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Metabolic and Molecular Approaches to the Study of Bacterial Communities in Wetlands of the Alberta Athabasca Oil Sands Region

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Metabolic and Molecular Approaches to the Study of Bacterial Communities in Wetlands of the Alberta Athabasca Oil Sands Region

by

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Abstract

Bacterial communities in wetlands from the Athabasca region (Alberta, Canada) were surveyed and their utility as bio-indicators for wetland reclamation was assessed. Sediment samples were collected from wetlands categorized as: (1) natural (off mining leases), (2) reference (on mining sites but not directly impacted by oil sands processed material (OSPM)), and (3) OSPM (directly affected by OSPM). Wetlands of the latter two groups ranged in age from 11 to 24 years. Analysis involved community level physiological profiling (CLPP) with BIOLOG™ EcoPlates, and denaturing gradient gel electrophoresis (DGGE). Multivariate analysis applied to data from both techniques revealed differences in the bacterial communities based on site type; although overlap between groups occurred. Principal component analysis (PCA) and cluster analysis applied to CLPP data revealed a trend in which communities in OSPM sites were most similar to each other, followed by those in natural sites, and communities in reference wetlands were variable. The vegetated areas of a wetland displayed increased functional richness and diversity (as measured by CLPP) compared to non-vegetated areas. DGGE analysis applied to the total bacterial community revealed the highest number of operational taxonomic units (OTUs) in OSPM samples; the 2008 and 2009 OSPM samples contained an average of 22±4.5 and 22.4±5.1 OTUs, respectively, while reference sites contained 17.7±4.9 (2008) and 16.4±1.8 (2009) and natural sites contained 17.1±4.4 (2008) and 20.5±0.7 (2009). When DGGE was applied to the bacterial subgroups, γ-Proteobacteria and Actinomycetes, clustering effects based on site-type were more evident through PCA than when a total bacterial approach was taken. Within OSPM wetlands, γ-Proteobacteria populations were varied while Actinomycetes were similar across sites. Overall, there are clear functional (CLPP) and genetic (DGGE) differences between bacterial communities in OSPM and less impacted sites, although communities are not distinct, potentially reflecting the age of the wetlands studied and adaptation

of the communities to oil sands materials. Given that CLPP and DGGE could distinguish between communities based on site type, both may be useful for monitoring microbial communities in Athabasca wetlands throughout reclamation. In particular, CLPP and group-specific DGGE are recommended as tools for community monitoring.

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List of Abbreviations

ARDRA	amplified ribosomal DNA restriction analysis
ARISA	automated ribosomal intergenic spacer analysis
A_T	annealing temperature
AWCD	average well colour development
CCA	canonical correspondence analysis
CLPP	community level physiological profiling
CSUP	carbon source utilization pattern
CT	consolidated/composite tailings
Ct	cycle threshold
DGGE	denaturing gradient gel electrophoresis
dNTP	deoxyribonucleotide triphosphate
dO	dissolved oxygen
E. coli	*Escherichia coli*
EPL	End Pit Lake
FISH	fluorescent *in situ* hybridization
GC	guanine cytosine
MFT	mature fine tailings
NA	naphthenic acids
OSPM	oil sands processed materials
OSPW	oil sands process-affected water
OTU	operational taxonomic unit
PAC	polycyclic aromatic compounds
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid analysis
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SRB	sulphate reducing bacteria
SSCP	single strand conformation polymorphism

TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
TV	tetrazolium violet
UPGMA	unweighted pair group method with arithmetic means
UV	ultraviolet

Chapter 1
General Introduction

1.1 The Athabasca Oil Sands Industry

1.1.1 Oil Sands Mining and Associated Problems

Oil sands are naturally-occurring deposits of bituminous sand. Bitumen, the viscous oil present in these sands, can be extracted from the surrounding sand, water, and clay to be used as a profitable source of petroleum (CAPP, 2005). As technology to recover bitumen improves, and as traditional oil sources are depleted, the oil sands industry is increasingly economically profitable and ecologically important. Canada is a leader in this rapidly expanding industry with one third of all oil in the country coming from oil sands (CAPP, 2005). The Athabasca oil sands (located near Fort McMurray, AB, Canada) contain over 1.7 trillion barrels of oil and are the largest and most accessible oil sands in the province (CAPP, 2005). Two of the largest oil sands mining and refinery operators in the world, Syncrude Canada Ltd. and Suncor Energy Inc., are currently extracting from the oil sands in this region. In this thesis, Syncrude Canada Ltd. and Suncor Energy will hereafter be referred to as Syncrude and Suncor.

The production of crude oil from these deposits generally involves a caustic hot-water extraction method whereby large amounts of hot water and caustics (NaOH) are added to the mined material (Leung *et al.*, 2003). In addition to being an energy-demanding process, this method produces large amounts of waste; approximately 4 m^3 of fluid tailings is produced for every cubic meter of mined oil material (Holowenko *et al.*, 2000). The waste, often referred to as tailings slurries, contains solids (sands and clays), oil sands process-affected water (OSPW), and un-recovered bitumen (Holowenko *et al.*, 2000). As part of the Alberta government's "zero discharge" policy, industries keep all wastes on site, predominantly in large tailings ponds (Pollet

and Bendell-Young, 2000). Although the majority (>70%) of water produced through extraction is recycled back into the extraction process (Peters *et al.*, 2007), the net amount of waste contained in these ponds is increasing; estimates suggest that 10^9 m^3 of tailings water will accumulate in ponds on the Syncrude lease by 2025 (Rogers *et al.*, 1999).

Another problematic aspect associated with oil sands mining is that OSPW is acutely toxic to aquatic organisms such as fish, amphibians, and zooplankton. Lai *et al.* (1996) reported that all fathead minnows (*Pimephales promelas*) exposed to newly produced OSPW died within 48 hours. Other researchers noted that the growth and development of toad (*Bufo boreas*) and frog (*Rana sylvatica*) tadpoles were hindered by process-affected waters (Pollet and Bendell-Young, 2000). Repeated low-dose exposures to OSPW may also have adverse health effects on small mammals (Rogers *et al.*, 2001). Toxicity of OSPW has largely been attributed to naphthenic acids (NA) (e.g. Del Rio *et al.*, 2006), although polycyclic aromatic compounds (PACs) are also of concern (e.g. Smits *et al.*, 2000). NA are low molecular weight organic acids released from bitumen during extraction and that dissolve in process waters where they can accumulate to concentrations over 100 mg/L (Leung *et al.*, 2003). Under aerobic conditions, NA can be removed from OSPW by microbial degradation. Del Rio *et al.* (2006) found, for example, that *Pseudomonas* species extensively degraded commercial NA. Such findings suggest it may be possible to exploit microorganisms to benefit reclamation.

1.1.2 Reclamation Strategies

Due to the environmental damage caused by the mining of oil sands, developers are required to dispose of tailings materials while reclaiming mining sites to at least the equivalent of their prior land capability. The land must be reclaimed in order to support its intended future uses and to ensure that it will be self-sustaining without the need for long-term management (CAPP, 2005).

The production of 4 m^3 of waste for every cubic meter of mined oil material necessitates a strategy to reduce the amount of tailings before it can be effectively disposed of (Fedorak *et al.*, 2003). Without treatment, tailings undergo slow sedimentation rates; 5 to 10 years are required for tailings to reach 30% solids (Mackinnon, 1989 as cited by Penner, 2006) at which point they are referred to as mature fine tailings (MFT) (Chalatumyk *et al.*, 2002). Further densification of tailings to a solidity that is suitable for use in land reclamation is estimated to take up to 150 years (Eckert *et al.*, 1996, as cited by Fedorak *et al.*, 2002). To accelerate this process, divalent cations (Ca^{2+}) in the form of gypsum ($CaSO_4 \cdot 2H_2O$) are added to the tailings to form consolidated or composite tailings (CT). The CT slurry releases water more quickly to achieve 62-70% solid content within a few hours (McKenna, 1998, as cited by Fedorak *et al.*, 2002). The released process water contains high concentrations of sulphate (>1000mg/L) and the MFT, CT, and process waters contain high levels of salt (Fedorak *et al.*, 2003). Process waters and MFT also contain high levels of dissolved organics such as NA (Leung *et al.*, 2003). The formation of CT is the most commonly used densification strategy but other flocculent aids, such as polyacrylamide, have similarly been evaluated for their ability to speed the densification process (Sworska *et al.*, 2000). Following the densification of tailings, reclamation efforts are possible.

Two key reclamation strategies currently being assessed for reclaiming terrestrial and aquatic environments respectively are the dry and wet landscape approaches. The wet landscape approach is of particular importance for this study. It involves transferring MFT to an abandoned mine pit, allowing tailings to settle over time, and subsequently capping them with clean or process-affected water (Fedorak *et al.*, 2002; Figure 1). A number of experimental test ponds have been created on the Syncrude and Suncor lease sites to demonstrate this approach and allow researchers to investigate the environmental sustainability of the End Pit Lakes (EPL). The wet landscape approach may prove useful in reducing the amount of waste and in recreating viable ecosystems; though at present, more research is needed to determine the long term sustainability of these systems.

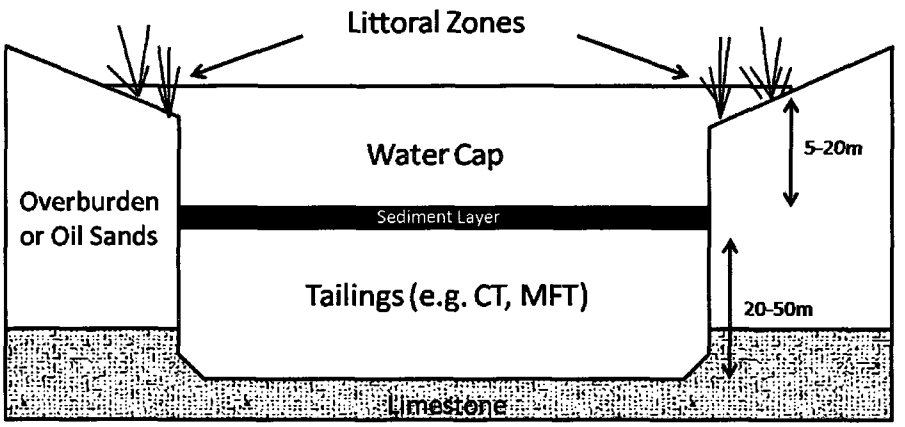


Figure 1.1 Schematic of an End Pit Lake (EPL) used in the wet landscape reclamation approach. Adapted from Penner (2006).

1.1.3 Wetlands in the Athabasca Region

Wetlands are abundant in areas of Alberta where oil sands are found and, as such, are an important part of reclamation. In the Athabasca oil sands region, bogs, fens, and marshes are common, with bog and fen peatlands being the predominant wetland types (Oil Sands Wetlands Working Group, 2000). These water bodies are integral in maintaining productive environments: they increase landscape diversity, provide habitats for a variety of aquatic and terrestrial species, protect and improve the quality of surface and ground water, control soil erosion, and provide flood control and carbon sequestration (Oil Sands Wetlands Working Group, 2000). Wetlands are also important for their economic and heritage values (Oil Sands Wetlands Working Group, 2000).

On-site wetlands can be affected in various ways by OSPM. When they are directly over oil sands, natural wetlands may be removed completely to access the bitumen below. Those not directly excavated can be affected by dyke seepages, surface runoff, and contamination of

groundwater (Nix *et al.*, 1993). Experimental test ponds are contaminated directly by the utilization of OSPM and/or OSPW in their construction (Del Rio *et al.*, 2006). As a result of contamination, impacted wetlands are expected to be different from non-impacted sites. They will be exposed to higher levels of bitumen (MacKinnon and Sethi, 1993, as cited by Hadwin *et al.*, 2006) and up to 30 times higher levels of NA (Pollet and Bendell-Young 2000; Holowenko *et al.*, 2000). They might also differ from non-impacted sites since they are established on land that has been disturbed through mining and that may be quite barren in terms of vegetation (Hadwin *et al.*, 2006). The result is that these impacted wetlands are less able to carry out their important functions (e.g. Bendell-Young *et al.*, 2000), and natural wetlands, as well as experimental test ponds, will require time to mitigate the effects associated with oil sands mining and its associated waste.

1.2 Microbial Communities in Natural and Disturbed Environments

1.2.1 Microbial Community Structure and Function

Microorganisms are not readily visible to the unaided eye and include bacteria, viruses, algae, protozoa, and fungi (Atlas and Bartha, 1998). In nature, microorganisms rarely live in isolation. Instead, cells live in association with other cells to form populations which, in turn, interact with other microbial populations to form communities (Madigan and Matinko, 2006). The ecosystem is subsequently defined by the interaction of microbial communities with communities of macroorganisms and the environment (Madigan and Martinko, 2006). In this thesis, the terms "microbial community" or "microorganisms" refers primarily to bacterial assortments although, viruses, algae, protozoa, and fungi were not specifically excluded in the biochemical based studies. In molecular-based analyses, however, bacteria were specifically examined through the use of bacterial-specific molecular primers.

In terrestrial and aquatic environments, microbial communities carry out irreplaceable roles. Microorganisms are the primary consumers of organic carbon and are integral in cycling carbon and energy towards upper trophic levels (e.g. Azam *et al.*, 1983). In particular, microorganisms carry out major roles in carbon, hydrogen, oxygen, nitrogen, sulphur, phosphorus, and iron biogeochemical cycles (Madigan and Martinko, 2006). In detrital-based food webs, microorganisms provide a food source for other organisms by releasing mineral nutrients from detritus (e.g. Ford, 1993). Also, nutrients contained in bacterial cells have been shown to be effectively recycled into the food web following their consumption by microbial grazers (e.g. Berman *et al.*, 1987 as cited by Daly 2007).

Microorganisms are highly valuable in disturbed environments given their capabilities to degrade environmental contaminants and reduce toxicity. For example, microbial biodegradation of hydrocarbons in soils has been well documented (e.g. Chaineau *et al.*, 2005) and in aquatic environments, microbially-mediated biodegradation is the major mechanism of removing spilled oil (Benka-Coker, 1997). Because of their ability to metabolize and reduce the toxicity associated with environmental contaminants, it is not surprising that there is a great interest in microorganisms for bioremediation or bioaugmentation purposes. Bacterial members of the microbial community are often associated with microbial bioremediation (e.g. Chaineau *et al.*, 2005) but fungi are also important (e.g. Leitão, 2009).

1.2.2 Methods of Microbial Community Profiling

The characterization of microbial communities has proven to be challenging, largely because of the vast diversity of microbial populations in the environment, taxonomic ambiguity, and methodological limitations (Kirk *et al.*, 2004). Using traditional cultivation techniques it is estimated that 1% of soil bacterial populations can be cultured, and it is not known whether this fraction is representative of the total community (Torsvik *et al.*, 1998). Nevertheless, there are a

number of methods commonly used to study microbial communities in natural and disturbed environments and recent reviews have detailed these approaches (see Kirk *et al.*, 2004; Malik *et al.*, 2008).

Available techniques for studying microbial communities can roughly be divided into two categories: biochemical-based and molecular-based. Biochemical-based approaches include traditional plate counts, as well as community level physiological profiling (CLPP) and fatty acid-based techniques (Kirk *et al.*, 2004). CLPP is the characterization of heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs) (Lehman *et al.*, 1995). It has been successfully applied to study the potential metabolic diversity of microbial communities from a range of natural and contaminated sites (e.g. Viti *et al.,* 2008; Costa *et al.*, 2007; Tiquia *et al.,* 2008; Chabaud *et al.*, 2008; Lõhmus *et al.*, 2006; Fuller *et al.*, 1997; Derry *et al.*, 1998; Merkley *et al.*, 2004; Nelson and Mele, 2007; Weber *et al.*, 2008).

Molecular-based methods for profiling environmental microbial communities include: denaturing and temperature gradient gel electrophoresis (DGGE and TGGE), fluorescent *in situ* hybridization (FISH), single strand conformation polymorphism (SSCP), nucleic acid reassociation and hybridization, amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA), and automated ribosomal intergenic spacer analysis (ARISA), as well as others (Kirk *et al.*, 2004; Malik *et al.*, 2008). Many of these approaches (e.g. DGGE, TGGE, ARDRA, RISA, ARISA, and T-RFLP) involve the use of the genes coding for ribosomal RNAs which are among the most conserved macromolecules in living systems (Atlas and Bartha, 1998). In prokaryotes, rRNA genes are arranged in operons and microorganisms can contain from one to fifteen of these operons in their genomes (Louws *et al.*, 1999; Figure 1.2). Prokaryotic rRNA genes such as 16S rRNA are useful in bacterial characterization studies given their ubiquity in prokaryotes and the presence of highly conserved regions (useful for primer design) interspaced with variable ones (useful for phylogenic comparisons) (Head *et al.*, 1998). Additionally,

evolutionary relationships can be reasonably predicted using this gene since there does not appear to be lateral 16S rRNA gene transfer between microorganisms and the 16S rRNA gene is an appropriate length for inferring phylogenetic relationships without making sequencing an unreasonable effort (Pace *et al.*, 1986). 16S rRNA genes can be used in several ways to understand the microbial community structure in environmental habitats. In DGGE, total bacterial DNA is extracted, 16S rRNA genes are amplified using PCR, and the PCR fragments are separated according to base pair sequence along a linearly increasing gradient of denaturant (Muyzer *et al.*, 1993). The utility of DGGE as a microbial profiling method is evident in its application to a wide range of environmental habitats (e.g. Leckie *et al.*, 2004; Rønn *et al.*, 2002; Qu *et al.*, 2008; Massieux *et al.*, 2004; Nicomrat *et al.*, 2006; Ogino *et al.*, 2001).

