at a Petroleum-Hydrocarbon Spill Site: Laboratory and Field Studies. Journal of Environmental Engineering-Asce 2010;136(1):54-67.
164. Chen KF, Kao CM, Hsieh CY, Chen SC, Chen YL. Natural biodegradation of MTBE under different environmental conditions: Microcosm and microbial identification studies. Bulletin of Environmental Contamination and Toxicology 2005;74(2):356-364.
165. Fahy A, McGenity TJ, Timmis KN, Ball AS. Heterogeneous aerobic benzene-degrading communities in oxygen-depleted groundwaters. Fems Microbiology Ecology 2006;58(2):260-270.
166. Paslawski JC, Headley JV, Hill GA, Nemati M. Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid. Biodegradation 2009;20(1):125-133.
167. Wiegel J. The Genus Xanthobacter. Prokaryotes: A Handbook on the Biology of Bacteria, Vol 5, Third Edition: Proteobacteria: alpha and beta subclasses 2006.
168. Janssen DB, Scheper A, Dijkhuizen L, Witholt B. Degradation of halogenated aliphatic-compounds by xanthobacter-autotrophicus gj10. Applied and Environmental Microbiology 1985;49(3):673-677.
169. Agrawal A, Lal B. Rapid detection and quantification of bisulfite reductase genes in oil field samples using real-time PCR. Fems Microbiology Ecology 2009;69(2):301-312.
170. Muyzer G, Stams AJM. The ecology and biotechnology of sulphate-reducing bacteria. Nature Reviews Microbiology 2008;6(6):441-454.
171. Wilms R, Sass H, Kopke B, Cypionka H, Engelen B. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. Fems Microbiology Ecology 2007;59(3):611-621.
172. Callbeck CM, Dong XL, Chatterjee I, Agrawal A, Caffrey SM, Sensen CW, Voordouw G. Microbial community succession in a bioreactor modeling a souring low-temperature oil reservoir subjected to nitrate injection. Applied Microbiology and Biotechnology 2011;91(3):799-810.

173. Narihiro T, Sekiguchi Y. Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea. Microbial Biotechnology 2011;4(5):585-602.
174. Steinberg LM, Regan JM. mcrA-Targeted Real-Time Quantitative PCR Method To Examine Methanogen Communities. Applied and Environmental Microbiology 2009;75(13):4435-4442.
175. Kozubal MA, Dlakic M, Macur RE, Inskeep WP. Terminal Oxidase Diversity and Function in "Metallosphaera yellowstonensis": Gene Expression and Protein Modeling Suggest Mechanisms of Fe(II) Oxidation in the Sulfolobales. Applied and Environmental Microbiology 2011;77(5):1844-1853.
176. Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. Journal of Bacteriology 1998;180(11):2975-2982.
177. Thauer RK. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology-Uk 1998;144:2377-2406.
178. Kappler U. Bacterial sulfite-oxidizing enzymes. Biochimica Et Biophysica Acta-Bioenergetics 2011;1807(1):1-10.
179. Smith CJ, Nedwell DB, Dong LF, Osborn AM. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. Environmental Microbiology 2006;8(5):804-815.
180. Theron J, Cloete TE. Molecular techniques for determining microbial diversity and community structure in natural environments. Critical Reviews in Microbiology 2000;26(1):37-57.
181. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. European Journal of Soil Science 2003;54(4):655-670.
182. Walker NJ. A technique whose time has come. Science 2002;296(5567):557-+.
183. Nayak BS, Levine AD, Cardoso A, Harwood VJ. Microbial population dynamics in laboratory-scale solid waste bioreactors in the presence or absence of biosolids. Journal of Applied Microbiology 2009;107(4):1330-1339.
184. Li W, Wang LY, Duan RY, Liu JF, Gu JD, Mu BZ. Microbial community characteristics of petroleum reservoir production water amended with n-alkanes and incubated under nitrate-, sulfate-reducing and methanogenic conditions. International Biodeterioration & Biodegradation 2012;69:87-96.
185. Luton PE, Wayne JM, Sharp RJ, Riley PW. The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. Microbiology-Sgm 2002;148:3521-3530.
186. Hallam SJ, Girguis PR, Preston CM, Richardson PM, DeLong EF. Identification of methyl coenzyme M reductase A (mcrA) genes associated with methane-oxidizing archaea. Applied and Environmental Microbiology 2003;69(9):5483-5491.
187. Head IM, Jones DM, Larter SR. Biological activity in the deep subsurface and the origin of heavy oil. Nature 2003;426(6964):344-352.

188. Knittel K, Boetius A. Anaerobic Oxidation of Methane: Progress with an Unknown Process. Annual Review of Microbiology 2009;63:311-334.
189. Lloyd KG, Lapham L, Teske A. Anaerobic methane-oxidizing community of ANME-1b archaea in hypersaline Gulf of Mexico sediments. Applied and Environmental Microbiology 2006;72(11):7218-7230.
190. Lovley DR, Phillips EJP. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Applied and Environmental Microbiology 1994;60(7):2394-2399.
191. vandenEnde FP, Meier J, vanGemerden H. Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. Fems Microbiology Ecology 1997;23(1):65-80.
192. Peduzzi S, Tonolla M, Hahn D. Isolation and characterization of aggregate-forming sulfate-reducing and purple sulfur bacteria from the chemocline of meromictic Lake Cadagno, Switzerland. Fems Microbiology Ecology 2003;45(1):29-37.
193. Beller HR, Chain PSG, Letain TE, Chakicherla A, Larimer FW, Richardson PM, Coleman MA, Wood AP, Kelly DP. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium Thiobacillus denitrificans. Journal of Bacteriology 2006;188(4):1473-1488.
194. Sugio T, Domatsu C, Munakata O, Tano T, Imai K. Role of a ferric ion-reducing system in sulfur oxidation of thiobacillus-ferrooxidans. Applied and Environmental Microbiology 1985;49(6):1401-1406.
195. Bosch J, Lee K-Y, Jordan G, Kim K-W, Meckenstock RU. Anaerobic, Nitrate-Dependent Oxidation of Pyrite Nanoparticles by Thiobacillus denitrificans. Environmental Science & Technology 2012;46(4):2095-2101.
196. Ferry JG, Smith PH, Wolfe RS. Methanospirillum, a new genus of methanogenic bacteria, and characterization of methanospirillum-hungatii sp-nov. International Journal of Systematic Bacteriology 1974;24(4):465-469.
197. Ficker M, Krastel K, Orlicky S, Edwards E. Molecular characterization of a toluene-degrading methanogenic consortium. Applied and Environmental Microbiology 1999;65(12):5576-5585.
198. Ulrich AC, Edwards EA. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environmental Microbiology 2003;5(2):92-102.
199. Leutwein C, Heider J. Anaerobic toluene-catabolic pathway in denitrifying Thauera aromatica: activation and beta-oxidation of the first intermediate, (R)-(+)-benzylsuccinate. Microbiology-Sgm 1999;145.
200. Allen MS, Welch KT, Prebyl BS, Baker DC, Meyers AJ, Sayler GS. Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium Thauera sp MZ1T. Environmental Microbiology 2004;6(8):780-790.
201. Agrawal A, Park HS, Nathoo S, Gieg LM, Jack TR, Miner K, Ertmoed R, Benko A, Voordouw G. Toluene Depletion in Produced Oil Contributes to Souring Control in a Field Subjected to Nitrate Injection. Environmental Science & Technology 2012;46(2):1285-1292.

