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# Usage of laboratory bench scale testing in environmental remediation strategies

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## Abstract

*The oil sands tailings ponds of the Athabasca region, in Northern Alberta, are complex slurries of residual bitumen, oil organics, naphtha diluent, water, sand, clay and heavy metals. These ponds present a unique, yet challenging environment to work with. Remediation of organic contaminant can occur via abiotic and biotic mechanisms. Acceleration of these processes can be achieved through amendments, such as addition of nutrients, surfactants and oxygen, to the contaminated environment which can stimulate microbial growth and metabolism as well as favourable abiotic reactions. Bioremediation efficacy varies greatly from site to site due to changing environmental conditions, soil characteristics, contaminant composition and complexity and indigenous microbial communities. Due to this complexity, assessment of the feasibility of implementing a biologically-based remediation solution and the establishment of optimal treatment conditions for specific sites is critical to ensure efficacy of the bioremediation process, especially in areas exposed to challenging conditions such as mine and oil sands tailings. The bioremediation potential of a site can be evaluated using laboratory or bench scale biotreatability testing, where field conditions are mimicked and the optimal conditions for contaminant degradation are identified. Advanced approaches to bioremediation may also involve the use of mixed species biofilms (microbial consortia). Biodegradation of a compound is often dependent on a microbial consortium, a mixture of species often including bacteria, fungi and archaea (extremophiles), as individual organisms can metabolise only a limited range of substrates. Bioremediation of tailings could be optimised through better understanding and utilisation of diverse microbial groups that are indigenous to mining environments, and through the use of biofilms. A few studies are presented to illustrate: i) the value of bench scale biotreatability testing; ii) the application of non-conventional microbial and molecular techniques; and iii) biofilm methodology; to gather a better understanding and to test the efficacy of bioremediation programmes prior to implementation in the field.*

## 1 Introduction

Microbial communities play a key role in the nutrient cycling of the soil ecosystem and provide a vast array of metabolic capabilities that can be applied to various aspects of the surface mining process, such as:

- Bioremediation of organic and inorganic contaminants generated through the mine life cycle, including metals, hydrocarbons (oil sands) and complex tailings.
- Recovery of valuable metals such gold, copper and uranium from mining waste, low-grade ores and metal-bearing minerals (biomining, or bioleaching) (Gadd, 2010).
- Measurement of success of soil ecosystem reclamation/restoration approaches on mine closure through estimation of soil health based on microbial abundance and diversity.
- Microbial communities from the Athabasca oil sands mining operations have been characterised and shown to be able to biodegrade compounds such as naphta, naphtenic acids (NAs) and hydrocarbons

(HCs), which indicates there is potential for in situ bioremediation in those sites (Hadwin et al., 2006; Sidique et al., 2006; Ramos-Padron et al., 2011).

Bioremediation comprises the use of living organisms to promote the degradation of pollutants to a non-toxic state or to levels below concentration limits established by regulatory guidelines (Vidali, 2001). Bioremediation efficacy varies greatly from site to site due to changing environmental conditions, soil characteristics, contaminant composition and complexity, and abundance and diversity of indigenous microbial communities (Heitzer and Sayler, 1993). Bench scale studies are designed to mimic field conditions as closely as possible under controlled laboratory settings, providing a better understanding of remediation strategies for particular sites before large scale field studies are undertaken. Multiple treatment options (temperature, nutrient, surfactants, redox agents) can be applied to the contaminated sample (soil, water, sediment, tailings), while reduction of contaminant levels are monitored through routine chemical analysis over time to assess which is the most effective treatment. These studies also allow for modelling of the time required to achieve elimination or reduction of contaminants to required regulatory levels (Atlas, 1995).

Until recently, characterisation of microorganisms living in the environment depended mainly on studies of pure single species grown in artificial media in a laboratory (Pace, 1997). It is estimated, however, that only 1 to 10% of environmental microbes can be grown under such conditions. The development and widespread application of culture-independent molecular (DNA-based) methods to microbial ecology have revealed the immense metabolic diversity of microorganisms (Stenuit et al., 2008).