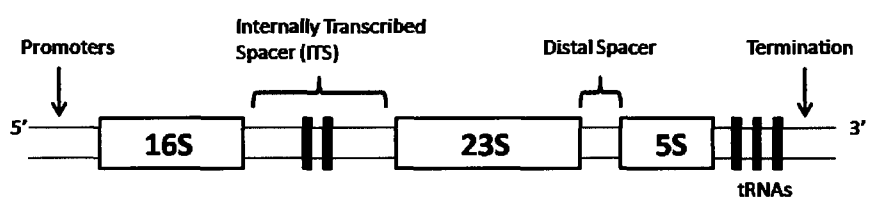


Figure 1.2 Schematic of genes in the bacterial rRNA operon. Adapted from Louws *et al.* (1999).

1.2.3 Microbial Communities in Wetlands of the Athabasca Oil Sands Region

There is a growing body of literature regarding the microbiota in oil sands-impacted sites. The majority of studies that have taken a microbiological standpoint have focused on unique groups such as methanogens (e.g. Holowenko *et al.*, 2000; Fedorak *et al.*, 2002) or bacteria with the ability to degrade NA (e.g. Lai *et al.*, 1996; Del Rio *et al.*, 2006). Methanogens have been

found to be active in large tailings ponds and are of concern because of their contribution to greenhouse gas pollution and, originally, because they were thought to impede reclamation (e.g. Holowenko *et al.*, 2000). Sulfate reducing bacteria (SRB) have also been identified in tailings ponds and found to inhibit methanogenesis through bacterial competition (Holowenko *et al.*, 2000). Bacteria with the ability to degrade NA are highly valuable in the oil sands industry since they can reduce the toxicity of OSPW. Realizing this, researchers are working towards understanding NA-degrading organisms with the long-term goal of manipulating them for use in reclamation. Del Rio *et al.* (2006), for example, found that co-cultures of *Pseudomonas putida* and *Pseudomonas fluorescens* are effective in removing more than 95% of commercial NA, whereas pure cultures of these organisms removed only 15%. This research team also found that, while degradation of monocyclic NA did not require exposure to OSPW, degradation of bicyclic NA required exposure in order to induce and/or select for microorganisms capable of more effectively performing this degradation. Other research groups have examined microbial communities indirectly through the characterization of carbon and energy flow through microbial food webs in impacted sites (e.g. Daly, 2007).

Fewer studies concerning the microbiology of oil sands-affected sites have taken a more inclusive community-characterization approach. In a report to Syncrude by Microbial Technologies (1999), extensive culture-based work was completed to obtain profiles of the microbial communities in the water, interface, and sediment of process-affected aquatic ecosystems including experimental ponds and tailings ponds. This work revealed the strongest presence of heterotrophic bacteria in the water samples, decomposers at the interface, and methanogens in the fine tails. The bacterial community in a site called "Demo pond", an experimental test pond, was also noticeably different from other experimental pits and the research group concluded it likely represented a transitional state from a tailings pond to a productive ecosystem. In addition, this group found that the sediment-water interface was

particularly important in the restoration of tailings ponds and commented that this habitat deserves further characterization.

A study published in 2006 by Hadwin and colleagues is the only one that has examined microbial communities as a whole specifically in wetlands of the Athabasca region. In this case, emphasis was placed on comparing the microbial community structures in sediments from wetlands exposed to varying amounts of NA (Hadwin *et al.*, 2006). They found that the amount of NA present had a strong influence on the composition of the bacterial community, and that exposure to low levels of NA was sufficient to cause a shift in the community structure to one that is capable of degrading this organic acid.

It is expected, that there are a range of microbial groups present in less-impacted, and natural wetlands in the Athabasca region. For example, heterotrophic and autotrophic bacteria that are important in nutrient cycling are expected as well as photoautrophic bacteria such as cyanobacteria and purple and green anaerobes. Methanotrophic bacteria and methanogenic archaea that maintain a controlling influence on methane release are strongly expected given previous literature (e.g. Holowenko *et al.*, 2000). A number of fungal genera, algae, and protozoa are also expected in healthy wetland ecosystems and recently, the presence of fungi and protozoa in wetlands in the Athabasca region has been documented (Legg, 2009). It is expected that the composition of microbial communities in impacted wetlands is slightly different given the variations in physicochemical, toxicological, and ecological parameters. For example, fewer plant-associated microbial taxa and an increase in microbial degraders such as *Pseudomas* spp. is predicted. Further, a reduced diversity is expected in impacted sites because adaptation to oil sands material is expected to be the predominant force shaping community structure. As a result of the predominantly physically controlled environment in impacted sites, there is expected to be a decreased pressure towards the evolution of integrated and balanced species interactions (Atlas and Bartha, 1998).

1.3 Microbial Community Structure as an Indicator for Reclamation

In order to evaluate the success of reclamation of wetlands affected by oil sands mining, it will be important to monitor the progression of wetlands from oil sands-impacted to more natural states. The ability to efficiently monitor this progression depends on the development of a suite of tools for this purpose. A number of groups have been working towards identifying suitable chemical or biological indicators to monitor progression of ecosystem health as reclamation of wetlands in the Athabasca region proceeds. For example, two separate groups have recently evaluated testate amoebae, a group of protists, as a potential bio-indicator group for wetland re-establishment (McCarthy *et al.*, 2008; Legg 2009). Other groups are assessing macrophytes, chironomids, and environmental variables for their potential as bio-indicators in wetland reclamation. This study is the first to focus on bacterial communities as potential bio-indicators for these systems.

It is expected that bacterial communities may be important predictors of the status of wetlands in the Athabasca region for a number of reasons, including their position at the bottom of the food chain, their key roles in ecosystem health, and their ability to degrade environmental contaminants (e.g. Parkinson and Coleman, 1991; Aelion and Bradley, 1991). In addition, bacteria reproduce quickly (Baldwin *et al.*, 2008) and have high surface area to volume ratios (Merkley *et al.*, 2004) and therefore, are expected to respond rapidly to environmental changes. It has been established that in the oil sands region bacteria have the capacity to degrade NA (e.g. Del Rio *et al.*, 2006) and bacterial community structure differs as a result of degree of impact from NA (Hadwin *et al.*, 2006). Given their capacity to degrade contaminants and participate in essential ecological processes, it is clear that bacteria will play an important functional role in reclaiming wetlands in the Athabasca oil sands region. If it can be confirmed that bacterial communities differ as a function of impact from oil sands materials, and monitoring tools are

effective in quickly and accurately detecting this difference, then bacterial community profiles may be sensitive indicators for monitoring wetland reclamation.

As discussed previously, monitoring bacterial communities in complex environments, however, can be challenging. An alternative approach to monitoring bacterial communities directly may be to determine if a measurable relationship exists between bacteria and members of the broader microbial community such as testate amoebae, a group of protozoa which are more easily monitored than bacteria. If a reliable relationship is identified, then an alternative and more easily monitored microbial group may serve as an indicator for the bacterial fraction of the community, and in turn, for wetland reclamation in the oil sands industry.

1.4 Thesis Objectives

The primary objective of this thesis was to characterize the bacterial communities present in a group of wetlands in the Athabasca region that have been variably affected by oil sands materials. To accomplish this goal, two techniques, BIOLOG™ (a metabolic approach) and DGGE (a molecular approach), were optimized for their specific application to sediments from wetlands in the Athabasca region. Overall this study will contribute to the limited knowledge of microbial communities in wetlands of the Athabasca region. Also, this study will be the first to address whether the bacterial fraction of the microbial community might serve as a useful bio-indicator for use in wetland reclamation. In general, it was hypothesized that bacterial communities would be measurably different in impacted and non-impacted sites and that increased bacterial diversity may reflect more productive wetland environments.

This study will also support a broader research initiative by a group from the University of Waterloo in which the primary goal is to establish associations between bacterial communities and members of the broader microbial community, including testate amoebae and fungi, in oil sands-affected wetlands. The research described herein will contribute to this long-term project

by providing more in-depth bacterial analysis, but no direct exploration of other microbial populations is included in the current work. This more inclusive project will be the first to integrate information from multiple microbial subgroups and efforts from this project are expected to aid in the identification of an appropriate indicator organism for monitoring wetland ecosystem performance.

1.5 Thesis Outline

This thesis is organized into 5 main chapters. The first chapter (General Introduction) reviews background information relevant to the thesis project. Athabasca mining practices and reclamation challenges are outlined followed by a discussion of microbial communities and their potential utility as bio-indicators. Chapter 2 is intended to provide a description of sampling sites and the general sampling methodology pertinent to Chapters 3, 4, and 5. In Chapter 3, I discuss three initial experiments that were conducted to optimize BIOLOG™ and DGGE for their application to sediments from Athabasca wetlands. Chapters 4 and 5 discuss the application of BIOLOG™ and DGGE, respectively, to the characterization of bacterial communities in wetlands of the Athabasca oil sands region. In both of these chapters, the utility of bacterial communities (monitored through the respective technique) as bio-indicators for wetland reclamation is discussed. Chapter 6 is a general discussion that synthesizes key results and conclusions from chapters 4 and 5 and to a lesser extent, chapter 3. References are provided in Chapter 7.

Chapter 2
Site Description and Sampling Methodology

2.1 Site Description and Sampling Protocol

Sampling took place in the Athabasca oil sands region near Fort McMurray, Alberta (56.66° N 111.21° W) on sites leased by Syncrude and Suncor. Sampling was completed by Allison Legg in July and August of 2007 and 2008 and Hao Chen in June 2009. Specifically, a grab sampling technique was used to collect sediment samples from the sediment-water interface of wetland sites. Wetlands were chosen to capture an accurate representation of wetlands in the Athabasca region. Samples were obtained from: (1) natural wetlands that are off mining leases and un-affected by mining, (2) reference wetlands that are on mining leases but have had no OSPM directly incorporated, and (3) OSPM wetlands which are on mining leases and have been directly affected by OSPM through oil sands process affected waters or composite tailings (CT). Three or six representative field plots (1 X 1 meter) were identified in each wetland and one sediment sample from the sediment-water interface (approximately 4 cm deep) was collected for each plot. Approximately 100 g of sediment was collected for each plot by hand (with clean latex gloves) and placed in shallow, sterile plastic containers (250 ml volume). Care was taken to keep the sediment samples in the same, natural orientation as it was in the wetland. In the 2008 field season, six samples were collected from wetlands that had distinct zones of emergent vegetation; three samples were collected from a common area characterized by emergent vegetation and three samples were collected from a common area lacking emergent vegetation. Note that many of the wetland sites lacked emergent vegetation completely in which case only three samples from a common, non-vegetated area were collected. Few vegetated areas were sampled in 2007 and none in 2009. For the 2008 and 2009 seasons, triplicate samples within a commonly identified area (vegetated versus non-vegetated) were separated by approximately 1 m each. All samples were

stored at 4 °C for shipment to Wilfrid Laurier University and held at this temperature until processed.

For each site, information pertaining to vegetation and various ecological parameters - water table depth, pH, conductivity, soil moisture content- was recorded (Appendix A). Also, visual observations and calculations of percent dry weight were completed within 1 week of receipt of samples at the lab (Appendix A) by drying a known amount of the sediment sample for 48h in a 44°C incubator.

2.2 Storage, Usage of Samples, and Quality Control

Sediment samples collected in the 2007 field seasons were used to test and optimize key methodological components such as cell extraction and DNA extraction techniques (Chapter 3) and to explore other potentially applicable methodology (e.g. phospholipid fatty acid analysis (PLFA) and fluorescent *in situ* hybridization (FISH)). A considerable effort was put towards validating key methodological components for the chosen techniques (CLPP and DGGE) given the expected complexities associated with Athabasca wetland sediment samples. The 2007 samples remained at 4 °C for 6 months prior to their use; culture-based methods prior to the use of these refrigerated samples confirmed the presence of a strong population of active microbial cells (data not shown). A summary of the wetlands sampled in the 2007 field season is provided in Table 2.1.

Samples used in the core studies described in Chapter 4 and 5 were primarily those collected during the 2008 field season; a summary of these samples is provided in Table 2.2. In addition to the 2008 samples, a reduced number of samples collected in June 2009 were included in part of the DGGE analysis described in Chapter 5; available information pertaining to these samples is summarized in Table 2.3. The 2008 and 2009 samples were subjected to 4 °C storage for no more than 2 weeks prior to their use with one exception (see Chapter 5).

In all instances, samples were handled aseptically and appropriate positive and negative controls were used (see respective chapters for a description of the specific controls). Other quality control parameters are discussed in the appropriate chapters.

Table 2.1 Summary of Athabasca wetlands sampled in July and August of 2007

Wetland (code)	Samples	Lease Area	Age in 2008 (Years)	Status (OSPM, Natural, Reference)	Type of OSPM
Mike's Pond (MP-1)[a]	MP-1	Syncrude	16	OSPM	Process water
4 Meter Consolidated Tailings (4MCT-1)[a]	4MCT-1	Suncor	11	OSPM	CT and Process Water
Test Pond 9 (TP9)[a]	TP9-1	Syncrude	15	OSPM	Process water
Natural Wetland (NW)[a]	NW-1	Suncor	22	OSPM	Process water
Demo Pond (DP)[b]	DP-1	Syncrude	15	OSPM	CT
Beaver Pond (BP)[c]	BP-1	Syncrude		Natural	n/a
Bill's Lake (BL)[b]	BL-1	Syncrude	12	Reference	n/a
High Sulfate (HS)[a]	HS-1	Suncor	24	Reference	n/a
Shallow Wetland[a] (SW)	SW-1, SW-3	Syncrude	16	Reference	n/a
South Ditch (SD)[c]	SD-1	Syncrude	15	Reference	n/a
South West Corner Waste Area 11 (SWC)[c]	SWC-1	Suncor		Reference	n/a
Small Beaver (SB)[c]	SB-1	Syncrude	~28	Reference	n/a

[a] information regarding lease area, age, status, OSPM, for these wetlands was obtained from Daly, 2007
[b] information regarding lease area, age, status, OSPM for these wetlands was obtained from Golder Associates Ltd., 2002
[c] information regarding lease area, age, status, OSPM for these wetlands was obtained through communication with A. Legg (2007)
blank= unknown

Table 2.2 Summary of Athabasca wetlands sampled in July and August of 2008

Wetland (code)	Samples	Lease Area	Age in 2008 (Years)	Status (OSPM, Natural, Reference)	Type of OSPM
Test Pond 9 (TP9)[a]	TP9-$1_{1, 2, 3}$	Syncrude	16	OSPM	Process water
Natural Wetland (NW)[a]	NW-$1_{1, 2, 3}$	Suncor	22	OSPM	Process water
Duck Pond (DU)[c]	DU-$1_{1, 2, 3}$			Natural	n/a
Loon Lake (LL)[c]	LL-$1_{1, 2, 3}$	Suncor		Natural	n/a
Maclean Creek (MC)[c]	MC-$1_{1, 2, 3}$			Natural	n/a
Shallow Wetland (SW)[a]	SW-$1_{1, 2, 3}$ SW-$2_{1, 2, 3}$	Syncrude	17	Reference	n/a
Mike's Pond (MP)[a]	MP-$1_{1, 2, 3}$	Syncrude	17	OSPM	Process water
4 Meter Consolidated Tailings (4M)[a]	4M-$1_{1, 2, 3}$ 4M-$2_{1, 2, 3}$	Suncor	11	OSPM	CT and Process Water
Demo Pond (DP)[b]	DP-$1_{1, 2, 3}$	Syncrude	15	OSPM	CT
Beaver Pond (BP)[c]	BP-$1_{1, 2, 3}$	Syncrude		Natural	n/a
Bill's Lake (BL)[b]	BL-$1_{1, 2, 3}$ BL-$2_{1, 2, 3}$	Syncrude	12	Reference	n/a
High Sulfate (HS)[a]	HS-$1_{1, 2, 3}$ HS-$2_{1, 2, 3}$	Suncor	24	Reference	n/a