202. Shokrollahzadeh S, Azizmohseni F, Golmohammad F, Shokouhi H, Khademhaghighat F. Biodegradation potential and bacterial diversity of a petrochemical wastewater treatment plant in Iran. Bioresource Technology 2008;99(14):6127-6133.
203. Meijer WG, Nienhuis-Kuiper ME, Hansen TA. Fermentative bacteria from estuarine mud: phylogenetic position of Acidaminobacter hydrogenoformans and description of a new type of Gram-negative, propionigenic bacterium as Propionibacter pelophilus gen, nov., sp nov. International Journal of Systematic Bacteriology 1999;49:1039-1044.
204. Zhang SY, Wang QF, Xie SG. Molecular characterization of phenanthrene-degrading methanogenic communities in leachate-contaminated aquifer sediment. International Journal of Environmental Science and Technology 2012;9(4):705-712.
205. Shimizu S, Ueno A, Ishijima Y. Microbial Communities Associated with Acetate-Rich Gas-Petroleum Reservoir Surface Facilities. Bioscience Biotechnology and Biochemistry 2011;75(9):1835-1837.
206. Dhal PK IE, Kazy SK, Sar P. Culture-independent molecular analysis of bacterial diversity in uranium-ore/-mine waste-contaminated and non-contaminated sites from uranium mines. Biotech 2011;1:261–272.
207. Scott AC, MacKinnon MD, Fedorak PM. Naphthenic acids in athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environmental Science & Technology 2005;39(21):8388-8394.
208. Straub KL, Schonhuber WA, Buchholz-Cleven BEE, Schink B. Diversity of ferrous iron-oxidizing, nitrate-reducing bacteria and their involvement in oxygen-independent iron cycling. Geomicrobiology Journal 2004;21(6):371-378.
209. Schippers A, Jozsa PG, Sand W, Kovacs ZM, Jelea M. Microbiological pyrite oxidation in a mine tailings heap and its relevance to the death of vegetation. Geomicrobiology Journal 2000;17(2):151-162.
210. Manconi I, Carucci A, Lens P. Combined removal of sulfur compounds and nitrate by autotrophic denitrification in bioaugmented activated sludge system. Biotechnology and Bioengineering 2007;98(3):551-560.
211. Muyzer G, Hottentrager S, Teske A, Waver C. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA. A new molecular approach to analyze the genetic diversity of mixed microbial communities. In: Akkermans A, van Elsas J, de Bruijn F, editors. Molecular microbial ecology manual. Dordrecht, The Netherlands: Kluwer Academic Publishing; 1996.
212. Muyzer G, Dewaal EC, Uitterlinden AG. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-rna. Applied and Environmental Microbiology 1993;59(3):695-700.
213. Lovley DR, Phillips EJP. Organic-matter mineralization with reduction of ferric iron in anaerobic sediments. Applied and Environmental Microbiology 1986;51(4):683-689.

Appendix One: List of primers used for 16 S rRNA gene amplification

Primer	*Sequence (5' → 3')*	*references*
1392r	ACGGGCGGTGTCTRC	152
27f	AGAGTTTGATCCTGGCTCAG	152
454 T-FB	adaptor sequence of CTATGCGCCTTGCCAGCCCGCTCAG.	55
454 T-RA	adaptor sequence of CGTATCGCCTCCCTCGCGCCATCAG	55
926f	AAACTYAAAKGAATTGACGG	152
341f	CCTACGGGAGGCAGCAG	211
534r	ATTACCGCGGCTGCTGGCA	212

Appendix Two: Microbial rank identified in pond 6

Table 0-1 Microbial rank identified in pond 6 2008

#groupName	*2 mbs*	*5 mbs*	*6 mbs*	*8 mbs*	*9 mbs*	*11 mbs*	*12 mbs*	*14 mbs*	*15 mbs*	*17 mbs*	*18 mbs*
#totalCount	**2860**	**6402**	**6296**	**6905**	**5581**	**3606**	**4937**	**3767**	**4855**	**5665**	**4980**
#taxonomic term	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Archaea;Euryarchaeota;Methanomicrobia; Methanosarcinales;:Methanosaetaceae; Methanosaeta	50.6	16.8	16.8	23.2	17.1	18.8	20.8	14.0	20.1	9.7	14.0
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus _Methanoregula;	23.4	12.1	8.7	14.7	12.1	11.6	12.5	6.1	9.8	4.8	7.4
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured;	0.3	6.0	5.7	5.4	5.6	5.8	6.1	6.3	6.3	5.6	6.9
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea;	8.8	7.9	8.5	7.3	4.0	3.6	3.5	1.8	2.7	1.2	2.1
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Syntrophus;	0.2	5.1	7.1	7.1	3.4	1.7	2.8	2.1	2.7	1.4	3.1
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;Leptolinea;	0.4	3.5	2.7	3.3	3.1	3.0	3.0	3.0	3.8	2.5	3.1
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfocapsa;	0.3	2.2	1.1	2.1	4.3	3.6	4.1	6.9	3.2	1.3	1.7
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera;	0.3	1.5	2.8	1.3	3.6	3.3	1.6	4.2	1.6	2.6	3.0
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Smithella;	0.1	4.7	4.2	3.3	3.6	3.0	2.4	1.0	2.0	0.4	0.6
Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus;	0.0	0.1	1.1	0.0	0.1	0.0	0.1	13.1	1.1	3.8	2.9
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Brachymonas;	0.0	0.1	0.6	0.1	0.1	0.1	0.0	0.7	2.3	7.8	10.1
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Pelotomaculum;	0.1	1.1	0.4	0.7	2.0	5.0	5.1	2.4	3.7	0.3	0.5
Bacteria;Actinobacteria;Actinobacteria;subActinobacteridae;Actinomycetales;subMicrococcineae;	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	16.7	1.8
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;vadinHA17;	0.1	1.7	1.7	2.2	1.8	2.0	1.8	1.8	1.9	1.2	1.8
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Acidovorax;	0.8	1.0	3.5	0.5	0.6	0.7	0.3	1.4	0.8	4.0	4.1
Bacteria;Candidate_division_WS6;	0.9	1.7	1.1	1.3	1.4	1.2	1.9	1.1	1.2	0.9	0.8
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;	1.1	1.5	1.1	1.0	1.5	1.0	0.6	0.9	0.9	1.7	1.9
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae;uncultured;	0.0	1.1	1.3	1.1	1.6	1.8	1.3	2.1	1.4	0.5	0.9
Archaea;Euryarchaeota;Thermoplasmata;Thermoplasmatales;AMOS1A-4113-D04;	0.6	0.8	1.3	1.0	0.5	1.3	1.2	1.0	2.4	0.6	1.8
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Rhodoferax;	0.1	0.7	2.5	0.9	1.3	1.4	1.2	0.7	1.0	1.4	1.4
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;uncultured;	0.1	1.7	1.1	1.4	1.7	1.1	1.5	1.0	1.4	0.4	0.9
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;Methanomethylovorans;	0.3	0.2	0.3	0.2	0.3	0.3	0.6	1.2	1.1	5.6	1.2
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobacteraceae;	0.1	0.7	0.3	0.9	1.5	1.4	1.5	1.6	2.0	0.4	0.9
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfurivibrio;	0.1	1.0	0.3	0.5	2.3	3.5	2.0	0.4	0.7	0.1	0.2
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	1.5	0.8	3.3	0.3	0.4	1.0	0.4	0.7	0.2	0.8	1.4
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Geobacteraceae;Geobacter;	0.1	0.8	0.8	1.0	1.7	1.7	1.5	0.7	0.6	0.2	0.2
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococcaceae;	0.4	0.9	0.3	0.4	0.9	2.4	1.9	1.0	0.9	0.1	0.1
Archaea;Euryarchaeota;Thermoplasmata;WCHA1-57;	1.2	1.2	0.8	0.9	0.8	1.0	0.9	0.7	0.9	0.4	0.5
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;uncultured;	0.0	0.1	0.4	0.1	0.1	0.0	0.0	1.7	0.2	2.9	3.6
Bacteria;Proteobacteria;Betaproteobacteria;	0.1	0.1	0.7	0.0	0.0	0.0	0.1	2.2	0.5	2.2	3.1
Bacteria;	0.1	0.5	0.6	0.6	0.7	1.0	0.9	0.9	1.7	0.4	0.5