Polymerase Chain Reaction (PCR) has been successfully used for evaluation of microbial diversity and for the detection of specific microbes of interest in environmental samples. The 16S rDNA is routinely used in environmental microbiology studies as it is almost identical amongst all bacterial groups, allowing for detection of all representative species in microbial populations found in a particular site. Each bacterial species also contains small unique ("signature") 16S DNA sequences that can be used to detect specific groups (DeLong and Pace, 2001; Kirk et al., 2004; Head et al., 1998). These sequences can be separated by Denaturing Gradient Gel Electrophoresis (DGGE), which allows for differentiation of DNA fragments based on differences in their sequences (Muyzer et al., 1993). DGGE generates a pattern of bands of DNA similar to a "bar code" (fingerprint), which provides a "snapshot" of the genetic diversity in a complex microbial ecosystem. DNA fingerprinting enables monitoring of changes occurring in microbial communities undergoing environmental disturbances or subjected to different treatments in a timely and cost-effective manner. In addition, quantitative PCR (qPCR) can be used to estimate abundance of microorganisms in the environment (Stenuit et al., 2008).

An important concept in microbial ecology is that, in nature, often microorganisms grow as biofilms. Biofilms are microbial communities commonly composed of more than one species, attached to a surface and enveloped in a slimy, protective matrix. They are inherently more resistant to environmental and chemical stressors (such as heavy metals and organic pollutants) than their planktonic (non surface-attached) counterparts and therefore more likely to succeed in harsh contaminated environments (Hall-Stoodley et al., 2004). Microorganisms growing as biofilms also undergo a phenotypic shift, meaning that their metabolic activities are altered, and production of enzymes that are favourable to remediation are often increased (Singh et al 2006). Examples of biofilms include dental plaque, biofouling of ship hulls and pipelines, the slimy coverage of river rocks and moulds growing on walls (Costerton et al., 1987; Harrison et al., 2005a). Biofilms have successfully been applied in wastewater treatment operations (Nicolella et al., 2000), where mixed microbial biofilms (sometimes grown as flocs) are used to "clean up" activated sludges. Biofilms have been used to clean up crude oil, jet fuel, volatile organic compounds and chlorinated solvents (Schachter, 2003).

This paper presents examples of how the concepts discussed above can be applied in the laboratory to evaluate possible solutions to remediation needs in the oil sands and mining industry. The studies illustrate: i) the value of bench scale biotreatability testing; ii) the value of using non-conventional microbial and molecular techniques; and iii) the value of using biofilm methodology; to gather a better understanding and to test the efficacy of bioremediation programmes prior to implementation in the field.

# 2 Methodology

## 2.1 Microcosm setup

### *2.1.1 Tetrachloroethene (PCE) biotreatability study*

Tests were set up under anaerobic conditions using an Aldrich® AtmosBag (Sigma-Aldrich) purged with 100% Nitrogen gas (N2). The microcosms were set in 250 mL amber bottles with Teflon lined septa caps. Each microcosm contained a ratio of soil to water of 3:1 (60 g of soil (wet weight) and 20 mL of site water added on top). Two different carbon sources (lactate and molasses) and a reducing agent, nano-scale zero-valent iron (nZVI), were used as amendments. Lactate amendment was added at 50, 150 and 250 mg/L (lactic acid, Fluka, Biochemika) and the molasses was added at 50, 250 and 500 mg/L (Crosby Molasses Company Ltd.). The concentration of nZVI to be added as amendment was determined by measuring the redox potential of nZVI solutions at various concentrations with a YSI 565 MPS meter (Multiparameter System). A concentration of 4 g/L was established as necessary to achieve a redox potential of -400 mV. Microcosms were incubated in an environmental test chamber at 10 ± 2°C for 90 days in darkness. Samples were taken for chemical and bacterial analysis every 30 days.