[a] information regarding lease area, age, wetland type, and OSPM type obtained from Daly, 2007
[b] information regarding lease area, age, wetland type, and OSPM type obtained from Golder Associates Ltd., 2002
[c] information regarding lease area, age, wetland type, OSPM type obtained through communication with A. Legg (2008)
blank= unknown

Table 2.3 Summary of Athabasca wetlands sampled in June of 2009

Wetland (code)[d]	Samples	Lease Area	Age in 2008 (Years)	Status (OSPM, Natural, Reference)	Type of OSPM
Mike's Pond (MP)[a]	MP-1, 2, 3	Syncrude	16	OSPM	Process water
4 Meter Consolidated Tailings (4M)[a]	4M-1, 2, 3	Suncor	11	OSPM	CT and Process Water
Test Pond 9 (TP9)[a]	TP9-1, 2, 3	Syncrude	15	OSPM	Process water
Natural Wetland (NW)[a]	NW-1, 2, 3	Suncor	22	OSPM	Process water
Demo Pond (DP)[b]	DP-1, 2, 3	Syncrude	15	OSPM	CT
Beaver Pond (BP)[c]	BP-1, 2, 3	Syncrude		Natural	n/a
Bill's Lake (BL)[b]	BL-1, 2, 3	Syncrude	12	Reference	n/a
High Sulfate (HS)[a]	HS-1, 2, 3	Suncor	24	Reference	n/a
Shallow Wetland (SW)[a]	SW-1, 2, 3	Syncrude	16	Reference	n/a

[a] information regarding lease area, age, wetland type, and OSPM type obtained from Daly, 2007
[b] information regarding lease area, age, wetland type, and OSPM type obtained from Golder Associates Ltd., 2002
[c] information regarding lease area, age, wetland type, OSPM type obtained through communication with A. Legg (2008)
[d] all samples taken in 2009 were from non-vegetated sites
[e] % dry weight, and visual descriptions courtesy of Heather McCormick, 2009

Chapter 3
Optimization of Methods for the Application of CLPP and DGGE to the Study of Bacterial Communities in Wetlands of the Athabasca Oil Sands Region

3.1 Introduction

There is a particular interest in understanding microbial community structure and function in disturbed environments given the vital roles microorganisms play in regulating nutrient transformations, decomposing litter, maintaining structural and hydrological properties, and degrading and detoxifying environmental contaminants (e.g. Parkinson and Coleman, 1991; Aelion and Bradley, 1991). Microbial ecologists, however, face a number of challenges in characterizing microbial communities in natural environments. One problematic aspect is that environmental habitats vary widely in many characteristics including soil texture, moisture, and organic content, and as a result each environment presents a unique challenge for microbiological study. Methods must first be carefully tested and, if necessary, modified to enhance their efficiency for the specific habitat under investigation.

For this project, two common microbial profiling methods were used to assess the microbial communities in wetlands of the Athabasca region. These methods were: (1) community level physiological profiling (CLPP) with BIOLOG™ EcoPlates and (2) denaturing gradient gel electrophoresis (DGGE). Before detailed analyses were completed, three preliminary studies were carried out to assess important aspects of both CLPP and DGGE for their application to sediment from oil sands-affected wetlands. The first study assessed the efficiency of different methods for cell extraction and has direct applications to CLPP with BIOLOG™ EcoPlates. The two remaining studies were considered important for DGGE and involved: (1) assessing the efficacy of the Powersoil™ DNA Isolation Kit (Medicorp Inc., Montreal, Canada) for extracting DNA

from contaminated and non-contaminated samples, and (2) monitoring the persistence of extracellular DNA in wetland sediment samples.

3.1.1 Extraction of Microbial Cells for CLPP with BIOLOG™ EcoPlates

CLPP with BIOLOG™ EcoPlates allows for the characterization of heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs) (Lehman *et al.*, 1995). This approach involves inoculating a cell suspension into a BIOLOG™ microplate and subsequently monitoring colour production, which occurs as a result of microbial respiration of the substrates. The requirement for a cell suspension necessitates that if working with soil, sediment or biofilm, a preliminary step is employed to separate cells from their environmental matrix. This initial disruption step is critical not only for technical reasons (e.g. many techniques, such as BIOLOG™, require a liquid inoculum), but because microorganisms can often produce extracellular polysaccharides and form strong chemical and ionic bonds with soil, allowing strong attachments to occur (Ogram *et al.*, 2007). If a proper detachment step is not used, downstream results may reflect a bias towards organisms that are only loosely associated with soil (Ogram *et al.*, 2007).

Over the past two decades, a number of protocols have been developed to separate microbial cells from soil or sediment (e.g. Torsvik, 2004; Kallmeyer *et al.*, 2008). However, these methods are far from standardized, a likely indication that the best protocol depends on the nature of the soil type (Amalfitano and Fazi, 2008). Researchers using the BIOLOG™ approach have relied on a number of techniques to separate microbial isolates from their environmental matrix with the most commonly used methods involving a combination of agitation in the presence of buffer, saline, or a weak detergent, and subsequent centrifugation and dilution steps (e.g. Derry *et al.*, 1998; Chabaud *et al.*, 2008; Classen *et al.*, 2003). Hadwin *et al.* (2006), who applied CLPP with BIOLOG™ EcoPlates to study microbial communities in oil sands impacted wetlands, used

centrifugation and dilution of samples to obtain a liquid inoculum. In the current study, it was thought that a more thorough extraction method, combined with less dilution of samples, might be more effective in extracting bacterial cells from the sediment matrix. In turn, this might allow for further insights into the metabolic potential of the microbial communities in Athabasca wetlands.

The objective of this study was to evaluate methods of extracting bacterial cells from the Athabasca wetland sediment samples, and to determine the most appropriate protocol for application to CLPP with BIOLOG™ EcoPlates.

3.1.2 Extraction and Purification of DNA for PCR-Based Techniques

The extraction of DNA is the first step in all molecular analyses of microbial communities including the PCR-based method, DGGE. Environmental samples are expected to contain complex mixtures of microorganisms and organic compounds and, as with cell extractions, methods to extract DNA must be carefully considered in order to extract genetic material from a representative fraction of the microbial community. There are two major approaches for extracting microbial DNA from soil and sediment environments: the direct approach and the indirect approach. In the direct approach, cells are lysed *in situ* and the DNA is subsequently extracted, while the indirect approach involves separate sequential cell extraction and DNA extraction steps (Roose-Amsaleg *et al.*, 2001). The direct lysis approach, which is often preferred because it is more time-efficient and results in a better recovery (Roose-Amsaleg *et al.*, 2001), was employed in this study. Perhaps the largest disadvantage of the direct lysis approach is that other organic soil components, such as humic and fulvic acids, are extracted in addition to genomic DNA. Given that these contaminants can interfere with PCR processes by binding to enzymes involved in the reaction or directly to the DNA (von Wintzingerode *et al.*, 1997; Cullen and Hirsch, 1998), an effective DNA purification step is critical.

In this study, a DNA Extraction Kit (Powersoil™ DNA Isolation Kit) that has previously been shown to be effective in extracting microbial DNA from complex environments (e.g. Wakelin *et al.*, 2008) was chosen and tested for its efficiency. It was of particular importance to ensure that the method was effective in extracting a relatively consistent fraction of pre-inoculated DNA from diverse wetland environments and that organic contaminants were adequately removed in all samples such that PCR inhibition did not disrupt the reaction.

3.1.3 Persistence of Extracellular DNA in Wetland Environments

Major limitations of DNA-based techniques have been well described in the literature (e.g. Suzuki and Giovannoni, 1996; Kirk *et al.*, 2004). One issue less often addressed, is the difficulty in distinguishing live cells (microbial DNA) from dead cells (extracellular DNA). Although in most cases, nucleic acids released into soil, sediment, or water, are quickly degraded by DNases and RNases (e.g. Romanowski *et al.*, 1991), DNA can sometimes be stable in the environment, and persist for days to weeks. Novitsky (1986) found that extracellular DNA from dead biomass was degraded 60-70% after 14 days when introduced into sandy sediment samples. Using a method by Ogram *et al.* (1987), designed to extract extracellular DNA only, another group detected naked *E. coli* EL1003 DNA over a 40 day period (Recorbet *et al.*, 1993). Furthermore, based on studies that have examined the proportion of intact cells to dead cells, the amount of extracellular DNA released into environments may be substantial. For example, by comparing total direct counts to the number of dead or live bacteria (using dual fluorochrome staining Sybr Green I-propidium iodide), Luna *et al.* (2002) found that cells with intact membranes accounted for only 26-30% of the total bacterial population in different marine sediments. Another group found that, using the LIVE/DEAD® *Bac*Light™ bacterial viability kit and fluorescent in situ hybridization (FISH), only 24% of the total bacterial population in a

eutrophic river in Germany had an intact membrane, although the authors acknowledge that this value may be an underestimate (Freese *et al.*, 2006).

Although the issue of distinguishing microbial DNA from extracellular DNA is recognized (e.g. Leckie, 2005; Kozdrój and van Elsas, 2000), few studies have taken an experimental approach towards understanding the persistence of extracellular DNA in the environment. Those that have addressed this issue have mostly focused on the persistence of DNA in soil (Recorbet *et al.*, 1993, England *et al.*, 1997) while rarely has the persistence of extracellular DNA in sediments been addressed (e.g. Novitsky, 1986). No studies, to the author's knowledge, have examined the persistence of extracellular DNA in wetland environments. Understanding if, and how, extracellular DNA persists in wetlands may have important applications for many areas of research.

For the purposes of this study, the objective was to understand if extracellular DNA can persist in sediment samples from wetland environments in order to interpret data from molecular techniques (DGGE) accordingly. If extracellular DNA persists in wetlands, then any interpretation that bands on DGGE gels are representative of viable species must be done cautiously, and ideally, the issue of distinguishing live from dead fractions of the microbial community would be further addressed.

3.2 Materials and Methods

3.2.1 Wetland Sediment Samples

A description of the wetlands sampled in the 2007 field season is provided in Table 2.1. A select number of these samples were used in experiments to evaluate cell extraction and DNA extraction methodologies. Specifically, samples from wetlands MP, 4MCT, BP, and BL were used in both the cell extraction and DNA extraction experiments. Additional samples from the

following wetlands were used for the DNA extraction experiment: DP, SWC, and SB. In order to evaluate the persistence of extracellular DNA, sediment samples from a local wetland (Cambridge, Ontario) were used instead of 2007 Athabasca wetland samples.

3.2.2 Extraction of Microbial Cells for CLPP with BIOLOG™ EcoPlates

In order to identify a suitable cell extraction protocol for application to oil sands-affected sediment samples, a preliminary study was carried out on 2007 Athabasca wetland samples. A protocol was adapted from Insam and Goberna (2004), Calbrix *et al.* (2005), and Atlas (1993) involving mechanical treatments with a dispersal medium and centrifugation to release the bacterial fraction from the associated soil, water, and organic content. Given that the most appropriate mechanical treatment and dispersal agent depends on the nature of the sample (Amalfitano and Fazi, 2008), three mechanical treatments and two dispersal agents were tested. The three mechanical treatments involved homogenization using: (1) a Stomacher®400 Circulator (Seward Laboratory Systems Inc.) for 30 seconds at low speed; (2) a vortex mixer at maximum speed for 1 minute and; (3) rotary shaking in an Erlenmeyer flask with glass beads for 15 minutes at 150 rpm. The dispersal agents used were either 0.85% (w/v) physiological saline or 0.1% (w/v) sodium pyrophosphate. Centrifugation was carried out using a Beckman Avanti® J-301 High Performance Centrifuge with the JA 30.50 Ti Fixed Angle rotor for smaller volumes and JLA 10.500 Fixed Angle rotor for larger volumes. The six treatments were tested on samples from two OSPM-affected sites (4MCT and MP), one reference site (BL), and one natural site (BP). Specifically, 2.5 g of sediment was suspended in 22.5 mL of cold saline or sodium pyrophosphate and homogenized via stomacher, vortex mixer, or rotary shaker with glass beads, as described above, three times with 1 minute cooling on ice between each treatment. Slurries were centrifuged for 15 minutes at 1,000 x g and 8 °C and the supernatant was decanted and stored at 4 °C until further processing. Soil pellets were disrupted using the original method of

mechanical treatment with 22.5 mL of saline or sodium pyrophosphate and the mechanical treatment and slow centrifuge step was repeated. Supernatants were combined, stored at 4 °C, and the soil pellet was transferred back to the vortex, stomacher, or rotary shaker for a third mechanical treatment and centrifuge spin. Supernatants from the three slow spins were combined and centrifuged for 30 minutes at 10,000 x g and 8 °C. The supernatant was discarded and the bacterial pellet was re-suspended in 22.5 mL of physiological saline.

Immediately following extraction, the number of cells recovered through the different treatments was compared by serial dilution and plate count analysis conducted in triplicate on R2A (Difco™) medium; plates were incubated at room temperature for 48 hours and counts between 30 and 300 were considered for analysis. BIOLOG™ GN plates, which are used most commonly for the classification of gram negative organisms but contain the same chemical constituents as EcoPlates, were incubated with the extracted cell suspensions to determine if natural nutrient sources had been adequately removed; GN plates were incubated at room temperature for 9 days.

3.2.3 Extraction of DNA for PCR-Based Techniques

An experiment was carried out to test if the chosen extraction method, Powersoil™ DNA Isolation Kit, was effective at extracting DNA from OSPM-affected as well as non-affected samples. An overnight culture of *Escherichia coli* O157:H7 (ATCC 43895) was grown in 50 mL of LB broth-Lennox (BioShop, Burlington, Canada) at 37 °C. By spread plating on LB agar plates (BioShop, Burlington, Canada) and using values between 30 and 300 for analysis, the CFU/mL of the pure culture was determined to be approximately 1.0×10^{10}. *E. coli* O157:H7 was chosen for this study as well as that described in section 3.2.4 because of the expectation that this strain of bacteria would not be present in high quantities in the wetland sediment samples. Subsets of three OSPM-affected samples, three reference samples, and one natural sample, collected in 2007, were

inoculated with 1 mL of the overnight *E. coli* culture and thoroughly mixed. Total DNA was immediately extracted from the samples, as well as from the *E. coli* pure culture, using the Powersoil™ DNA Isolation Kit following the wet soil protocol. Using a Bio-Rad iCycler IQ, real-time PCR was used to compare the amount of *E. coli* O157:H7 recovered from the samples following a protocol from Lee *et al.* (2008) in which the *tir* (translocated intimin receptor) gene of *E. coli* O157:H7 is targeted through PCR. PCR reactions were performed in 20 µL volumes containing 1 µM of each primer, ECOOH-R (5'GGCGGCGTCTGAGATAACA-3') and ECOOH-F (5'-TCGAGCGGACCATGATCA-3'), 0.25 µM of the TaqMan molecular probe ECOOH-Pr (5'-AGAACTTCAAATCCATCATT-3') labeled with the fluorophore, FAM-490, 2X Fynzyme DyNamo PCR master mix (New England Biolabs) solution, and 5 µL of template DNA. The PCR protocol was as follows: an initial denaturation for 15 minutes at 95 °C, followed by 55 cycles of a 20 second denaturation at 95 °C, and 1 minute annealing and extension at 65 °C.

3.2.4 Persistence of Extracellular DNA in Wetland Environments

Sediment samples for this study were obtained via grab-sampling from a wetland located in Cambridge, Ontario in April of 2008. This local wetland was chosen because fresh samples from wetlands in Fort McMurray, Alberta were not available at the time this experiment was started, and year-old samples from the Athabasca region were thought to potentially have too few actively growing microbial cells to give reliable results. Four treatment conditions were tested and will be referred to as: (1) inoculated, autoclaved sediment; (2) inoculated, non-autoclaved sediment; (3) un-inoculated, autoclaved sediment; (4) un-inoculated, non-autoclaved sediment. All treatments were done in duplicate with the exception of treatment 3 which was completed once. The non-autoclaved samples were allowed to equilibrate at room temperature for 1 week prior to the commencement of the experiment. The autoclaved samples were autoclaved for 30 minutes at 121 °C. Note that for the purpose of this experiment, duplicate trials of condition 1

were labeled samples 1a and 1b, duplicate trials of condition 2 were designated samples 2a and 2b, the condition 3 trial was designated 3a, and duplicate trials of condition 4 were designated samples 4a and 4b (Table 3.1).