Table 0-2 Microbial rank identified in pond 6 2010

#groupName	*3 mbs*	*6 mbs*	*9 mbs*	*12 mbs*	*15 mbs*	*18 mbs*	*21 mbs*	*22 mbs*
#totalCount	**7600**	**5379**	**6287**	**6012**	**6880**	**5880**	**7242**	**7030**
#taxonomic term	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Archaea;Euryarchaeota;Methanomicrobia; Methanosarcinales;:Methanosaetaceae; Methanosaeta	42.6	31.5	35.7	25.3	39.1	37.0	38.3	38.3
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus _Methanoregula;	19.7	20.5	14.7	9.5	16.2	9.0	11.5	16.3
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;Methanosarcina;	1.2	7.0	9.7	14.4	3.3	6.8	4.0	1.6
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	0.5	0.7	0.9	0.6	7.7	5.6	7.4	8.7
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea;	7.5	5.8	3.2	1.9	2.9	2.2	3.8	4.4
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter;	0.4	2.4	3.2	6.2	1.9	2.5	2.5	0.8
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;	0.8	2.4	3.9	4.5	1.0	3.1	2.2	0.7
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured;	1.4	0.9	0.4	0.6	3.0	1.6	1.6	2.0
Archaea;Euryarchaeota;Methanobacteria;Methanobacteriales;Methanobacteriaceae;Methanobacterium;	0.6	1.9	0.9	0.5	1.1	1.4	1.5	3.3
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Diaphorobacter;	0.3	2.5	1.6	2.0	0.2	2.4	0.8	0.2
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobacteraceae;	0.5	0.6	0.7	0.8	1.9	0.9	1.5	2.5
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;Methanomethylovorans;	0.5	0.4	0.5	0.0	1.9	1.7	1.3	2.6
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophobacteraceae;Desulfoglaeba;	0.0	0.0	0.2	0.1	1.6	1.4	2.8	2.4
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;	1.3	1.3	1.4	1.4	0.6	0.5	0.6	1.2
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;Leptolinea;	1.1	0.5	0.2	0.2	1.6	0.8	1.2	1.4
Bacteria;Proteobacteria;Betaproteobacteria;	0.1	0.6	1.2	1.6	0.5	1.4	0.9	0.3
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Rhizobium;	0.4	0.9	1.2	2.1	0.4	0.8	0.5	0.2
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;Cupriavidus;	0.1	1.1	1.5	1.7	0.3	0.8	0.6	0.2
Archaea;Euryarchaeota;Thermoplasmata;WCHA1-57;	0.9	1.1	0.7	0.2	1.3	0.9	0.3	0.7
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Syntrophus;	2.1	1.0	0.4	0.5	0.4	0.1	0.2	1.3

Table 0-3 Microbial rank identified in pond 6 2011

#groupName	*3.5 mbs*	*4 mbs*	*7 mbs*	*10 mbs*	*13 mbs*	*16 mbs*	*18 mbs*
#totalCount	**6628**	**7381**	**4941**	**3771**	**5189**	**5844**	**6830**
#taxonomic term	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Archaea;Euryarchaeota;Methanomicrobia; Methanosarcinales;:Methanosaetaceae; Methanosaeta	18.2	18.5	21.6	27.3	16.4	14.9	8.2
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus _Methanoregula;	15.4	12.9	24.5	18.6	15.5	11.6	6.0
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter;	0.8	1.2	2.8	6.5	7.7	15.6	3.5
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Syntrophus;	11.1	10.1	4.1	3.3	1.9	0.5	0.5
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea;	7.1	5.7	6.2	3.6	3.4	1.8	1.1
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured;	4.9	4.8	4.4	2.9	3.2	2.8	2.8
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobacteraceae;	2.3	2.8	4.5	2.3	7.1	2.6	1.3
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Aquabacterium;	0.1	0.2	0.5	0.5	0.8	0.5	13.8
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfocapsa;	3.3	2.8	1.5	1.2	2.9	0.9	2.5
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;Leptolinea;	2.4	2.2	1.5	1.6	1.1	1.2	1.3
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae;uncultured;	2.0	1.8	1.0	1.6	1.2	1.8	1.3
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;	0.6	1.1	0.6	0.6	0.7	1.0	5.0
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	0.3	0.2	0.7	1.6	1.5	2.8	1.4
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Rhizobium;	0.2	0.2	0.5	1.1	1.8	3.3	1.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Delftia;	0.2	0.3	0.6	1.5	1.3	3.0	0.9
Bacteria;	0.8	0.9	0.7	1.9	1.0	1.2	1.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Acidovorax;	0.3	0.5	0.3	0.7	1.2	1.4	3.1
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;Cupriavidus;	0.2	0.1	1.1	1.6	1.6	2.3	0.5
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;vadinHA17;	1.8	1.7	1.5	0.3	0.8	0.4	0.6
Bacteria;Caldiserica;Caldisericia;Caldisericales;Caldisericaceae;Caldisericum;	0.5	0.6	0.7	1.2	1.8	1.2	1.1
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Smithella;	2.2	1.9	0.6	0.3	0.3	0.4	1.2
Bacteria;Candidate_division_OP8;	0.5	0.6	0.8	1.0	1.8	1.3	0.8