### *2.1.2 Oil sands tailings pond study*

Experimental tailing ponds from the Athabasca oil sands region (Alberta, Canada) were sampled in the fall of 2008 and spring and summer of 2009. The samples collected in 2008 were used to set up microcosms in the laboratory with sediments from the experimental tailing ponds capped with their respective pond water. The microcosms were set up in duplicate, under aerobic and anaerobic conditions (to mimic sediment conditions under shallow and deep water) and incubated in the dark at 20 ± 2°C. Anaerobic conditions were maintained by flushing the vessels weekly with nitrogen and resealing. Each anaerobic test vessel was equipped with an anaerobic indicator strip (BD BBL). The microcosms were sampled at 10 and 20 months after initiation and compared to field samples analysed at time of collection for microbial diversity based on DNA techniques.

## 2.2 DNA extraction

DNA was extracted from different substrates (soil, tailings, water) using commercially available DNA isolation kits, with or without incorporating a bead beating step. The kits used were the MO Bio Power Soil™ DNA isolation kit (Carlsbad, CA), or MP Bio Soil DNA Extraction™ kit (Solon, OH) for soil, sediment and microcosm samples and the MP Bio Power Water DNA Extraction™ kit (Solon, OH) for water samples, following manufacturers' instructions.

## 2.3 Polymerase chain reaction (PCR)

PCR is a standard technique consisting of an enzymatic reaction that creates millions of copies of a specific DNA fragment. PCR reactions consists of a mixture of template DNA, a polymerase enzyme, small DNA fragments called "primers" that attach to specific targets in the template DNA, and nucleotides, water and buffer (Mullis and Faloona, 1987). PCR was used to amplify a section of the 16S ribosomal DNA gene (which is almost identical in sequence for all microbial species) from the genomic DNA extracted from environmental and tailings samples. DNA Primers based on identical fragments of the 16S gene – universal primers – allow for detection of all representative species of bacteria or archaea in a population. Bacterial and archaeal species also contain unique 16S DNA sequences that can be used as "signatures" for species-specific detection. Universal bacterial primers, universal archaeal primers and specific primers for the 16S gene of Dehalococcoides spp. were used for detection, profiling and quantification of microbial populations.

Quantitative PCR (also called Real Time – RT – PCR or qPCR) allows for monitoring of the progress of a PCR reaction based on the detection and quantification of a fluorescent dye that binds to the target DNA template. The amount of fluorescence emitted at each amplification cycle allows for calculation of the initial amount of target template, which in turn can be used to estimate the abundance of the microorganism based on gene copy numbers (Stenuit et al., 2008) The SYBR® Green detection method (BioRad) was used to quantify 16S rRNA gene copy numbers of total bacteria and Dehalococcoides spp.

### 2.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a particular type of gel electrophoresis that separates DNA fragments based on differences in their sequence, which changes the rate at which the two DNA strands separate under denaturing chemical conditions when heat and electric current is applied. DGGE gels were prepared using 6–8% polyacrylamide with a denaturant gradient of 30–60% of urea (Fisher Scientific) and formamide (Sigma-Aldrich) (Muyzer, 1993). Gels were run in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 60°C and 60 V for 17 hours. DNA bands were visualised through fluorescent staining with SYBR® Gold (Invitrogen), visualised on a UV-transilluminator and digitally photographed using the BioRad GelDoc system (Bio-Rad Laboratories).

### 2.5 454 Pyrosequencing

PCR-amplified 16S rDNA fragments (5 ng/uL) were processed at Genome Quebec and McGill University Innovation Centre, in Montreal, Quebec, Canada for pyrosequencing. Sequencing was performed with a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation), following manufacturer's instructions. The 16S rDNA data were processed using the Phoenix pipeline developed in the Sun Center of Excellence for Visual Genomics (COE) in Calgary.

### 2.6 Biofilm culturing

Biofilms were grown using the MBEC Device (Innovotech, Inc., Edmonton, Canada) (formerly Calgary Biofilm Device) following published procedures (Ceri et al., 1999, 2001, Harrison et al., 2005b, 2010) through direct incubation with oil sands tailings material. Briefly, wells of a 96 well microtitre plate were filled with desired tailing inoculum (soil or water) along with a specific growth medium. The plates were incubated on a gyro rotary shaker at 150 rpm to stimulate biofilm attachment and growth on the device surface.