Table 3.1 Designation of sample types for survival of extracellular DNA experiment

Condition Description	Condition Number	Sample(s)
Inoculated/Autoclaved	1	a, b
Inoculated/Non-Autoclaved	2	a, b
Un-Inoculated/Autoclaved	3	a
Un-Inoculated/Non-Autoclaved	4	a, b

A pure culture of *E. coli* O157:H7 (ATCC 43895) was grown for 24 hours at 37 °C in 50 mL of LB broth Lennox (BioShop, Burlington, Canada). Cells were pelleted by centrifugation at 8,000 x g and 4 °C for 10 minutes using a Beckman Avanti® J-301 High Performance Centrifuge with the JA 30.50 Ti Fixed Angle rotor. The supernatant was replaced with 50 mL of 10 mM tris buffer (pH 8.0). Using counts within the range of 30-300 for analysis, plate counts revealed approximately 1.0×10^{10} CFU/mL in the overnight culture. Cells were lysed by heating in an 80 °C water bath for 60 minutes; plating of the heat-treated culture revealed no viable CFU in the 10^{-1} dilution. Sterile 50 mL polypropylene tubes containing 25 g of sediment (sterile or non-sterile) were inoculated with 1 mL of the 10 mM tris/lysed cell solution. Treatments designated "un-inoculated" received 1 mL of sterile ultrapure water. In all instances, the 1 mL solution (tris/cells or water) was distributed throughout the sediment. All samples were incubated at room temperature on a slow speed rotary shaker.

Immediately following inoculation, as well as at defined time intervals, total DNA was extracted from each of the sediment samples using the Powersoil™ DNA Isolation Kit with the following modifications: (1) the listed wet soil approach was followed, and (2) bead-beating on the vortex shaker was carried out for 5 minutes. Note that multiple DNA extractions from a sample were designated as 1d (day 1), 2d (day 2), 5d (day 5), or 15d (day 15). DNA was stored at -20°C until the final extraction was collected. Real-time PCR with a TaqMan molecular probe ECOOH-Pr (5'-AGAACTTCAAATCCATCATT-3') labeled with the fluorophore, FAM-490, and primers ECOOH-F (5'-TCGAGCGGACCATGATCA-3') and ECOOH-R (5'GGCGGCGTCTGAGATAACA-3') was used to compare the amount of *E. coli* O157:H7 extracted at the different time intervals (Lee *et al.*, 2008). The PCR reaction mix included 1μM each of the reverse and forward primers, 0.25 μM of the molecular probe, 2X concentrated Fynzyme DyNamo master mix solution, and 5 μL of template DNA. The PCR protocol involved an initial denaturation for 15 minutes at 95 °C, and 55 cycles of a 20 second denaturation at 95 °C and 1 minute annealing and extension at 65 °C.

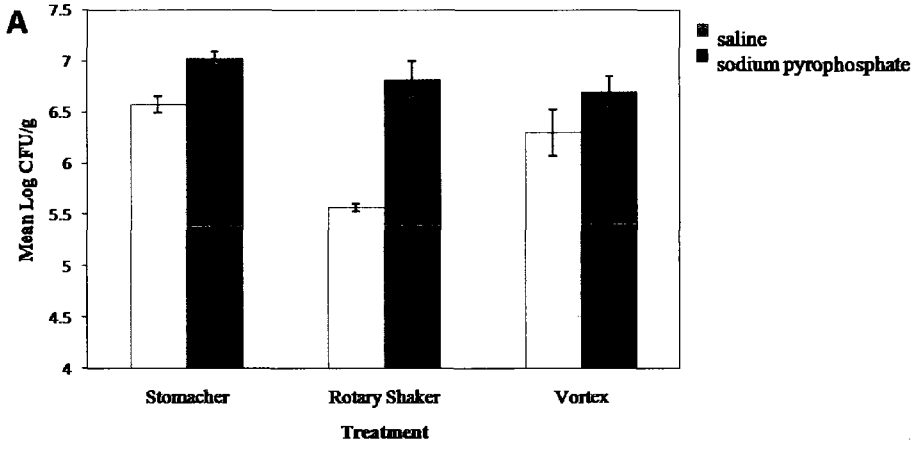
3.3 Results and Discussion

3.3.1 Extraction of Microbial Cells for CLPP with BIOLOG™ EcoPlates

An increase in the number of cells extracted was seen for samples when sodium pyrophosphate, as opposed to saline, was used as the extraction medium (Figure 3.1). Sodium pyrophosphate is a chelating agent that aids in loosening the strong electrostatic and chemical forces that bind the cells to the soil particles (Amalfitano and Fazi, 2008). In contrast, saline is expected to act as a stable isotonic medium for microbial cells and likely does not play a significant role in detachment. When sodium pyrophosphate treatments were considered, no particular physical treatment appeared consistently better at extracting cells from the sediment

samples. The most effective mechanical treatment appears to vary with respect to the specific sample under investigation and may reflect differences in soil properties (Figure 3.1).

In all cases no colour development was noted to have occurred over the 9 day incubation in the control well of BIOLOG™ GN microplates suggesting that all of the cell extraction protocols were effective in removing nutrient sources from the environmental samples (Figure 3.2).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Mean Log CFU/g
Stomacher
Rotary Shaker
Vortex
Treatment
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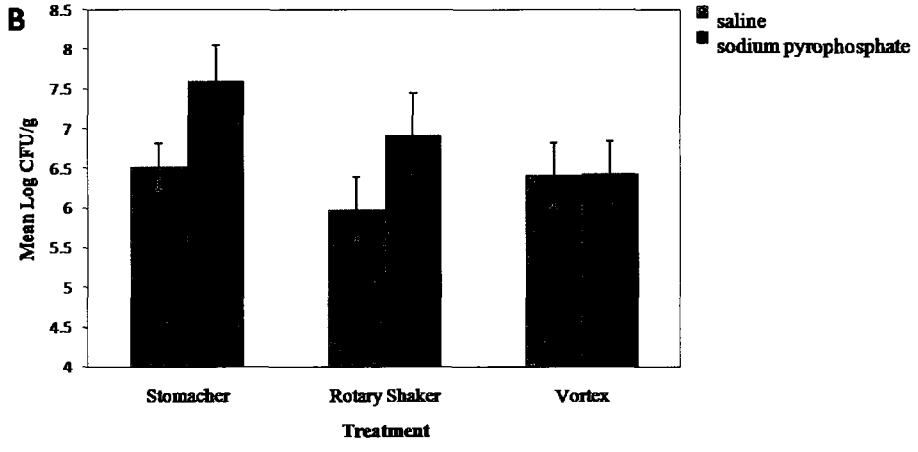
sodium pyrophosphate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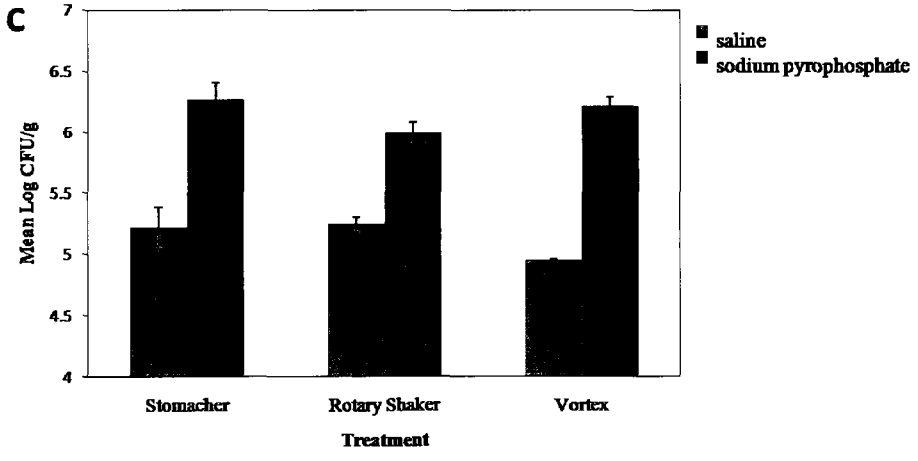
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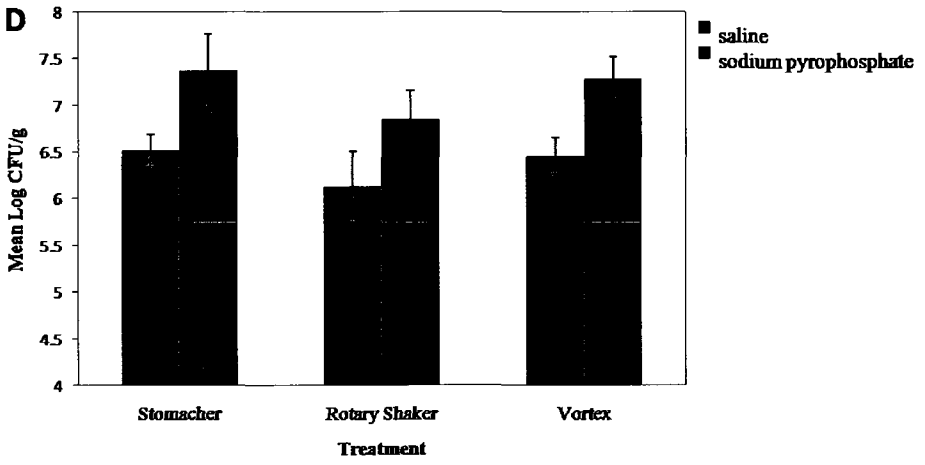


Figure 3.1 Comparison of mean log number of CFU/g extracted from sample 4MCT-1 (A), BL-1 (B), MP-1 (C), and BP-1 (D) when three different mechanical treatments (stomacher, vortex, or rotary shaker) and two different extraction mediums (physiological saline or 0.1% sodium pyrophosphate) were used. Cell suspensions were plated on R2A agar. Mean log CFU/g are based on three replicates. Error bars represent standard error of the means.

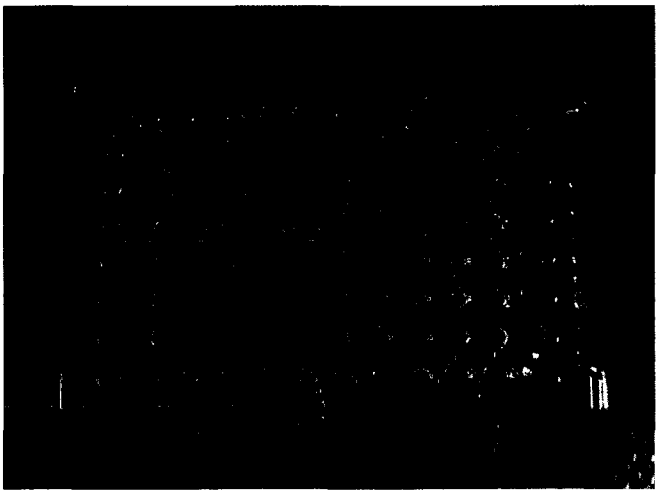


Figure 3.2 BIOLOG™ GN microplate >9 days after inoculation with a saline cell suspension extracted from an Athabasca wetland sediment sample. No colour development was seen in the control well (uppermost, left well) on any plates. The control well contains the same chemical constituents as other wells but lacks a carbon source.

It was determined that a cell extraction procedure involving sodium pyrophosphate and vortexing would be appropriate for CLPP based on the combined treatments' relative consistency in extracting a large number of cells from the different samples. Vortexing was chosen as the mechanical treatment in large part because of its ease of application and, therefore, the ability to process a large number of samples in a short period of time using this approach. It is expected that the protocol developed in this study may be an improvement over that used by Hadwin *et al.* (2006). This group used small sample volumes and high dilutions potentially losing some rare members of the population. Also, this group did not appear to make a significant attempt at extracting cells from the associated sediment and, as a consequence, their results may reflect the metabolic potential of microorganisms that were unattached or loosely associated with sediment particles.

This is a very brief evaluation on the subject of microbial cell extraction from sediment environments. There are recent published research articles devoted entirely to this concept (e.g.

Amalfitano and Fazi, 2008; Kallmeyer *et al.*, 2008) and problems associated with extracting microbial cells from environmental systems remains a significant challenge faced by environmental microbiologists. In a more in depth study of this kind, it would be advisable to obtain a baseline of comparison for the number of cells in untreated samples, to explore an increased number of treatments (e.g. an alternate chemical such as the detergent, Tween, and an alternate physical treatment such as use of an ultrasonic baths), to explore alternative centrifugation options (e.g. using a density gradient maker), and to evaluate the effect of these treatments on cell lysis and cell activity.

3.3.2 Extraction of DNA for PCR-Based Techniques

A fairly consistent amount of DNA was recovered from all samples when the Powersoil™ DNA Isolation Kit was used, irrespective of whether the sample was from an OSPM-affected or un-affected wetland. Real-time PCR was used to detect the relative amount of DNA using a TaqMan fluorescent probe. Briefly, fluorescence increases proportionally to the amount of DNA and given that theoretically, DNA doubles with every PCR cycle, after "n" cycles there is 2^n as much DNA. The Ct represents the point when fluorescence crosses the baseline threshold but is still in the linear phase of the reaction; Ct values are useful comparisons within single reactions but should not be compared across different experiments.

As shown in Figure 3.3, a relatively consistent amount of DNA was recoverable from diverse sample types. Some variation was noted between the quantity of *E. coli* O157:H7 DNA recovered from the different samples; the Ct values ranged from a low of 13.3 to a high of 14.9, suggesting a potential 3-fold difference in the amount of *E. coli* O157:H7 DNA recovered between the samples. Potential explanations for this variation might include different soil qualities, differences in the degree of predation of cells in the samples, or experimental errors such as inaccurate pipetting and inefficient distribution of the pathogenic *E. coli* DNA. No trend

was identified that would suggest that a higher amount of DNA was extracted from samples on the basis of sample type. For example, the Ct values for reference sites (n=4) ranged from 13.1 to 14.9, Ct values for natural sites (n=2) ranged from 14.0 to 14.1, and Ct values for OSPM sites (n=4) ranged from 12.8 to 14.3. Variation between replicates of the same sample, and between sample types, might again be explained by differences in soil quality, experimental error, and the difficulty in extracting DNA from a representative fraction of the sediment microbial community.

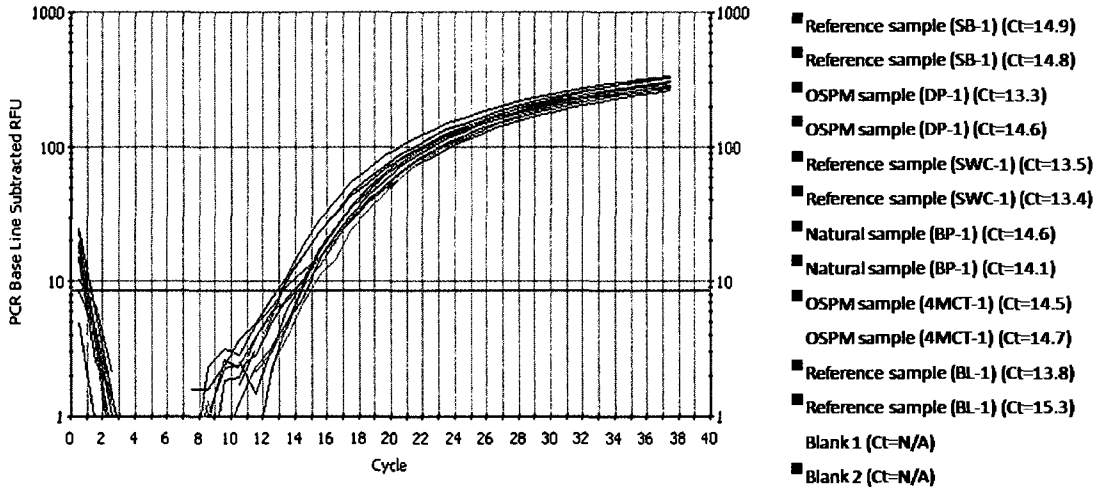


Figure 3.3 Evaluation of the Powersoil™ DNA Isolation Kit using real-time PCR to detect the relative amount of inoculated *E. coli* O157:H7 DNA re-extracted from OSPM impacted, reference, and natural samples. Note that the blank samples are not visible in this figure.

Ideally, optimization of DNA extraction from Athabasca sediment samples would involve testing of additional extraction protocols to determine which produced the most genomic DNA for 16S rRNA genetic analysis. Nevertheless, findings generated in this study suggested that the Powersoil™ DNA Isolation Kit would be suitable for this study because of its success in recovering a consistent percentage of *E. coli* O157:H7 from the various samples. The kits'

effectiveness in re-extracting a consistent fraction of target *E. coli* O157:H7 DNA from diverse samples suggests that PCR inhibitors were adequately removed from all of the samples tested.

3.3.3 Persistence of Extracellular DNA in Wetland Environments

Shown in Figure 3.4, are the standard curves produced through serial dilutions of the DNA product obtained from sample 1a and 1b on day 1. A similar standard curve was produced for sample 2a (data not shown). It is important to note that the logarithmic curves became erratic above a Ct value of approximately 36 in sample 1a, and that the standard curve generated for sample 1b displays nonspecific amplification of the negative control (*E. coli* ATCC 11229) beyond a similar cycle threshold. Thus, quantification of *E. coli* O157:H7 appears unreliable past a Ct value of about 36, or past a dilution of approximately 10^{-3} to 10^{-4} from the originally extracted DNA product.