Appendix Three: Microbial rank identified in pond 5

Table 0-4 Microbial rank identified in pond 5 2009

#groupName	*2.0* mbs	*2.4* mbs	*4.6* mbs	*6.1* mbs	*7.6* mbs	*9.1* mbs	*10.7* mbs	*12.2* mbs	*13.7* mbs	*15.2* mbs	*19.8* mbs	*25.0* mbs	*29.0* mbs
#totalCount	**4696**	**4246**	**4577**	**4092**	**4412**	**4467**	**4517**	**4258**	**4598**	**5435**	**3802**	**1469**	**5275**
#taxonomic term	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta;	15.4	15.7	19.5	31.2	28.5	10.7	9.8	14.2	12.5	13.7	11.7	5.0	12.7
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;	9.6	2.1	6.8	7.3	2.5	13.1	23.2	12.4	16.2	10.7	17.7	12.1	13.1
Bacteria;Proteobacteria;Betaproteobacteria;	9.7	13.4	9.2	4.1	3.3	8.9	11.4	10.8	15.4	15.1	8.8	13.7	10.1
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;uncultured;	5.8	10.1	3.4	4.2	0.9	2.7	9.3	9.7	12.8	9.0	6.7	13.0	5.1
Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus;	5.5	17.4	1.6	0.5	2.3	6.3	4.7	6.7	12.6	14.6	3.1	8.4	6.4
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Brachymonas;	0.8	0.5	21.3	3.5	2.4	10.0	3.5	1.1	0.4	1.8	4.2	1.2	3.6
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;	2.8	2.2	4.7	3.5	4.3	2.5	4.5	4.8	3.7	4.9	6.0	5.0	3.1
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea;	5.5	6.5	1.2	10.6	5.2	4.5	1.1	2.2	1.7	3.6	3.1	1.6	4.1
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus _Methanoregula;	12.3	11.9	1.0	3.7	6.7	0.6	0.1	1.5	0.2	0.3	2.2	2.0	2.3
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Acidovorax;	1.0	0.7	0.6	2.0	2.4	6.0	6.9	1.4	1.5	2.1	2.8	1.6	1.7
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;	1.4	2.3	2.3	1.4	0.8	2.1	2.9	4.3	4.1	2.3	1.2	3.4	1.9
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera;	0.7	0.2	4.4	4.4	6.8	3.2	1.9	1.8	0.8	0.6	0.7	2.2	1.1
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;	1.6	0.9	2.5	0.9	0.3	1.9	4.1	2.6	3.0	2.8	2.8	2.7	2.2
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Rhodoferax;	1.6	0.1	0.2	1.6	0.1	2.1	3.7	2.1	2.7	2.0	3.4	1.6	2.8
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	0.7	0.6	1.5	3.1	3.5	1.3	1.9	0.5	0.3	0.6	1.9	2.5	2.2
Bacteria;Proteobacteria;Gammaproteobacteria;	0.4	0.4	0.3	0.5	0.9	3.2	0.9	1.5	0.6	0.4	2.5	2.5	3.7
Bacteria;Proteobacteria;	0.6	0.7	0.5	1.2	0.9	0.8	1.0	1.1	1.0	0.6	2.3	2.8	2.6
Archaea;Euryarchaeota;Methanobacteria;Methanobacteriales;Methanobacteriaceae;Methanobacterium;	0.1	0.1	0.7	2.2	1.8	0.3	0.1	4.1	0.1	1.3	1.1	1.4	2.0
Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;	0.3	0.5	0.2	0.1	0.5	4.2	0.6	1.2	1.0	0.4	2.2	1.8	1.4
Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;	0.8	2.9	0.3	0.0	0.1	0.8	0.3	0.6	1.5	1.4	0.5	1.1	0.8
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Methyloversatilis;	0.1	0.1	0.1	0.2	0.2	1.0	0.6	2.2	0.7	1.7	1.7	1.2	1.3
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Desulfuromonadaceae;Desulfuromonas;	0.5	0.1	0.4	0.5	2.6	0.6	0.2	2.5	0.8	0.6	0.2	1.2	0.6
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;	1.8	1.4	1.0	1.0	1.4	0.4	0.1	0.4	0.2	0.2	0.3	0.1	0.6
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Desulfuromonadaceae;	0.1	0.1	0.5	0.6	1.9	0.3	0.2	1.0	0.6	0.8	0.2	0.8	0.6
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured;	0.8	0.8	0.7	0.2	1.4	0.0	0.1	0.4	0.2	0.2	1.3	0.3	0.3
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus;	0.4	0.1	4.8	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Archaea;Euryarchaeota;Thermoplasmata;Thermoplasmatales;AMOS1A-4113-D04;	4.6	0.5	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Legionellaceae;Legionella;	0.0	0.0	0.0	0.0	0.0	1.9	1.8	0.5	0.0	0.6	0.0	0.1	0.3
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Syntrophus;	1.8	0.7	0.3	0.4	1.0	0.1	0.0	0.1	0.0	0.3	0.1	0.0	0.1
Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;Smithella;	0.8	0.4	0.5	0.2	0.5	1.2	0.0	0.0	0.1	0.3	0.2	0.2	0.3
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;	0.4	0.0	0.1	0.0	0.2	0.2	0.3	0.6	0.5	0.4	0.5	1.0	0.5