## 3 Results

### 3.1 Biotreatability bench test of soil and groundwater contaminated with chlorinated compounds

The objective of this study was to assess the potential for biological reduction of tetrachloroethene (also known as perchlorotethylene; PCE) and other chlorinated aliphatic hydrocarbons in contaminated soil and groundwater through evaluation of various amendments. Two different carbon sources (lactate and molasses), a redox agent (nZVI) agent and their combinations were used as amendments (Table 1). The efficacy of the treatments was measured using both chemical and biological indicators. The detection and growth of Dehalococcoides ethenogenes strain 195, which has been identified as capable of degrading PCE to ethene (Moretti, 2005), was carried out through qPCR with specific primers. Growth of total bacteria was also measured through qPCR, using 16S DNA universal primers (Figure 1). Measurement of levels of PCE and other volatile organic compounds (VOCs) to compare the treatments efficacy in relation to non-treated controls was used as chemical indicator. The study was conducted over a period of 90 days, with sampling every 30 days.

The results showed there was a reduction in the PCE levels in the treatments throughout the duration of the test (Table 1). The removal efficiency of PCE using molasses as a co-substrate was higher than using lactate as a co-substrate. When molasses was used in conjunction with nZVI, the dechlorination process was improved for most treatments. Conversely, molasses was also the best co-substrate for the growth stimulation of the Dehalococcoides population (Figure 1).

**Table 1 Reduction of levels of Tetrachloroethene (PCE)**

| Treatment | Carbon Source (mg/L) | | | Result (mg/kg) | | | | Percent Reduction (%) |
|---|---|---|---|---|---|---|---|---|
| | Lactate | Molasses | nZVI | Day 0 | Day 30 | Day 60 | Day 90 | |
| AC | | | $^{-}$ | 0.79 | 1.10 | 0.51 | 0.49 | 38 |
| AC + nZVI | | | $^{+}$ | 0.75 | 0.76 | 0.48 | 0.45 | 40 |
| BC | | | $^{-}$ | 1.70 | 1.20 | 1.60 | 0.83 | 51 |
| BC + nZVI | | | $^{+}$ | 1.10 | 5.00 | 0.55 | 0.31 | 72 |
| 1 | 50 | | $^{-}$ | 1.10 | 1.80 | 1.10 | 0.40 | 64 |
| 2 | 50 | | $^{+}$ | 1.20 | 1.70 | 0.78 | 0.68 | 43 |
| 3 | 150 | | $^{-}$ | 1.10 | 1.10 | 0.52 | 0.41 | 63 |
| 4 | 150 | | $^{+}$ | 1.10 | 0.99 | 0.64 | 0.55 | 50 |
| 5 | 250 | | $^{-}$ | 1.40 | 1.40 | 1.10 | 0.45 | 68 |
| 6 | 250 | | $^{+}$ | 1.20 | 0.80 | 0.50 | 0.44 | 63 |
| 7 | | 50 | $^{-}$ | 1.20 | 2.30 | 0.59 | 1.10 | 8 |
| 8 | | 50 | $^{+}$ | 1.10 | 1.20 | 0.23 | 0.36 | 67 |
| 9 | | 250 | $^{-}$ | 1.40 | 2.20 | 0.74 | 0.69 | 51 |
| 10 | | 250 | $^{+}$ | 1.10 | 0.92 | 0.43 | 0.32 | 71 |
| 11 | | 500 | $^{-}$ | 1.40 | 1.80 | 0.69 | 0.39 | 72 |
| 12 | | 500 | $^{+}$ | 1.10 | 1.10 | 0.50 | 0.38 | 65 |

Notes: Reportable Detection Limit = 0.02 mg/Kg; nZVI, nanoscale zero-valent iron; AC, abiotic control; BC, biotic control.