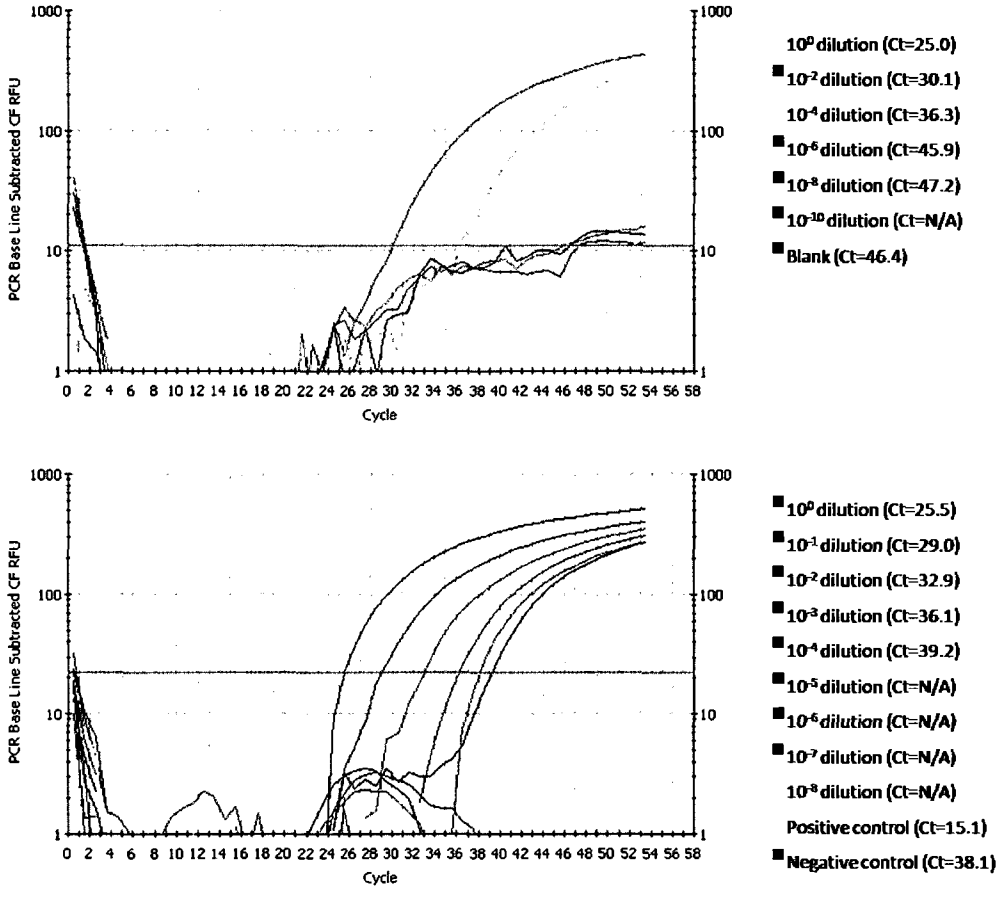


Figure 3.4 Standard curve generated with *E. coli* O157:H7 DNA in Sample 1a (1d) (top panel) and Sample 1b (1d) (bottom panel) using real-time PCR with a FAM-490 labeled *E. coli* O157:H7 TaqMan probe. Sample 1a represents the DNA extraction product from the day 1 extraction from sample 1a, an *E. coli* O157:H7 inoculated, autoclaved local wetland sample. Sample 1b represents the DNA extraction product from the day 1 extraction from sample 1b, an *E. coli* O157:H7 inoculated, autoclaved local wetland sample.

When DNA was monitored in the various experimental setups, little to no degradation of the inoculated, extracellular DNA occurred in autoclaved samples 1a and 1b over the course of the experiment (Figure 3.5). Although there is an apparent 2^8 –fold difference in the amount of DNA recovered from the pure *E. coli* O157:H7 samples when compared to samples 1a and 1b,

this difference can be attributed to inconsistencies in dilution factors; sediment samples were diluted by factor of approximately 0.003 compared to undiluted pure *E. coli* O157:H7 samples.

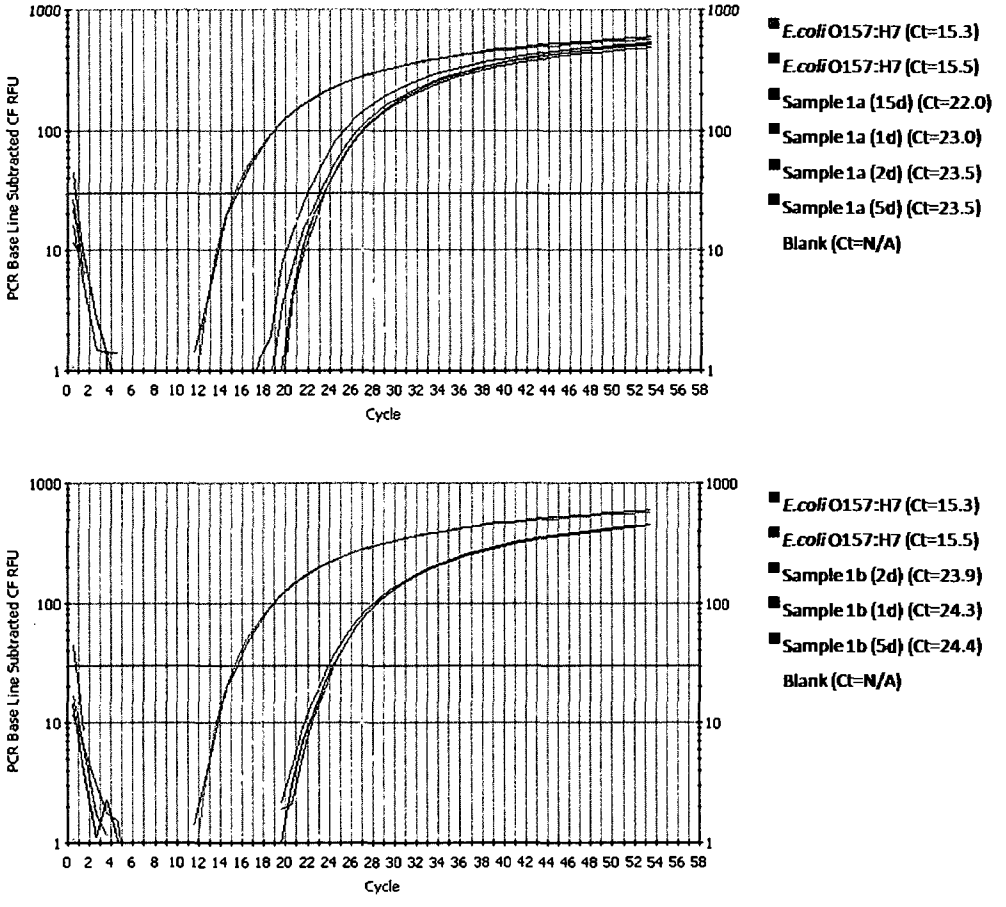


Figure 3.5 Real-time PCR with a FAM-490 labeled *E. coli* O157:H7 TaqMan probe showing the persistence of extracellular *E. coli* O157:H7 DNA in autoclaved sediment samples from a local wetland. Quantification is shown for Sample 1a (top panel) at Day 1, 2, 5, and 15 and Sample 1b (bottom panel) at Day 1, 2, and 5.

In contrast, obvious signs of DNA degradation over time were noted in non-autoclaved, inoculated samples 2a and 2b (Figure 3.6). The DNA inoculated into sample 2a, for example, was degraded by a factor of over 10 within the first few hours (day 1), and subsequently degraded by a

factor of 6.5 between day 1 and day 2, and a factor of 3.2 between days 2 and 5. A similar pattern of degradation was seen in sample 2b. The importance of viable microbial populations in the degradation of DNA can likely be attributed to the production of extracellular DNases by these organisms; for example, Lorenz *et al.* (1981) suggested that in sediments containing high bacterial activity, extracellular DNA could be degraded completely whereas in cases of low bacterial activity, naked DNA could persist. The rapid degradation of DNA seen within the first few hours prior to the initial extraction might be explained by the degradation of DNA that remains un-bound to sediment particles. Support comes from previous studies which have shown that DNA in an aqueous phase is much more available to attack by DNases than soil or sediment-bound genetic material (e.g. Lorenz *et al.*, 1981).

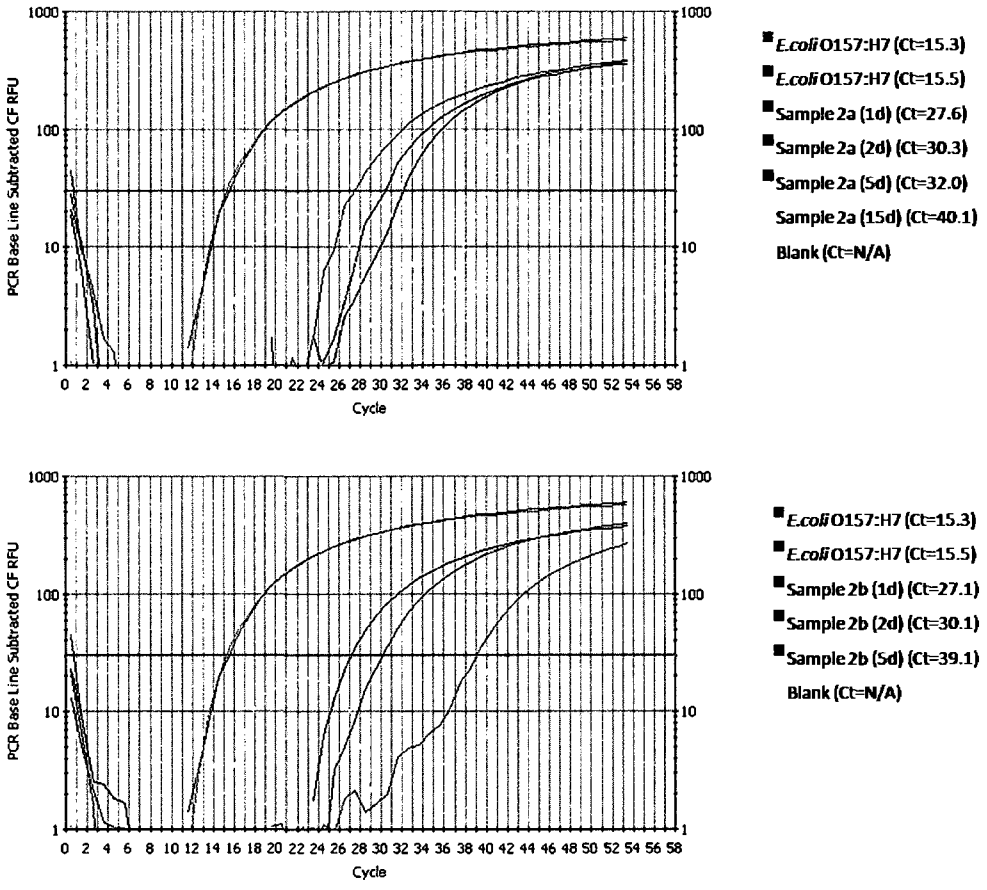


Figure 3.6 Real-time PCR with a FAM-490 labeled *E. coli* O157:H7 TaqMan probe showing the persistence of extracellular *E. coli* O157:H7 DNA in non-autoclaved sediment samples from a local wetland. Quantification is shown for Sample 2a (top panel) at Day 1, 2, 5, and 15 and Sample 2b (bottom panel) at Day 1, 2, and 5.

Although amplification is seen in sample 2a and 2b at day 15 and 5, respectively (Figure 3.6), it cannot be reliably concluded that extracellular DNA can persist in wetland samples for a period of these lengths. Amplification in both these instances occurs late in the PCR protocol with Ct values around 40. According to the standard curves generated with DNA extracted from samples 1a, 1b, and 2a (Figure 3.4), amplification at this point is unreliable and may be nonspecific. Results seen in Figure 3.7 also support this conclusion. In Figure 3.7, amplification

is seen in samples 4a and 4b to cross the threshold around cycle 38 despite these samples having been inoculated with sterile water and not DNA. The late cycle amplification seen in these samples might be attributed to a very low quantity of *E. coli* O157:H7 that exists in the wetland samples, or nonspecific amplification of another *E. coli* strain present in the samples. No amplification was seen in sample 3a, the un-inoculated, autoclaved sample.

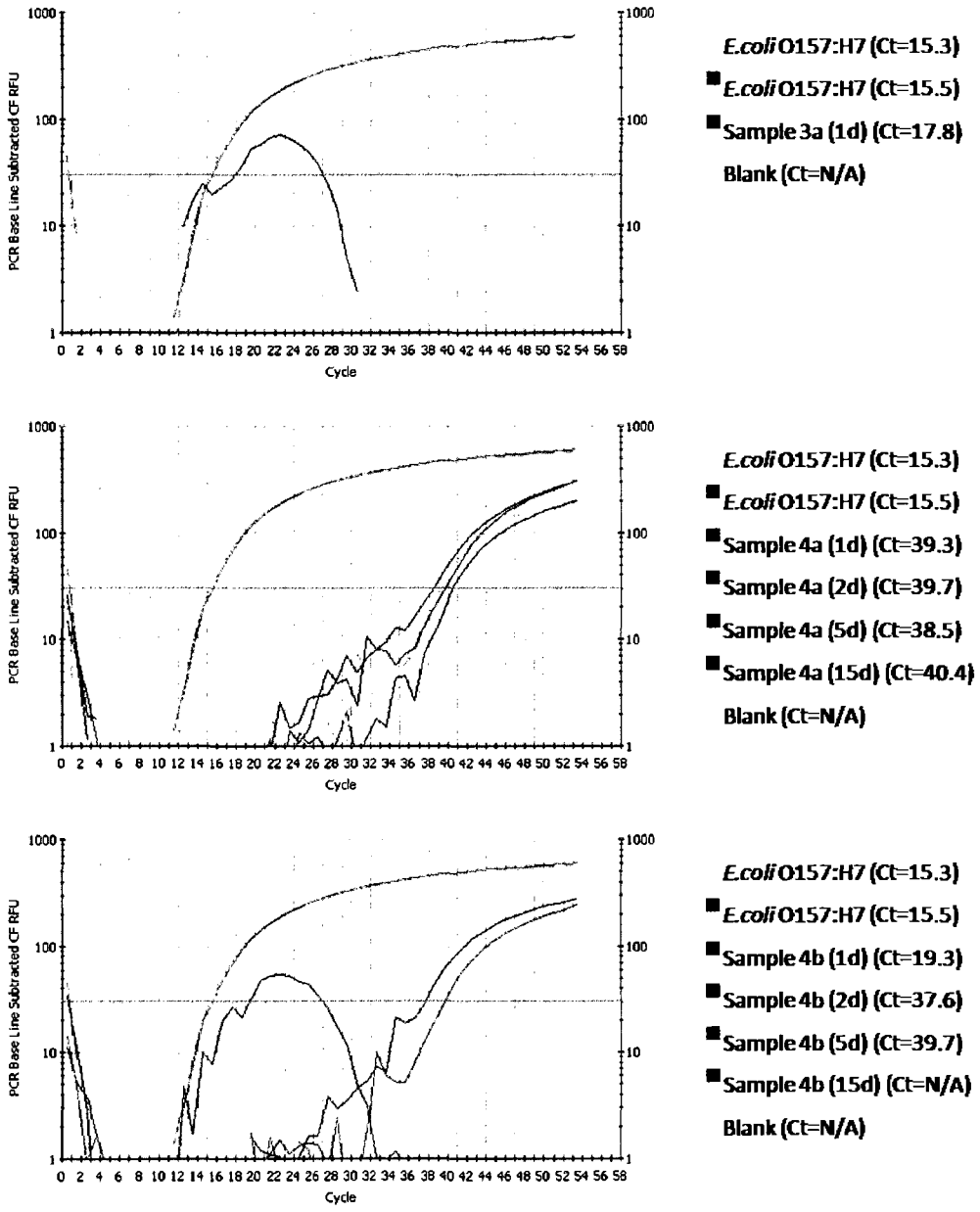


Figure 3.7 Real-time PCR with a FAM-490 labeled *E. coli* O157:H7 TaqMan probe showing the persistence of *E. coli* O157:H7 DNA in un-inoculated, autoclaved (Sample 3a; top panel) and un-inoculated, non-autoclaved (Sample 4a and 4b; middle and bottom panel) sediment samples. Quantification is shown for Sample 3a (top) at Day 1, Sample 4a (middle) at Day 1, 2, 5, and 15, and Sample 4b (bottom) at Day 1, 2, 5, and 15.