Table 0-5 Microbial rank identified in pond 5 2010

#groupName	*3*	*6*	*9*	*12*	*15*	*18*	*21*	*27*	*33*	*39*	*45*	*51*
	mbs	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**	**mbs**
#totalCount	**5504**	**2667**	**6412**	**2787**	**9845**	**7710**	**8825**	**8156**	**5291**	**8657**	**7638**	**5889**
#taxonomic term	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	24.7	56.5	26.0	51.8	61.3	16.7	25.7	32.7	20.1	40.7	42.5	21.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Acidovorax;	3.1	2.9	4.3	6.3	7.0	11.3	23.1	3.5	3.6	5.8	10.7	48.5
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta;	17.0	6.4	9.8	4.3	2.4	29.9	0.4	1.3	9.5	2.2	1.1	0.4
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;	1.5	3.1	2.0	3.1	2.0	9.4	12.8	2.3	4.2	3.5	2.3	13.7
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter;	0.5	2.6	0.7	1.5	1.1	0.7	0.3	14.1	21.3	3.5	7.9	0.6
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Desulfuromonadaceae;Desulfuromonas;	1.3	2.1	0.7	1.3	2.1	2.2	2.9	9.8	6.2	8.7	5.4	0.9
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Desulfuromonadaceae;	0.3	1.4	1.0	1.2	1.1	0.8	0.9	11.4	1.9	7.0	4.4	0.4
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Comamonas;	0.5	0.6	0.4	1.4	1.6	1.4	4.2	2.6	0.9	5.1	5.0	1.0
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera;	2.5	1.8	2.6	3.3	4.1	0.9	4.6	0.9	0.6	1.0	1.0	0.2
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea;	1.9	1.1	1.9	1.1	1.4	5.0	0.3	0.6	8.0	1.0	0.4	0.1
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured;	7.1	4.1	2.7	2.1	1.2	0.2	1.0	1.4	0.9	0.8	0.8	0.2
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus _Methanoregula;	3.9	0.8	5.4	2.0	0.2	0.3	0.2	0.3	2.5	0.2	0.4	0.1
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Hydrogenophaga;	1.1	4.4	1.6	0.3	0.4	0.1	4.3	0.3	0.5	0.3	0.6	0.2
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfomicrobiaceae;Desulfomicrobium;	0.1	0.8	2.1	1.0	1.2	0.1	0.1	1.7	0.5	3.7	1.2	0.2
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococcaceae;	1.2	0.5	3.0	1.4	1.5	0.8	0.0	0.8	0.3	1.8	0.4	0.1
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus;	1.1	0.0	7.2	2.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus;	0.5	0.3	1.0	0.7	1.1	0.6	1.3	0.4	0.2	0.2	1.6	1.5
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;Leptolinea;	4.7	0.6	1.2	0.6	0.3	0.1	0.3	0.3	0.4	0.4	0.4	0.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Rhodoferax;	0.5	0.4	0.5	0.8	0.4	0.1	2.6	0.8	0.5	1.3	0.8	0.2
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;	0.8	1.0	1.0	1.0	0.3	0.7	0.2	0.3	1.4	0.2	0.3	1.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Aquamonas;	0.1	1.1	0.3	0.4	1.0	0.7	1.8	0.2	0.3	0.5	0.6	0.1
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfocapsa;	1.7	0.2	1.1	0.4	0.4	0.3	0.3	0.2	0.5	1.2	0.6	0.0
Bacteria;Proteobacteria;Gammaproteobacteria;	0.1	0.2	0.0	0.3	0.1	0.9	0.1	0.2	3.3	0.0	0.2	1.0
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae;Acetobacterium;	0.0	0.0	0.0	0.0	0.0	0.1	0.0	5.9	0.0	0.1	0.1	0.0

Appendix Four: Naphthenic acid isolates DNA sequences

ER10 Complete sequence: ***Acidovorax*** **sp. strain ER10 (99 % max identity, query length 1307)**

CTTACNCNTGCAAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCGAACGGGTGAGTAATACATCGGAACGTG
CCCGATCGTGGGGGATAACGGAGCGAAAGCTTTGCTAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGG
CCTTGCGCGGACGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGTAGCTG
GTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA
CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGA
AAAGACTTCTTCTAATACAGGAGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATATAAGACAGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGTATAGCTAGAGTACGGTAGAGGGGGATGGAATTCCGCGTGTAGCA
GTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTTCACTGACT
CAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGAATCCT
TTAGAGATAGAGGAGTGCTCGAAAGAGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACATTTAGTTGGGCACTCTAATGAGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCTGGTA
CAGAGGGTTGCCAACCCGCGAGGGGGAGCCAATCCCATAAAGCCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTG
CGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGAA

ER19 Complete sequence: ***Xanthobacter*** **sp. strain ER19 (99 % max identity, query length 1249)**

CATGCAGTCGAGCGCCCAGCAATGGGAGCGGCAGACGGGTGAGTAACGCGTGGGGATCTGCCCGATGGTACGGAATAAT
TCCGGGAAACTGGGACTAATACCGTATGTGCCCGCAAGGGGAAAGATTTATCGCCATCGGATGAACCCGCGTCGGATTAG
CTAGTTGGTGTGGTAAAGGCGCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGT
GAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTA
ACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCAAGCGTTGCTCGGAATCACTGGGCGTAAAGCGCACGTAGGCGG
GTCGTTAAGTCAGAGGTGAAAGCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGGCGACCTAGAGTTCGAGAGAGGT
TGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGCCAACTGGCTCGA
TACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATGC
TAGCCGTTGGGGAGCTTGCTCTTCAGTGGCGCAGCTAACGCTTTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCA
GCCTTTGACATGGCAGGGCGATTTCCAGAGATGGATCTCTCTCAGCAATGAGCCTGCACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTGCCATCATTCAGTTGGGCA
CTCTAGGGGGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCT
ACACACGTGCTACAATGGTGGTGACAGTGGGATGCGAAAGGGCGACCTCTAGCAAATCTCCAAAAGCCATCTCAGTTCG
GATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGNGGATCAGC

ER28 Complete sequence: ***Pseudomonas putida*** **strain ER28 (100 % max identity, query length 1302)**

TGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAG
TGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGC
TATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG
GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG
AAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGT
AAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA
GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCG
GGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGT
AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTG
AGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAG
AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATG
GTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACT
CGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCATAA

Appendix Five: Diversity indexes for pond 6

Table 0-6 Taxonomic classification and estimated richness for pond 6 samples using 95% similarity values

sample	*# of reads*	*# of OTUs*	*Chao index*	*Shannon index*	*Simpson index*
Pond 6 2008 3.0 mbs	11350	1349	2801.7	5.78	0.01
Pond 6 2008 5.0 mbs	10612	1355	3122.5	5.63	0.02
Pond 6 2008 6.0 mbs	9860	1091	2454.8	5.42	0.02
Pond 6 2008 8.0 mbs	10824	1259	3027.2	5.27	0.03
Pond 6 2008 9.0 mbs	10273	1171	2759.5	5.53	0.02
Pond 6 2008 2.0 mbs	5222	888	2531.9	4.98	0.06
Pond 6 2008 11.0 mbs	6977	937	2141.7	5.81	0.01
Pond 6 2008 12.0 mbs	9511	1135	2543.3	5.84	0.01
Pond 6 2008 14.0 mbs	7637	909	2137.9	5.57	0.02
Pond 6 2008 15.0 mbs	9826	1165	2619.1	5.91	0.01
Pond 6 2008 17.0 mbs	9818	1064	2667.3	5.56	0.01
Pond 6 2008 18.0 mbs	9283	1061	2311.0	5.70	0.01
Pond 6 2010 3.0 mbs	12853	1640	4195.2	5.17	0.05
Pond 6 2010 6.0 mbs	8964	1302	3396.5	5.24	0.04
Pond 6 2010 9.0 mbs	10570	1537	3742.0	5.33	0.04
Pond 6 2010 12.0 mbs	10029	1463	3739.7	5.47	0.03
Pond 6 2010 15.0 mbs	11434	1499	3798.9	5.11	0.05
Pond 6 2010 18.0 mbs	9698	1481	3843.6	5.38	0.04
Pond 6 2010 21.0 mbs	12180	1757	4823.5	5.38	0.05
Pond 6 2010 22.0 mbs	11773	1587	3975.4	5.20	0.05
Pond 6 2011 3.5 mbs	10033	1452	3274.5	5.68	0.02
Pond 6 2011 4.0 mbs	11180	1645	3502.2	5.85	0.02
Pond 6 2011 7.0 mbs	7679	1109	2239.9	5.30	0.03
Pond 6 2011 10.0 mbs	5813	911	1905.9	5.21	0.03
Pond 6 2011 13.0 mbs	7951	1143	2409.6	5.58	0.02
Pond 6 2011 16.0 mbs	9687	1254	2797.3	5.56	0.02
Pond 6 2011 18.5 mbs	9838	1435	2915.2	5.87	0.01