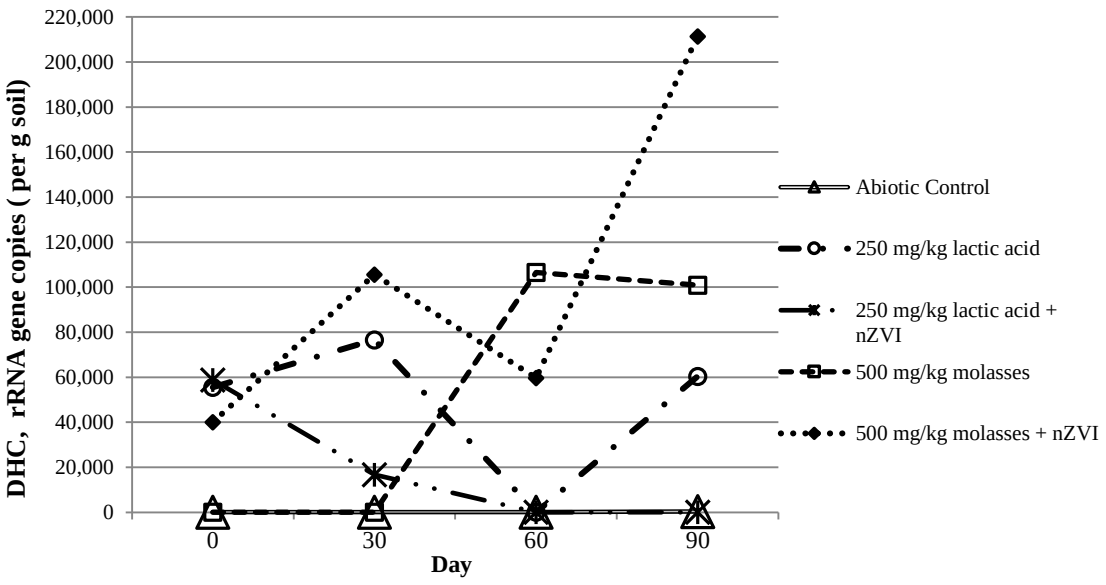


**Figure 1 Quantitative estimation of Dehalococcoides spp. populations using qPCR (Note: not all treatments described in Table 1 are shown in Figure 1)**

This bench scale approach combining conventional biotreatability studies with molecular techniques can be applied to different types of contaminants, including hydrocarbons (HC) and metals. In addition to qPCR, which is particularly useful for organisms like Dehalococcoides spp. that are very difficult to grow under laboratory conditions, other techniques such as MPN (most probable number) with specific substrates (HC, metals, etc.) can be used to estimate growth of specific microbial populations.

## 3.2 Microbial diversity and applications in oil sands tailings

### *3.2.1 Microbial profile of various oil sands tailings samples through PCR-DGGE*

Samples from various tailings ponds from the Athabasca oil sands were collected in the fall of 2008 and spring and summer of 2009. These tailings had been set up years ago in situ under various conditions: mature fine tailings (MFTs) only; MFTs in combination with various sources of capping water (tailings water, water from a lake, fertilised water, etc. Microbial communities (bacterial and archaeal) were analysed based on DNA fingerprinting (PCR-DGGE) for both sediment and water of each tailings pond (Figure 2).

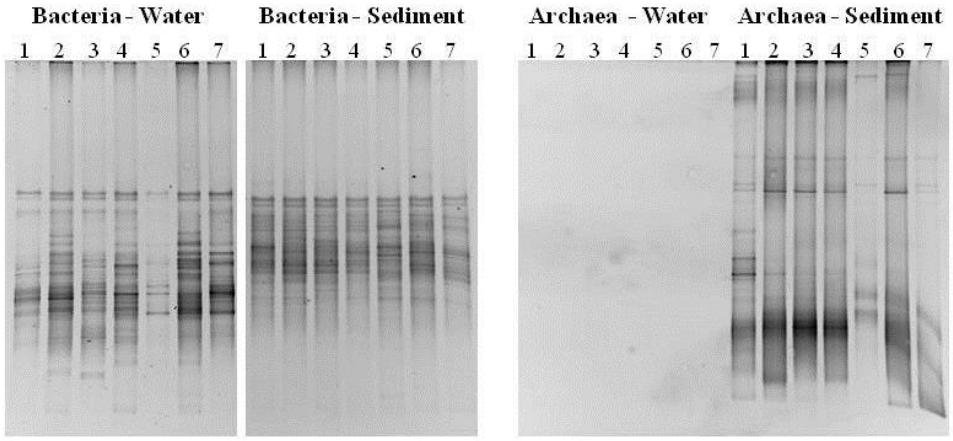


**Figure 2 Microbial profile of populations of bacteria and archaea in various oil sands tailings ponds sediment and water (Tailings 1 through 7, fall 2008 sampling event)**