This is a brief examination of the survival of extracellular DNA in wetland sediment samples. It is difficult to draw interpretations from PCR-DGGE results produced with oil sands wetland sediment samples in light of the results produced here given the inconsistencies in the samples tested (Athabasca wetland samples versus local wetland samples). Ideally, this experiment would have been repeated with the appropriate sample types as well as with additional replicates. Future studies regarding DNA persistence in wetlands should examine the process of DNA survival in additional sediment types with variations in clay, inorganic and organic soil content, and pH, factors which have previously been discussed to affect DNA adsorption (e.g. Aardema *et al.*, 1983; Recorbet *et al.*, 1993). DNA survival studies in wetland water samples may be warranted. Further, it would be of interest to know whether the persisting extracellular DNA can participate in genetic exchange via transformation.

3.4 Summary and Conclusions

Given the wide variability in environmental microbial niches, testing and optimization of methods is important. In this study, three preliminary experiments were conducted to address important methodological aspects of CLPP and PCR-DGGE.

The application of the BIOLOG™ approach requires the extraction of microbial isolates from their environmental matrix in a manner that is as un-biased as possible. An appropriate extraction methodology for the application of CLPP to sediments from oil sands-affected wetlands was identified by testing six different extraction treatments for their efficiency in recovering viable cells. Results indicated that sodium pyrophosphate is a superior extraction medium to simple saline, a finding which can likely be attributed to its role in destabilizing bonds between microorganisms and sediment particles. No clear differences were noted between the three mechanical treatments tested. As such, vortexing in the presence of sodium pyrophosphate

was chosen for further applications due to the combined treatments' consistency in extracting a large number of cells and its ease of application.

DNA extractions must also be carefully considered such that the genetic material from a representative fraction of the total microbial community is extracted. Additionally, DNA extraction protocols must be effective in removing PCR inhibitors from diverse sample types in order to limit downstream PCR effects. In an experiment to evaluate the efficiency of the Powersoil™ DNA Isolation Kit, it was determined that this method was effective in re-extracting a relatively consistent quantity of *E. coli* O157:H7 DNA from the diverse wetland samples an, presumably, in removing PCR inhibitors. As a result, this kit was considered to be appropriate for further molecular applications on these sample types.

Towards the goal of better understanding the degradation of extracellular DNA in wetland sediment samples, real-time PCR was used to monitor the quantity of an initial addition of *E. coli* O157:H7 DNA remaining in samples over time. Results suggested that significant DNA degradation was dependent on active microbial populations while abiotic degradation played a minor role. Interpretation of the significance of these findings for concurrent molecular work carried out on Athabasca wetland samples is hampered because the inconsistencies in the sample types used. At most, it can be hypothesized that an active turnover of extracellular DNA occurs in Athabasca wetland sediment samples as was shown in the samples collected from Cambridge, Ontario. As a consequence, the majority of recovered DNA from Athabasca wetlands is expected to be representative of viable species.

Chapter 4
Community Level Physiological Profiling of Bacterial Communities in Wetlands of the Athabasca Oil Sands Region

4.1 Introduction

While mining bitumen from the Athabasca oil sands is an economically important and increasingly expanding industry, it is also an energy-demanding process that has resulted in the disturbance of land and the production of large amounts of oil sands process-affected materials (OSPM) (Holowenko *et al.*, 2000). As part of their license-to-operate, oil sands developers are committed to reclaiming disturbed sites to a level of equivalent capability to the pre-development state (Pollet and Bendell-Young, 2000). A key reclamation strategy for lake and wetland environments is the wet landscape approach in which End Pit Lakes (EPLs) are created (Holowenko *et al.*, 2000). EPLs will contain varying amounts of OSPM and will require time to mitigate the effects associated with such materials but nevertheless, may represent an important reclamation strategy. In order to reclaim constructed, as well as opportunistic wetlands most efficiently, a clear understanding of the differences between impacted and non-impacted wetlands is necessary. Further, a means of assessing site progression from OSPM-stressed to a more natural state is important.

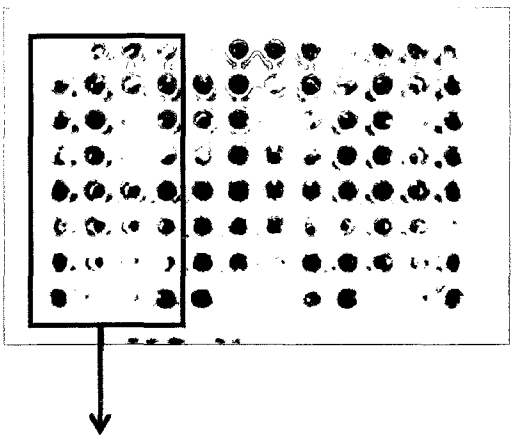
A great deal of information has been produced regarding the presence and function of specific groups of microorganisms in wetlands of the Athabasca region, including methanogens (e.g. Holowenko *et al.*, 2000; Fedorak *et al.*, 2002), and bacteria with the ability to degrade naphthenic acids (NA) (e.g. Nix and Martin, 1992; Lai *et al.*, 1996; Del Rio *et al.*, 2006). However, only Hadwin *et al.* (2006) have examined the microbial community structure as a whole in impacted wetlands. A more detailed understanding of the composition and function of microbial communities in OSPM-impacted and non-impacted wetlands is warranted given their potential utility in reclamation. Since microbial communities in OSPM-affected wetlands will be

established on land that has been disturbed by mining and exposed to higher levels of bitumen (MacKinnon and Sethi, 1993) and naphthenic acids (NAs) (Pollet and Bendell-Young, 2000), it was predicted, and later confirmed, that there are distinct microbial communities associated with OSPM-affected wetlands (Hadwin *et al.*, 2006). As a result, it may be possible to determine the status of a wetland by examining its microbial community profile.

Microbial communities may be important predictors of the status of disturbed landscapes given their position at the bottom of the food chain, their key roles in ecosystem health, and their ability to degrade environmental contaminants (e.g. Parkinson and Coleman, 1991; Aelion and Bradley, 1991). Despite this, studies associating microbial community structure with ecosystem functions have been impeded, likely a reflection of the difficulties associated with characterizing microbial communities (Garland and Mills, 1991). Classical plating techniques, which rely on the cultivation of organisms using specific media and conditions in the lab, can be time and labour intensive and only a small fraction of the bacterial population can be cultivated using current techniques (Torsvik *et al.*, 1998). Molecular approaches have become increasingly important in microbial ecology and offer the possibility of studying bacterial groups that resist cultivation but, these methods can be resource intensive and require specialized expertise. Although both classical plating techniques and molecular approaches can allow for different degrees of species identification, from some perspectives, identification may not be required. Arguably, it is diversity in the functional rather than taxonomic sense that provides information regarding the role of the community (Preston-Mafham *et al.*, 2002) and that is vital to the long-term stability of an ecosystem (Pankhurst *et al.*, 1996). Thus, studies aimed at understanding the overall stability of a microbial community would benefit from an easily applied and time efficient method that produces information regarding potential functional diversity. An attractive method towards this goal is community level physiological profiling (CLPP).

CLPP is the characterization of heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs) (Lehman *et al.*, 1995). Although many approaches can

be taken towards CLPP, most often CLPP refers to data collected using BIOLOG™ microplates, a method first described by Garland and Mills in 1991. BIOLOG™ microplates consist of 96 wells containing nutrients, a carbon source, and the redox dye tetrazolium violet (TV). When a sample of a mixed microbial community is inoculated into a microplate, microbial respiration causes the reduction of TV and subsequent production of a coloured compound which can be monitored using a spectrophotometer. BIOLOG™ manufactures several different microplates with the most popular being the GN2, GP2, and EcoPlate. Compared to the 95 carbon sources of the GN2 and GP2 plates, the EcoPlate, designed for environmental applications, contains 31 different carbon sources and a control well repeated in triplicate (Figure 4.1). The 31 substrates are deemed to be some of the most useful carbon sources for soil community analyses (Preston-Mafham *et al.*, 2002), including at least nine substrates that are constituents of plant root exudates (Campbell *et al.*, 1997). The utility of the EcoPlate is evident in its successful application to various environments including soil (e.g. Viti *et al.*, 2008), sediment (e.g. Costa *et al.*, 2007), freshwater (e.g. Tiquia *et al.*, 2008), biofilms (e.g. Chabaud *et al.*, 2008), and the rhizosphere (e.g. Lõhmus *et al.*, 2006).



A1 Water	A2 β-Methyl-D Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure 4.1 A BIOLOG™ EcoPlate and its associated carbon sources. The A1 control well and 31 carbon sources are repeated in triplicate on BIOLOG™ EcoPlates. The control well contains the same chemical constituents as the other wells but no carbon source.

Limitations of the BIOLOG™ approach have been well described in the literature (Garland, 1997; Konopka *et al.*, 1998; Preston-Mafham *et al.*, 2002). Notably, BIOLOG™ is selective for rapidly-growing microorganisms that actively respire under the imposed conditions (Winding and Hendriksen, 1997) and given the dependence of the technique on actively respiring cells, there is a need to reduce time between sampling and inoculation of plates. Also, inoculation

densities must be carefully considered due to the effect of the density of organisms on the rate and extent of colour response (Haack *et al.*, 1995). In more complex environments, such as sediment or soil, an appropriate cell extraction step is needed to detach cells from their associated environmental matrix (e.g. soil, water, organic content); this may detract from the overall simplicity of the CLPP approach. As discussed recently by Weber *et al.* (2007), data analysis and interpretation of BIOLOG™ data can also be particularly challenging.

Despite limitations, CLPP with BIOLOG™ microplates remains an attractive method for functional microbial community profiling, in particular for community comparisons (Preston-Mafham *et al.*, 2002), and can be complementary to other approaches (e.g. Nelson and Mele, 2007). The protocol is simple, efficient, and if data analysis is carried out properly, a large amount of relevant information can be produced. The BIOLOG™ approach has been applied successfully to many disturbed environments including, for example, soils disturbed by trichloroethylene and toluene (Fuller *et al.*, 1997), creosote-contaminated soils (Derry *et al.*, 1998), marshes disturbed by cattle grazing (Merkley *et al.*, 2004), plant rhizospheres affected by increased boron and sodium chloride concentrations (Nelson and Mele, 2007), and wetland mesocosms affected by acid mine drainage (Weber *et al.*, 2008). These studies offer encouragement for the application of CLPP to the assessment and comparison of bacterial communities in oil sands impacted and non-impacted wetlands. Although CLPP with BIOLOG™ EcoPlates has previously been applied to OSPM-stressed wetland samples (Hadwin *et al.*, 2006), the technique was repeated in this study because of the expectation that new information could be obtained with a modified approach.

The goal of this study was to provide more information regarding the microbial communities of OSPM-stressed and non-stressed environments in the Athabasca region. This was done using CLPP with BIOLOG™ EcoPlates as a first step towards assessing the utility of microbial community structure as an indicator for oil sands reclamation. More specifically, this study set out to: (1) obtain potential functional microbial community profiles of Athabasca

wetland sediment samples that differ in degree of OSPM contamination by using BIOLOG™ EcoPlates, (2) determine the suitability of CLPP with BIOLOG™ EcoPlates as a monitoring tool for remediation of OSPM-stressed wetlands, and (3) compare the potential functional microbial community results (BIOLOG™) to molecular-based results (DGGE) (see Chapter 6).

4.2 Materials and Methods

4.2.1 Wetland Sediment Samples

A description of the wetlands sampled in July and August of 2008 is provided in Table 2.2. CLPP was completed on all samples within 2 weeks of receipt of samples at the lab. Generally, protocols applying CLPP with BIOLOG™ EcoPlates involve three key steps: (1) obtaining a cell suspension by extracting microbial cells from the associated soil, water, and organic material; (2) inoculation and incubation of microplates, and subsequent monitoring of colour development; and (3) data analysis. These steps will be discussed separately.

4.2.2 Extraction of Microbial Cells

The sample inoculated into the microplate should be a cell suspension, therefore, if sampling soil, sediment, or biofilm, an appropriate extraction and homogenization protocol is required. Preliminary experiments were carried out on samples taken in the 2007 field season to assess the suitability of different methods for extracting microbial cells from oil sands-affected sediment samples (see Chapter 3). Ultimately, a protocol was developed that involved mechanical treatments using a vortex mixer in the presence of 0.1% (w/v) sodium pyrophosphate, and centrifugation. Specifically, 2.5 g of sediment was suspended in 22.5 mL of sodium pyrophosphate and homogenized by vortexing at maximum speed for 1 minute. This step was repeated a total of three times with 1 minute cooling on ice between each treatment. Sample

slurries were centrifuged for 15 minutes at 1,000 x g and 8 °C and the supernatant was decanted and stored at 4 °C until further processing. Soil pellets were resuspended in 22.5 mL of sodium pyrophosphate and the triplicate vortex treatment and slow centrifuge step was repeated. The supernatant was decanted and stored at 4 °C, and the soil pellet was vortexed for a third mechanical treatment in the presence of 22.5 mL of sodium pyrophosphate followed by the slow centrifuge step. Supernatants from the three slow spins were combined and together centrifuged for 30 minutes at 10,000 x g and 8 °C. The supernatant was discarded and the bacterial pellet was re-suspended in 22.5 mL of physiological saline. Centrifugation was carried out using a Beckman Avanti® J-301 High Performance Centrifuge with the JA 30.50 Ti Fixed Angle rotor for smaller volumes and JLA 10.500 Fixed Angle rotor for larger volumes.

4.2.3 Inoculation, Incubation, and Monitoring of EcoPlates

One or two BIOLOG™ EcoPlates were used for each wetland with the three independent samples for each identified wetland area (vegetated versus non-vegetated) incubated on the same plate. Immediately following cell extraction, the saline cell suspension was mixed and 150 µL of the suspension was added to each well of the EcoPlate. The prepared plates were incubated at room temperature under aerobic conditions and the absorbance read on a SpectraMax® 190 spectrophotometer (Molecular Devices) at 590 nm at time 0 and thereafter every 24 hours for 9 days. Serial dilutions and plate counts on R2A (Difco™) medium were also done following each cell extraction to obtain an estimate of the inoculum cell density. Plating was done in triplicate with incubation at room temperature for 48 hours, and counts between 30 and 300 considered for analysis.

4.2.4 Data Analysis

EcoPlate data were analyzed using three methods: (1) substrate-related diversity indices; (2) principal component analysis (PCA) and; (3) unweighted pair group method with arithmetic means (UPGMA) clustering analysis. Prior to analysis, the average well colour development (AWCD) was calculated for each sample at every 24 hour reading. All initial calculations were completed using Microsoft Excel®. AWCD is defined as:

$$AWCD = \frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)$$ (Lehman *et al.*, 1995)

where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the control well.

Also, a metric, representative of the activity in the wells and to be used for further data analysis, was determined. In this case, the most appropriate metric was determined to be an absorbance value for each well taken from a time point representing a specific AWCD reference value. The reference AWCD was chosen by comparing the AWCD, number of values above an absorbance of 2, and the standard deviation of the 31 absorbance values of each sample over time. A reference AWCD of 0.5 was chosen because of the few absorbance values above 2 and relatively large amount of variation between absorbance values at this point.

To reduce the bias due to differences in inoculum density, corrected time point absorbance was calculated for wells on each plate at the abovementioned reference AWCD. The corrected absorbance for well *k* is as follows:

$$\overline{A_k} = \frac{A_k - A_0}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)}$$ (Insam, 1997)

where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the control well. Negative values for corrected absorbance are meaningless and were coded as zeros.

4.2.4.1 Substrate-Related Diversity Indices

As suggested by Zak *et al.* (1994), BIOLOG™ data were assessed based on functional diversity indices. Calculations by Zak *et al.* (1994) were based on data from GN and GP microplates; here, calculations are based on data derived from EcoPlates. As indicated by Weber *et al.* (2008), the term "functional diversity" used by Zak *et al.* (1994) is replaced with "substrate diversity" for this study as it deals with substrate utilization patterns. Substrate-related diversity was assessed through three perspectives: richness, diversity, and evenness. Substrate richness (S) is the number of different substrates used by the microbial community and is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate diversity (H), also known as the Shannon index, is defined as:

$$H = -\sum p_i \ln (p_i)$$ (Zak *et al.*, 1994)

where p_i is the ratio of the activity of a particular substrate to the sum of activities on all substrates (at a particular corrected time point absorbance). Substrate evenness (E) is defined as the equitability of activities across all utilized substrates and is calculated as:

$$E = \frac{H}{H_{max}} = \frac{H}{\log S}$$ (Zak *et al.*, 1994)

where H is substrate diversity and S is substrate richness. Substrate-related diversity indices were analyzed over time for all substrates. Measures of richness, diversity, and evenness do not distinguish between the types of substrates utilized by the bacterial community and two microbial communities could display identical substrate-related diversity indices but use completely different substrates. In order to partially address this issue, analysis was also completed based on the chemical guilds present on EcoPlates (carbohydrate, carboxylic acid, amino acid, amines/amide, or polymer). All substrate-related diversity indices were calculated using Microsoft Excel®.