Appendix Six: Rarefaction curve of all pond 6 samples

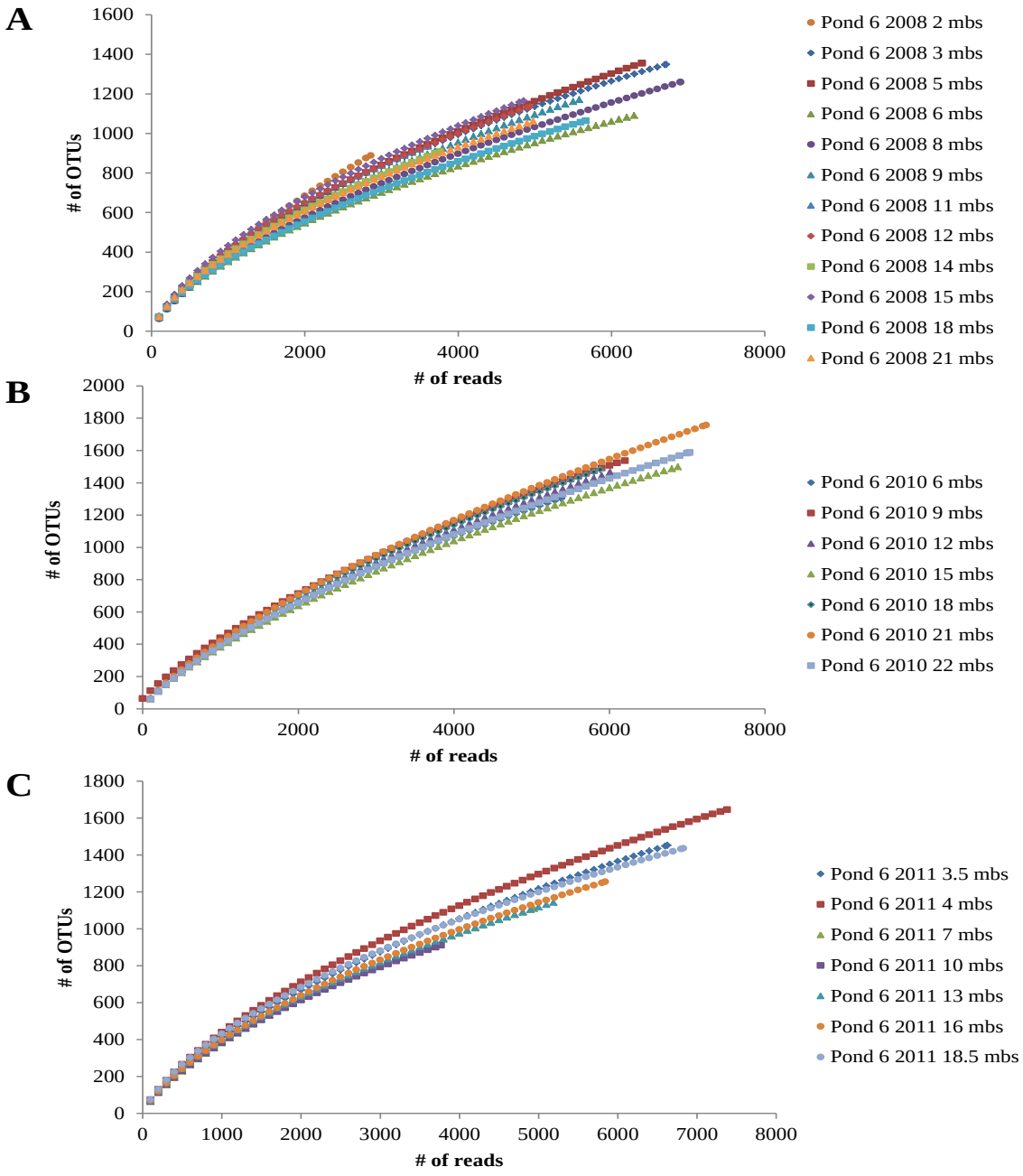


Figure 0-1 Rarefaction curves for the microbial community of Suncor pond 6 in the years (A) 2008, (B) 2010, and (C) 2011.

Appendix Seven: Diversity indexes for pond 5

Table 0-7 Taxonomic classification and estimated richness for pond 5 samples using 95% similarity values.

Sample	*# of reads*	*# of OTUs*	*chao index*	*shannon index*	*simpson index*
Pond 5 2009 1.8 mbs	4697	2059	6934.6	6.63	0.01
Pond 5 2009 2.4 mbs	4246	1514	4470.3	6.04	0.01
Pond 5 2009 4.6 mbs	4583	1868	6323.8	6.55	0.01
Pond 5 2009 6 mbs	4094	1845	6934.7	6.56	0.01
Pond 5 2009 8 mbs	4418	1850	6515.6	6.51	0.01
Pond 5 2009 9 mbs	4474	1925	5602.0	6.57	0.01
Pond 5 2009 11 mbs	4520	1953	5798.4	6.61	0.01
Pond 5 2009 12 mbs	4258	1825	5681.8	6.52	0.01
Pond 5 2009 14 mbs	4599	1616	4418.5	6.20	0.01
Pond 5 2009 15 mbs	5436	2239	7377.6	6.63	0.01
Pond 5 2009 20 mbs	3804	1989	7871.0	6.86	0.00
Pond 5 2009 25 mbs	1472	832	3476.2	6.13	0.01
Pond 5 2009 29 mbs	5276	2523	8660.1	6.98	0.00
Pond 5 2010 3 mbs	5507	1136	2494.0	5.69	0.01
Pond 5 2010 6 mbs	2669	562	1260.0	4.81	0.03
Pond 5 2010 9 mbs	6432	1571	4455.7	5.90	0.01
Pond 5 2010 12 mbs	2789	699	1796.5	5.07	0.02
Pond 5 2010 15 mbs	9845	883	2098.7	3.81	0.12
Pond 5 2010 18 mbs	7711	2459	7237.1	6.33	0.01
Pond 5 2010 21 mbs	8825	1123	2277.1	4.48	0.05
Pond 5 2010 27 mbs	8156	791	1721.1	4.10	0.06
Pond 5 2010 33 mbs	5293	1854	5919.0	6.22	0.01
Pond 5 2010 39 mbs	8658	690	1664.6	3.87	0.07
Pond 5 2010 45 mbs	7638	904	2266.8	4.33	0.06
Pond 5 2010 51 mbs	5889	1480	4274.6	4.81	0.10

Appendix Eight: Rarefaction curve pond 5.