The results presented in Figure 2, in addition to the same data obtained for samples collected from the same tailings in the spring and summer 2009 (data not shown) indicate that genetically diverse bacterial communities were present in both water and sediment phases of all tailings ponds. Bacterial profiles detected in the water phase were more variable amongst samples than sediment ones. The bacterial profiles of the tailings sediments were similar to one another (Figure 2) and overall did not show dramatic seasonal changes (data not shown) in their microbial profiles. The relative abundance of some bacterial groups, as revealed by the intensity of certain DNA bands on DGGE, varied seasonally, which was much more pronounced in water samples (data not shown).

Archaea were not detected in tailings water (Figure 2) from any tailings, in any of the sampling events, except for one faint band (indicative of low abundance) detected on Tailings 6 in the spring of 2009 (data not shown). Based on DGGE profiles, the richness of archaeal species was lower than the bacterial ones.

### *3.2.2 Microcosm studies of oil sands tailings under aerobic and anaerobic conditions*

Experimental tailings pond microcosms were created using the oil sands tailings sediment described above, capped with corresponding tailings water. The purpose of these microcosms was to evaluate the feasibility of developing a generic laboratory model for bioremediation studies of tailings in a bench scale setting. Duplicate sets of microcosms were developed, one incubated under aerobic conditions and the other under anaerobic conditions. After 3 months, visual changes in the sediment structure were observed in the microcosms. Distinct layers of colour, potentially iron oxides, were visible in the sediments of the aerobic microcosms (data not shown). The microcosms (water and sediments) were analysed after 10 and 20 months

of incubation using DNA profiling. Figure 3 shows results of bacterial population profiling in microcosm water and sediments after 20 months, under both aerobic and anaerobic conditions.

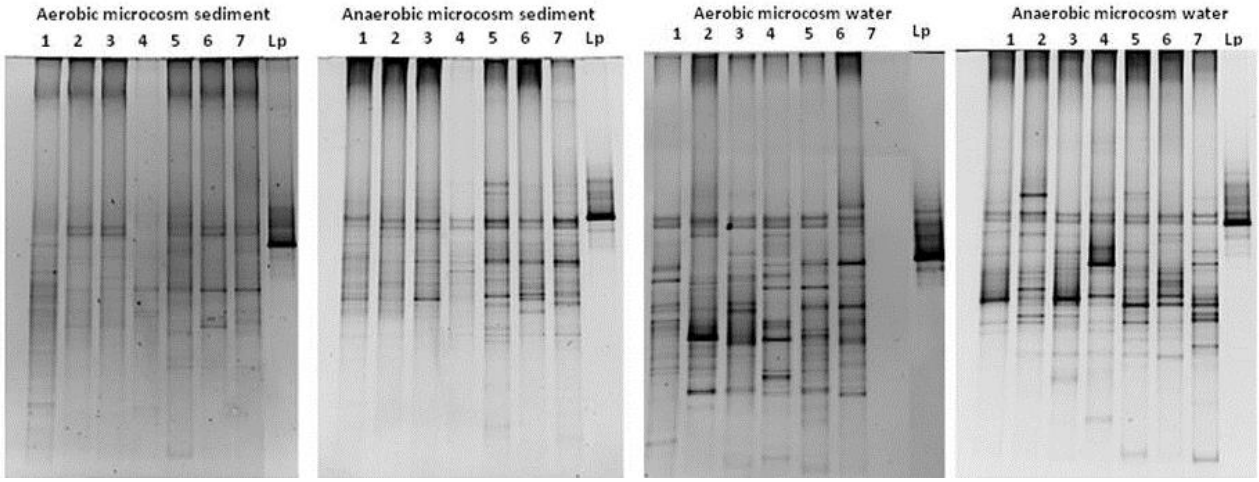


**Figure 3 Bacterial DNA profiling of anaerobic microcosms 20 months after initiation. The number indicates the source pond and Lp indicates control DNA from L pneumophila**

Overall, differences in the bacterial populations in sediments between the aerobic and anaerobic microcosms over a 20 month period were not pronounced. In contrast, the populations present in the water samples were more variable (Figure 3).