4.2.4.2 Principal Component Analysis (PCA)

PCA was used to ordinate objects from the 31 dimensional data set (31 carbon sources) onto a factor plane consisting of the first two extracted principal components (PCs). PCA assumes two fundamental principles: normality and homoscedasticity (homogeneity of variance). Therefore, following Weber *et al.* (2007), a natural logarithmic transform and Taylor power law transform were employed on the corrected absorbance values and the transformed data sets, in addition to the untransformed sets, were assessed for the two abovementioned criteria. Following transformations and testing, PCs were extracted from the covariance matrix of the data using Statistica 7.1.

4.2.4.3 Cluster Analysis

As recommended by Legendre and Legendre (1998), clustering analysis was performed to verify and validate the results obtained using PCA. An unweighted pair group method with arithmetic averages (UPGMA) clustering analysis was performed on the Euclidian distances for the Taylor transformed BIOLOG™ data (Statistica 7.1).

4.3 Results

4.3.1 Substrate-Related Diversity Indices

No detectable differences were noted when measures of substrate richness, diversity, and evenness were compared for all substrates as a function of wetland type (OSPM (n=18), reference (n=21), natural (n=9)), presence of vegetation (vegetated (n=15) non-vegetated (n=33)), or sampling date (July (n=24), August (n=24)). Patterns of richness over time followed a sigmoidal-type curve but a large amount of variation was noted between the richness of different samples. Measures of diversity increased over the first few days and began to plateau around day 5 but, again, a wide variation between diversity patterns was evident. In contrast, measures of evenness remained consistent over time and between samples (data not shown). As with measures of total substrates, no detectable differences were noted between utilization patterns when a guild analysis approach was used to compare wetland type (OSPM, reference, natural), presence of vegetation, and sampling date (data not shown).

Although substrate-related diversity indices could not distinguish wetland type, degree of vegetation, or sampling date, when all samples were considered, when a single wetland was evaluated it was found that patterns of diversity and richness were consistently different between samples from vegetated and non-vegetated areas (Figure 4.1 - 4.4). In all cases, samples taken from the vegetated area of a particular wetland displayed higher measures of richness and diversity than samples from the non-vegetated portion of the same wetland. The largest difference between measures of richness and diversity for vegetated and non-vegetated samples was noted for wetland 4MCT, the only oil sands-affected sample in this study with distinct vegetated and non-vegetated areas.

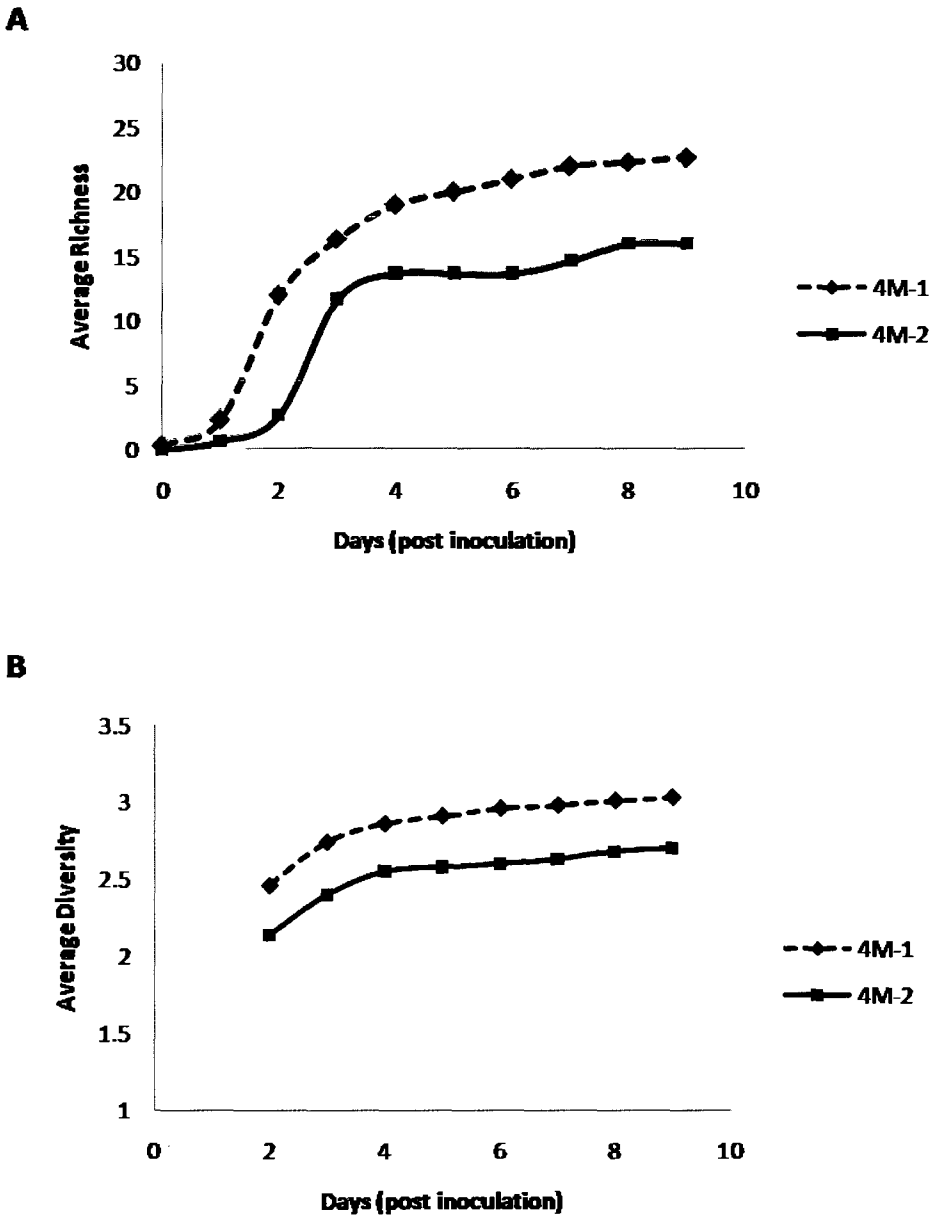


Figure 4.2 Average potential functional (A) richness and (B) diversity over time for vegetated (4M-1) and non-vegetated (4M-2) areas of wetland 4 Meter Consolidated Tailings (4M), an OSPM-affected wetland. Data points each represent the average of three samples.

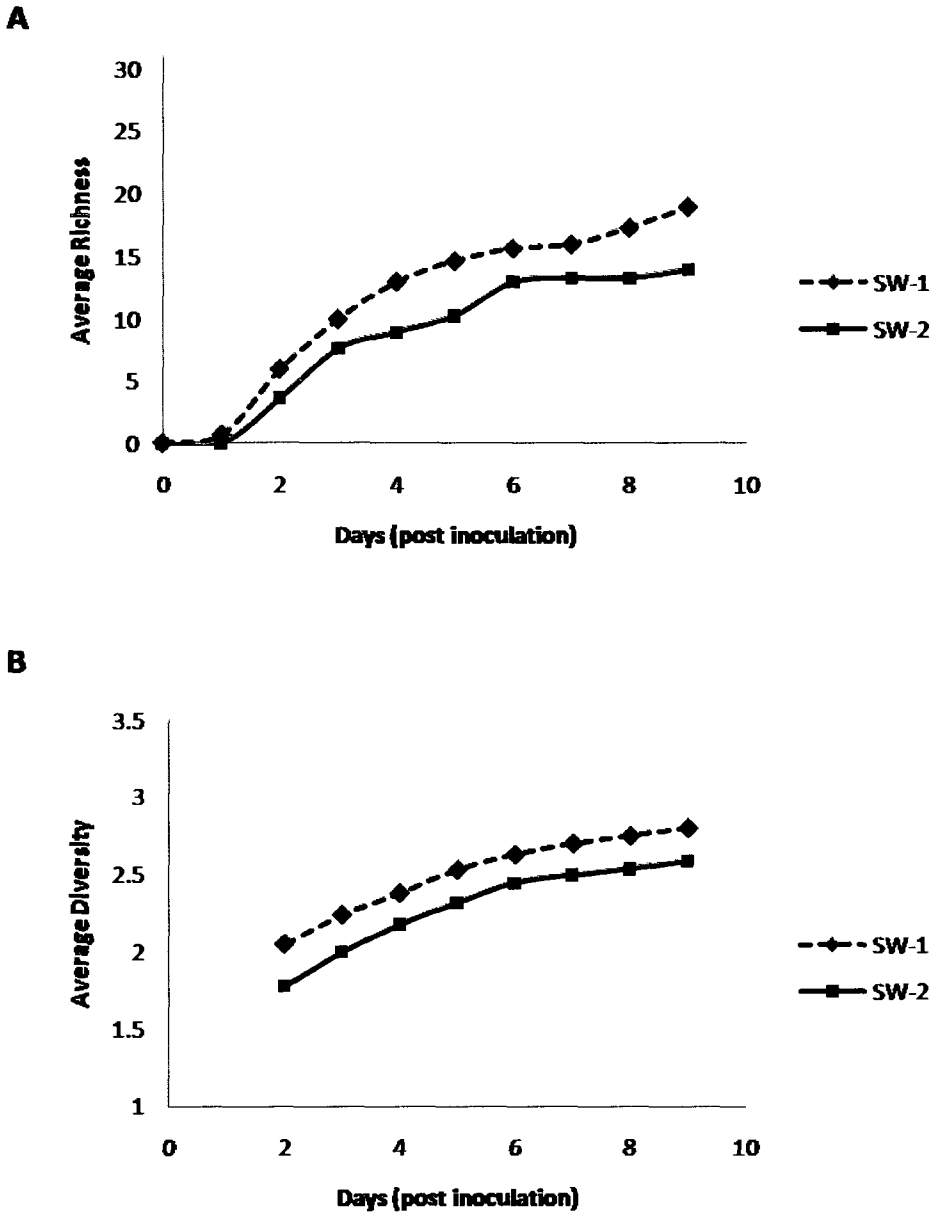


Figure 4.3 Average potential functional (A) richness and (B) diversity over time for vegetated (SW-1) and non-vegetated (SW-2) areas of wetland Shallow Wetland (SW), a reference wetland. Data points each represent the average of three samples.

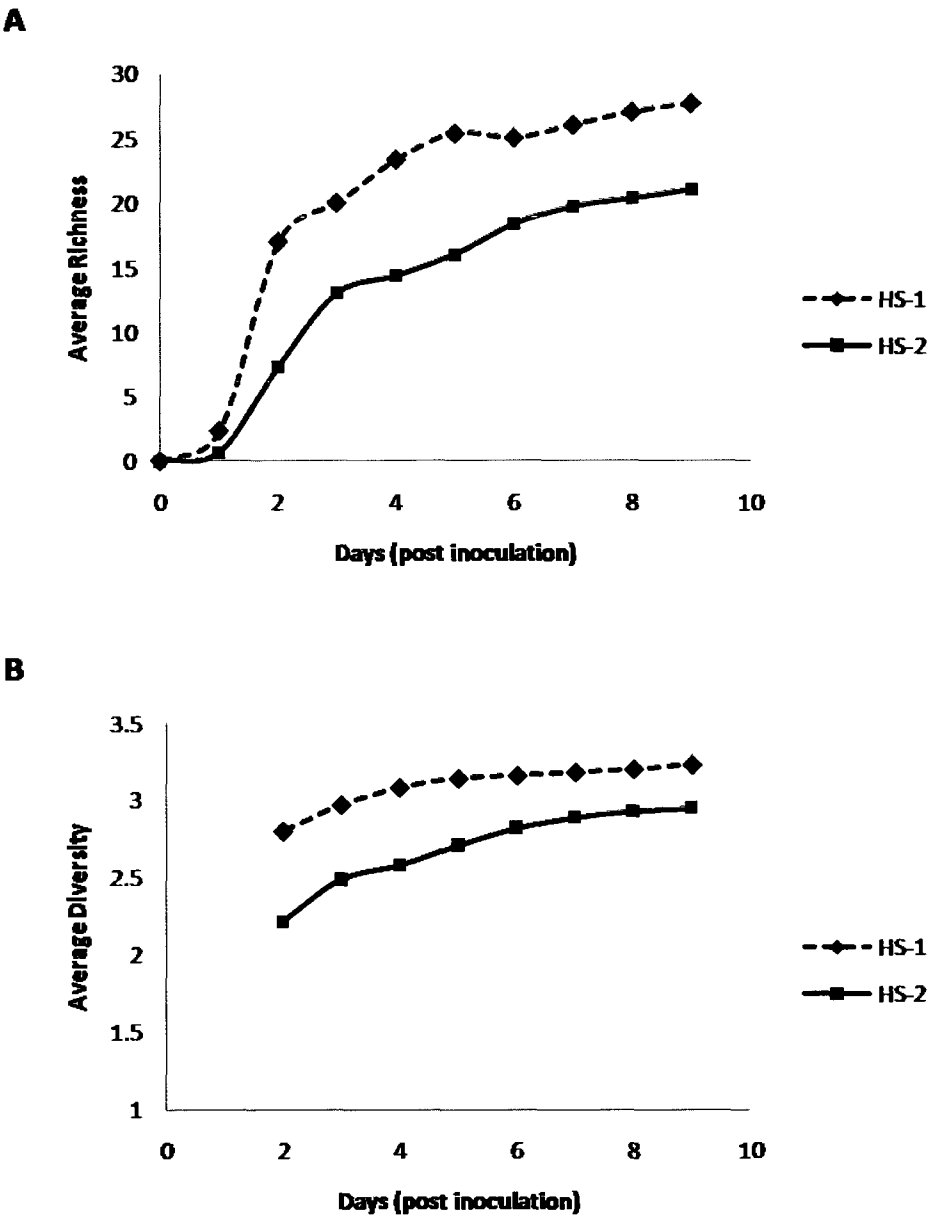


Figure 4.4 Average potential functional (A) richness and (B) diversity over time for vegetated (HS-1) and non-vegetated (HS-2) areas of wetland High Sulphate (HS), a reference wetland. Data points each represent the average of three samples.

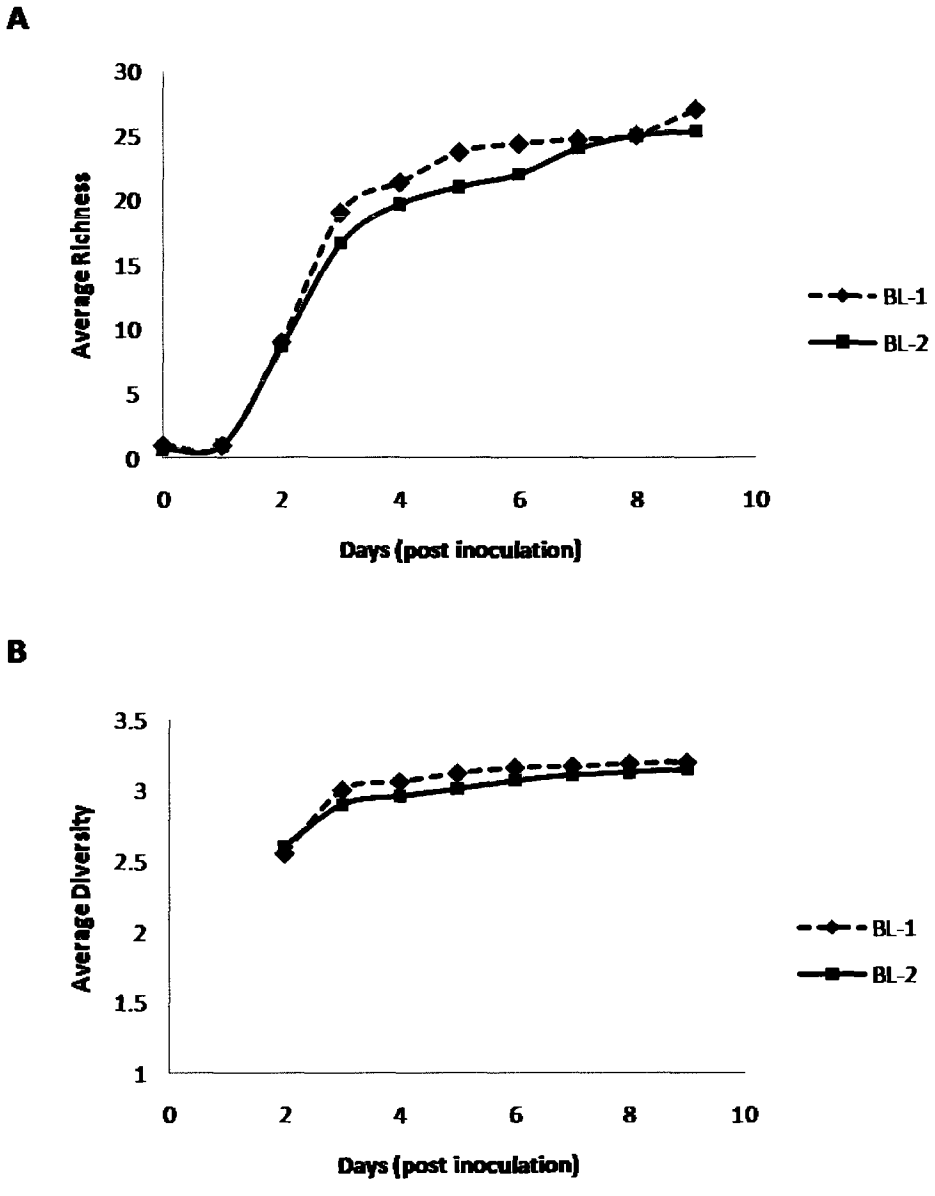


Figure 4.5 Average potential functional (A) richness and (B) diversity over time for vegetated (BL-1) and non-vegetated (BL-2) areas of wetland Bill's Lake (BL), a reference wetland. Data points each represent the average of three samples.