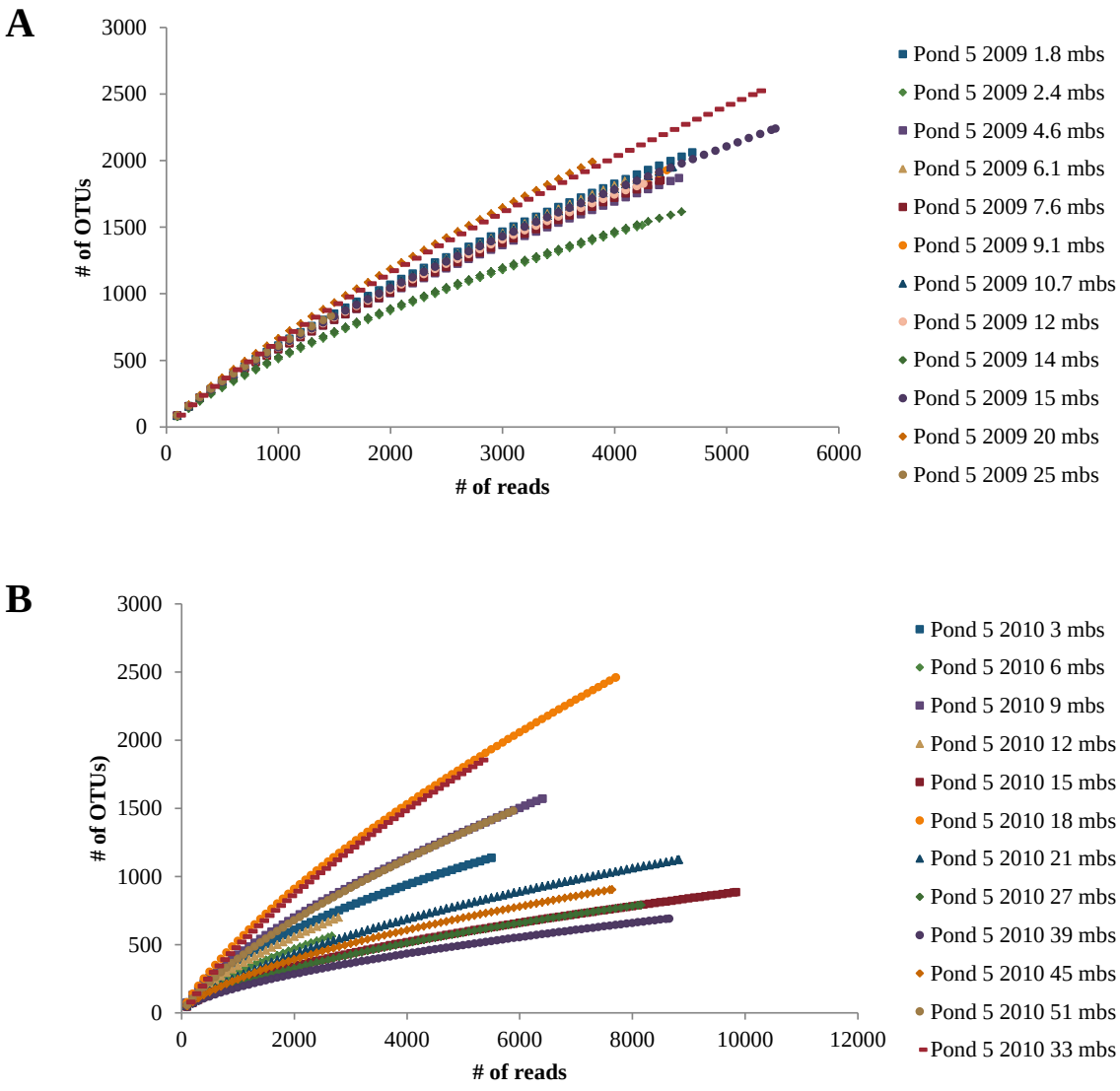


Figure 0-2 Rarefaction curves for the microbial community of Suncor pond 5 in the years (A) 2009, (B) 2010.

Appendix Nine: Diversity indexes for surface water samples

Table 0-8 Taxonomic classification, observed and estimated richness for TPW samples using 95% similarity values.

group	*# of reads*	*# of OTUs*	*Chao index*	*Shannon index*	*Simpson index*
P6 2008	10009	311	409.4	3.87	0.06
P5 2009	2827	278	439.6	4.33	0.03
P6 2011	6342	373	565.4	4.13	0.05

Appendix Ten: Rarefaction curves for surface water samples

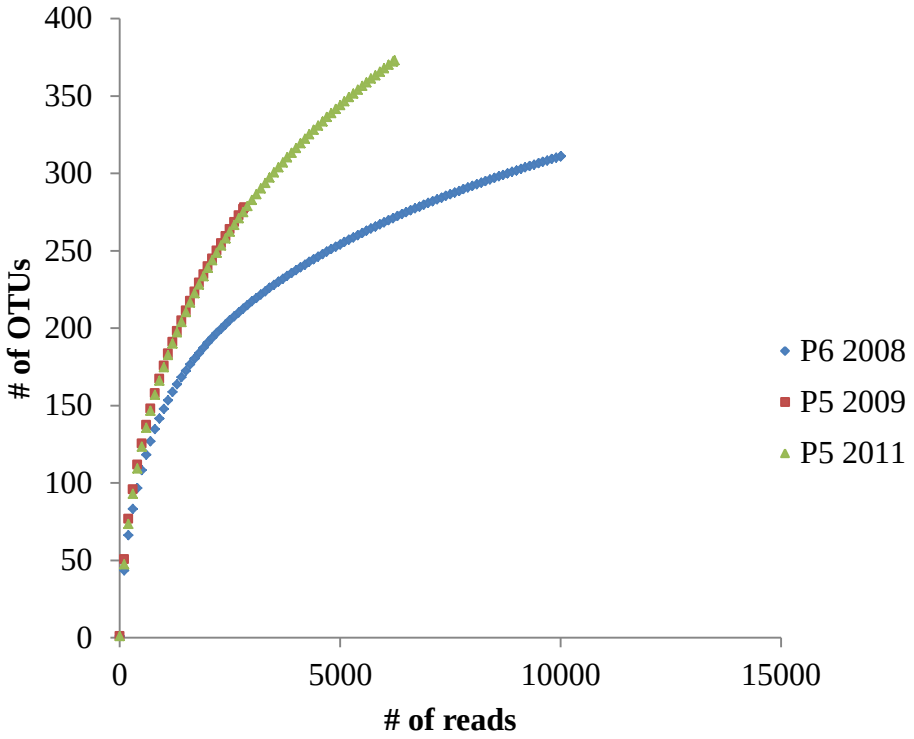


Figure 0-3 Rarefaction curves for the microbial community in TPW.