The microbial populations in the microcosms, especially in sediments, were also similar to the ones derived from the source material 20 months of incubation (Figures 2 and 3, comparison gel not shown). This indicates that the populations are stable and in equilibrium and that bench scale microcosm studies have good potential for bioremediation investigations on tailings. This consistency would allow for testing of different remediation treatments and evaluation of their effects on the microbial populations in relation to potential reduction of contaminants, similarly to the study described in section 3.1.

## 3.3 Biofilms and bioremediation of mine tailings

In this study, biofilms were initiated (seeded) from tailings ponds from the Athabasca Oils Sands for in vitro growth on the MBEC Device, following the general procedures outlined in Figure 4. The microbial profile of the biofilm population was determined based on PCR and DNA sequencing of 16s DNA through 454 DNA pyrosequencing, a high throughput sequencing tool used to identify a large number of microorganisms within a population. Sequencing of biofilms formed on MBEC device from oil sands tailings sediment sample revealed a diverse microbial population, with identification of many bacterial and a few archaea genera, some of which with known degradative potential for known contaminants (Table 3).

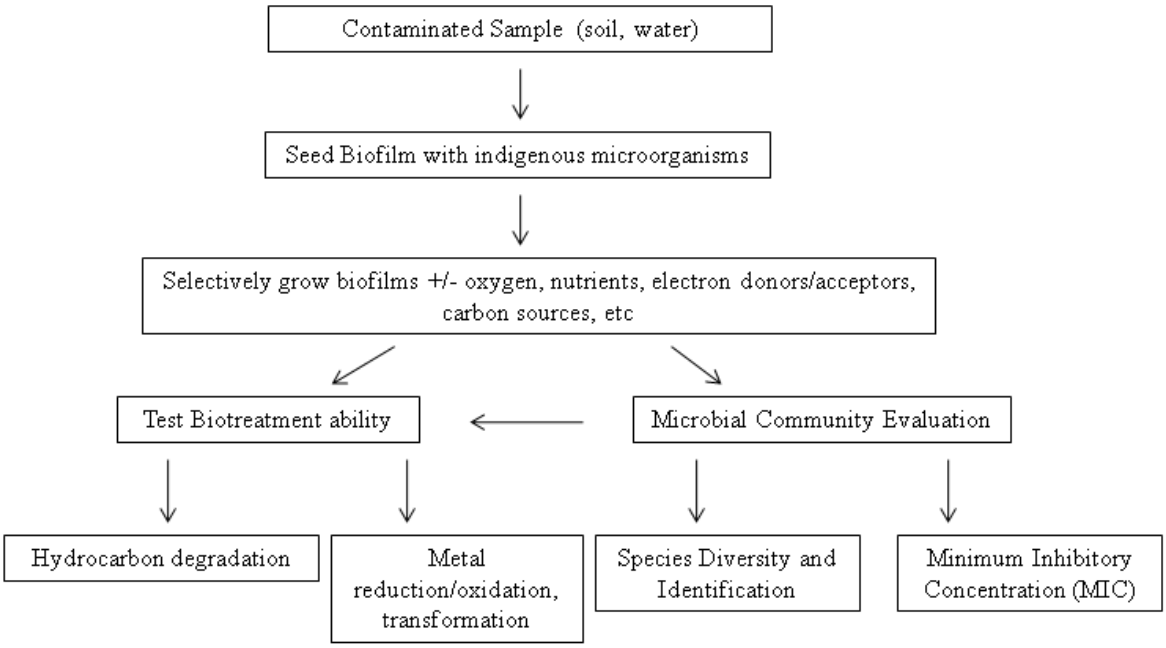


**Figure 4 Example flow chart of an in vitro biofilm study**

**Table 2 Microorganisms identified from an oil sands tailings sediment and two biofilms grown in vitro (as determined by 454 pyrosequencing of 16s DNA) and respective remediation potential**