Appendix Eleven: Sulfur metabolism in tailings from pond 6 2008 and 2010.

Obtained from metagenomic analysis by KEGG (http://www.genome.jp/kegg/)

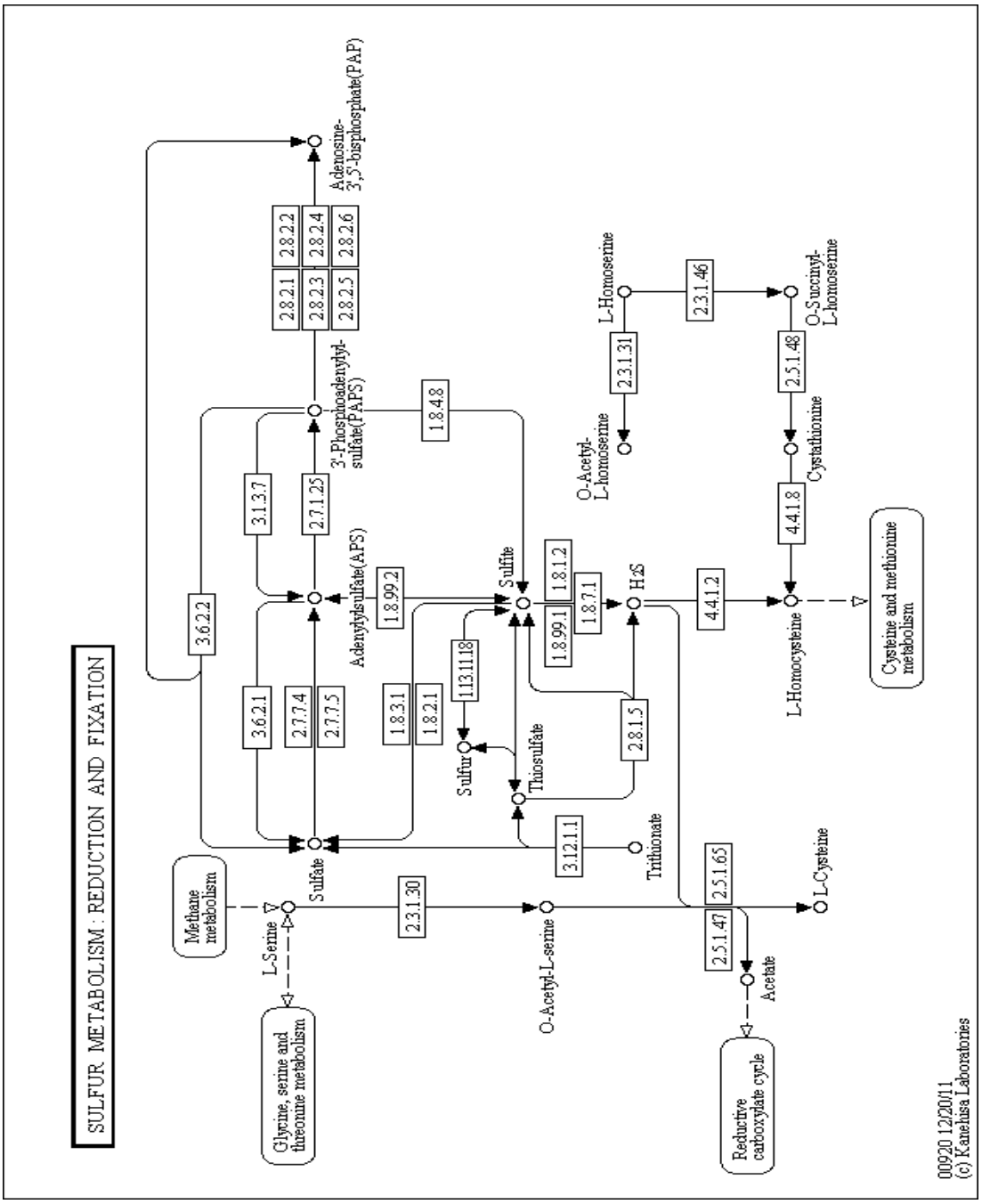


Figure 0-4 Sulfur metabolism in tailings ponds obtained by metagenomics.

Appendix Twelve: Iron concentration in oil sands tailings ponds

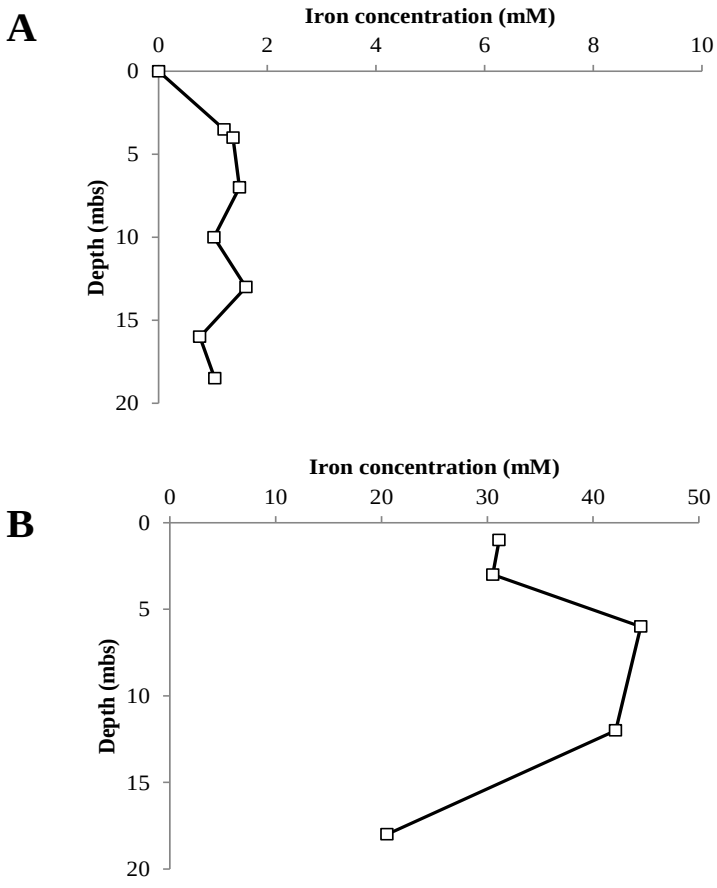


Figure 0-5 Iron concentration (Fe (II)) determined by the Ferrozine assay [213] in oil sands tailings. (A) pond 6 sampled in 2011, and (B) pond 6 sampled in 2012.