| | Number of Unique DNA Sequences | | | Example of Genera Found in Tailings Sediments | Metabolic Activities Associated with Organism | Reference |
|---|---|---|---|---|---|---|
| | Tailings Sediments | Aerobic Biofilm | Anaerobic Biofilm | | | |
| Archaea | 6 | 4 | 2 | | | |
| | | | | Methanosaeta | Methane production | Mizukami et al. (2006) |
| Bacteria | 139 | 134 | 111 | | | |
| | | | | Pseudomonas | - arsenate reduction<br>- uranium biomineralisation<br>- naphthenic acid degradation | Macur et al. (2001)<br>Choudhary and Sar (2011)<br>Del Rio et al. (2006) |
| | | | | Thiobacillus | - arsenate reduction<br>- thiosulfate oxidation | Macur et al. (2001)<br>Kellermann and Griebler (2009) |
| | | | | Acidovorax | - phenanthrene degradation<br>- iron oxidation | Singleton et al. (2009)<br>Byrne-Bailey et al. (2010) |

This approach can be used to isolate and grow representative biofilms from various contaminated environments (water, soil, sediment, tailings) for biofilm bioremediation modelling in the laboratory. The MBEC device can support the growth of 96 equivalent biofilms (Figure 5A) which can then be characterised by structure microscopy (Figure 5B), microbial diversity and metabolic activities that could be exploited for bioremediation approaches. Enrichment techniques can be employed to promote selective biofilm growth of particular organisms of interest (i.e. metal resistant and/or hydrocarbon degraders), to test their bioremediation capabilities. Using the MBEC device, this could easily be achieved by fitting the biofilm covered pegs directly into solutions or other matrices containing contaminants of interest in a standard 96-well plate.

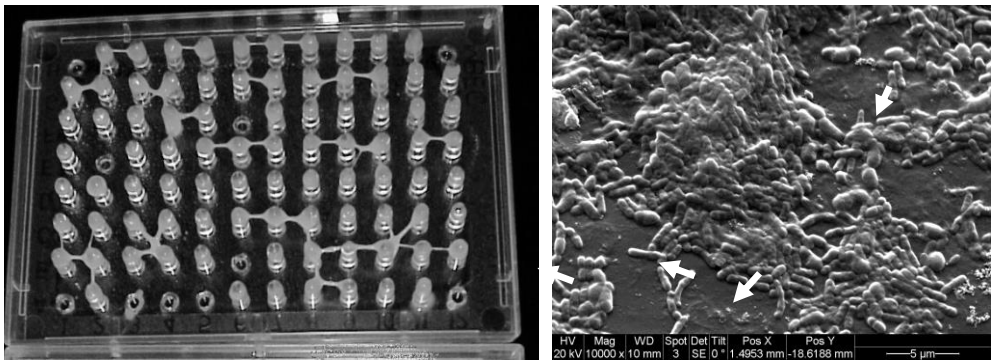


**Figure 5 Left: Biofilms grown on the pegs of the MBEC device; Right: Scanning electron micrograph (SEM) of a mixed species biofilm growing on the MBEC device. Arrows point to different cell morphologies**

# 4 Conclusions

The studies and techniques discussed in this paper highlight the valuable information that can be obtained from bench scale treatability testing prior to large scale field applications. The aim of these tests is to mimic the field conditions and determine the optimal conditions necessary for contaminate degradation, using a variety of traditional and innovative techniques to allow for maximum customisation of each study.

The bioremediation potential of a contaminated site can be greatly increased by understanding the indigenous microbial communities present and knowing the appropriate amendments to add to encourage the growth of the desired populations. Gathering this type of data in the laboratory can save time, money and resources, particularly in sites where there are challenging environmental conditions such as oil sands and mine tailings.

The results also suggest that biofilm-based technologies have the potential to be significantly more robust and efficient than traditionally developed biotechnologies. Further experiments should include exposure of biofilms formed in vitro directly from contaminated samples to known concentrations of contaminants and/or combinations to evaluate their bioremediation potential as a microbial consortium.

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