4.3.2 PCA Ordination

Normality and homoscedasticity were assessed for the untransformed, natural logarithmic transformed, and Taylor transformed data sets (Table 4.1). Normality was assessed through formal statistical tests of kurtosis and skewness. The standard errors and associated z values of kurtosis and skewness were calculated and the null hypothesis that the data are normally distributed was rejected with 95% confidence if $|z|>1.96$. Thus, in Table 4.1, the number of significant kurtosis and skewness values within normality reflects the number of values with $|z|<1.96$. A clear improvement of the number of values within normality is seen for both transformations, with the Taylor transformation indicating the most improvement. Homoscedasticity was assessed by calculating the variance ratio, the ratio of highest variance to the lowest variance. Lower variance ratio values reflect more homogeneity of variance between data sets. As seen in Table 4.1., improved homoscedasticity was seen by the significant reduction in variance ratio for both transformations, in particular for the Taylor transformed data set.

Table 4.1 Summary of normality and homoscedasticity statistical tests for untransformed, natural logarithmic transformed, and Taylor transformed data sets (n=48)

	Untransformed	LN(x+1) Transformed	Taylor Transformed
Mean Kurtosis z-value	7.03	3.59	1.10
Number of significant kurtosis values	12	18	23
Mean skewness z-value	4.83	2.46	0.23
Numbers of significant skewness values	7	14	23
Variance ratio	1555.32	215.77	13.434

As expected based on statistical tests, the transformations significantly improved the resolution of the PCA ordinations with the Taylor transformation being the most effective (Figure 4.5). In all PCA ordinations: the first letter indicates whether the sample is from an oil sands affected wetland (A), a reference wetland (B), or a natural site (C); the second letter indicates whether the sample was taken in July (J) or August (A); and presence of an asterisk indicates that the sample is from a vegetated area. The numbers refer to a specific wetland sample and the lower case a, b, or c reflects replicate samples from the same wetland. For example, BA-2a describes a reference wetland sample taken in August from a non-vegetated site and together, BA-2a, BA-2b, and BA-2c represent triplicate samples from a particular wetland which, in this case, is the wetland High Sulfate (HS). A table summarizing the descriptive title for each wetland is included in Appendix B.

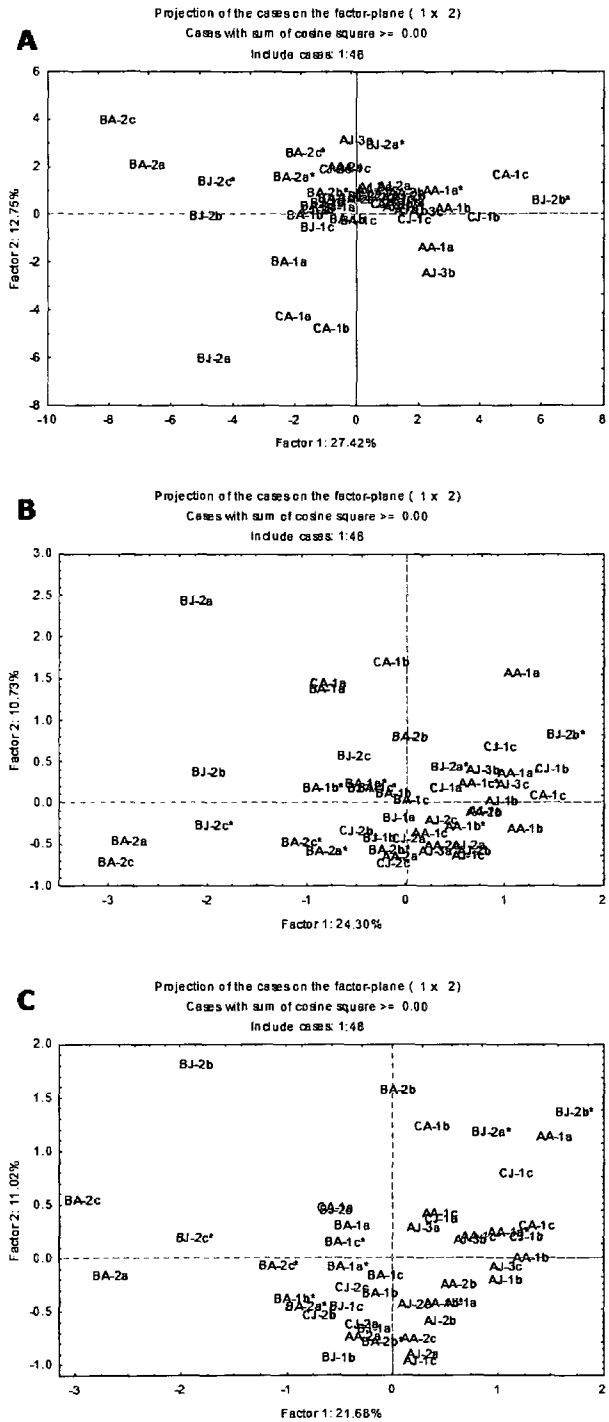


Figure 4.6 PCA ordinations for (A) untransformed, (B) natural logarithmic transformed, and (C) Taylor transformed BIOLOG™ EcoPlate data sets. Output generated using Statistica 7.1.

As seen in Figure 4.5, the untransformed data set preserves about 40% of the variance, while the logarithmic and Taylor transformed sets preserve about 35% and 33% respectively. Figure 4.5c shows a grouping effect of samples from oil sands-affected wetlands (indicated by the first letter, A), while reference and natural sites are more spread out. There does not appear to be a grouping effect based on sampling date or vegetation status. When the Taylor transformed data sets are analyzed with reduced samples, groupings are clarified. It appears that natural sites are more closely grouped than reference wetlands which do not show a grouping effect (Figure 4.6), but that oil sands-affected samples group more closely than natural ones (Figure 4.7). Oil sands-affected sites appear quite distinct from reference sites with the first principal component accounting for much of the variation between the two groups (Figure 4.8).

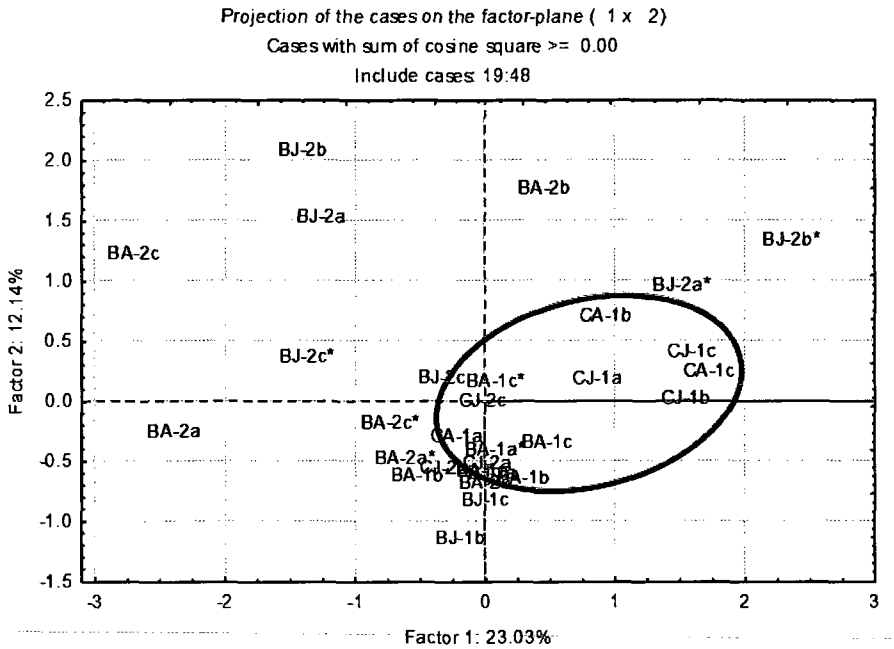


Figure 4.7 PCA ordination for Taylor transformed BIOLOG™ data comparing reference (first letter, B) and natural (first letter, C) sites. Output generated using Statistica 7.1.

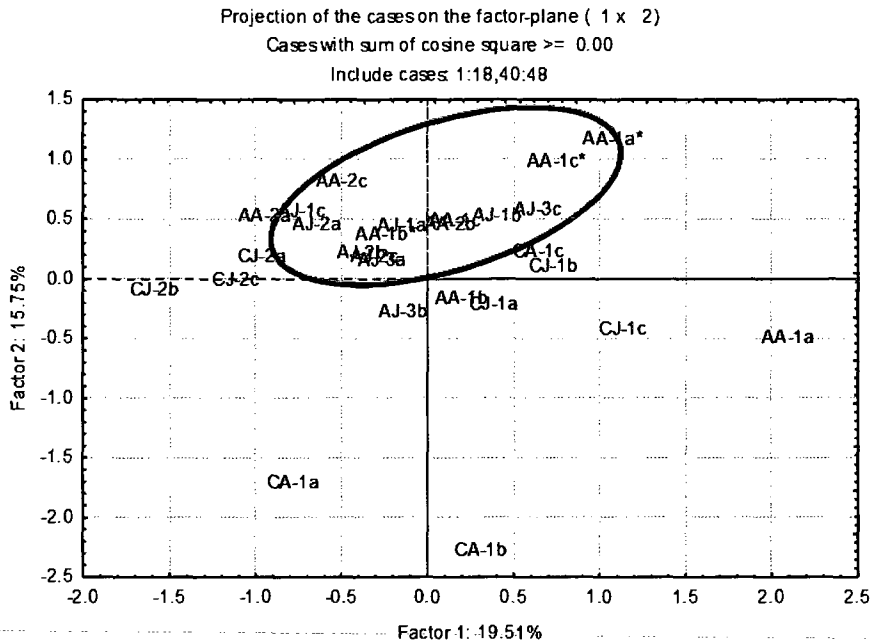


Figure 4.8 PCA ordination for Taylor transformed BIOLOG™ data comparing oil sands-affected (first letter, A) and natural (first letter, C) sites. Output generated using Statistica 7.1.

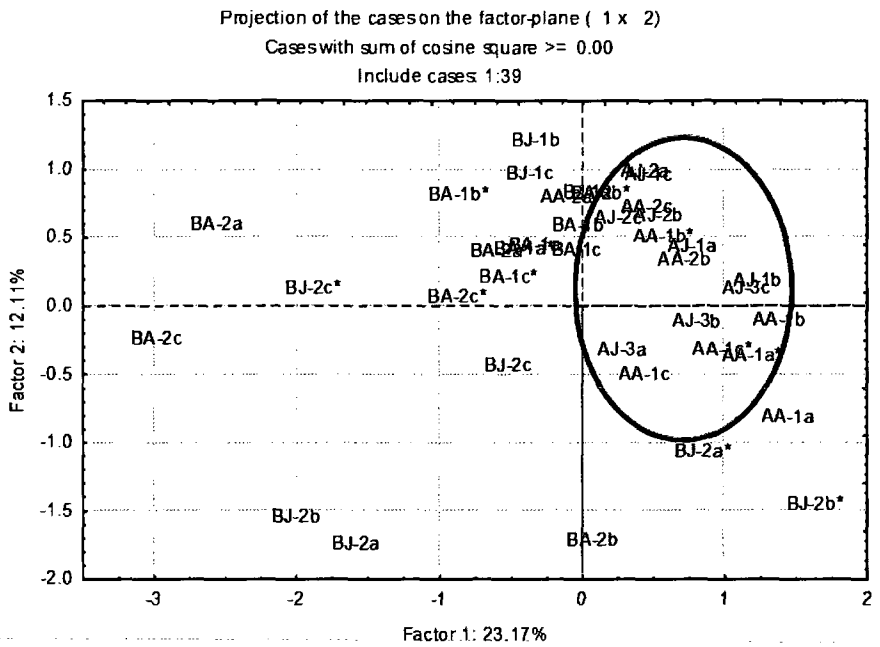


Figure 4.9 PCA ordination for Taylor transformed BIOLOG™ data comparing oil sands-affected (first letter, A) and reference (first letter, B) sites. Output generated using Statistica 7.1.

4.3.3 Cluster Analysis

UPGMA clustering analysis was performed on the Taylor transformed BIOLOG™ data to confirm if the same grouping effect seen with PCA was observed based on Euclidean distance in the 31-dimensional space. Figure 4.9 shows a similar grouping effect as found with the PCA ordination (Figure 4.5c). OSPM sites group moderately, while reference and natural sites are more spread out. When oil sands-affected and reference sites were compared without including natural sites, the distinction between OSPM-sites and reference sites is more evident (Figure 4.10). Euclidean linkage distances between oil sands-affected sites are generally shorter than those between reference sites, suggesting a higher similarity between oil-sands affected communities than between communities from reference wetlands.

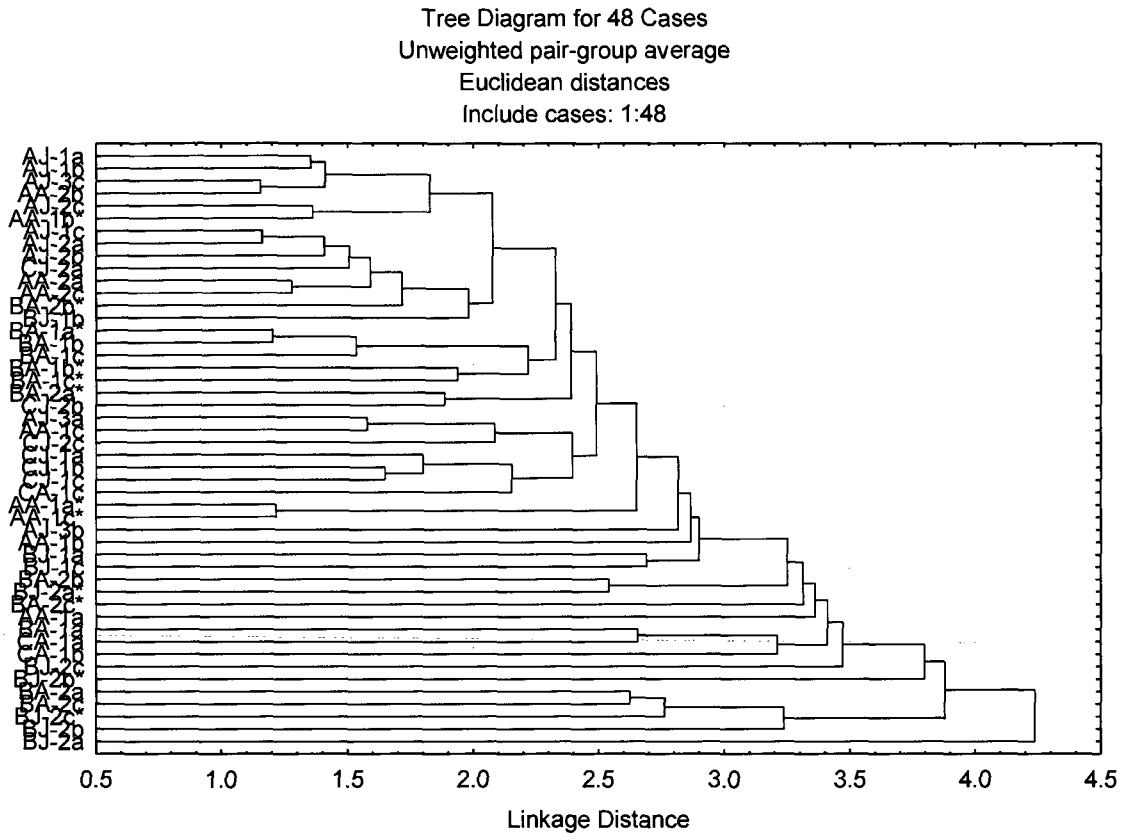


Figure 4.10 UPGMA dendrogram clustering analysis for Taylor transformed BIOLOG™ data comparing all wetland types. OSPM wetlands are indicated by the first letter, A, reference wetlands by the first letter, B, and natural wetlands by the first letter, C. Scale indicates Euclidean distance. Output generated using Statistica 7.1.

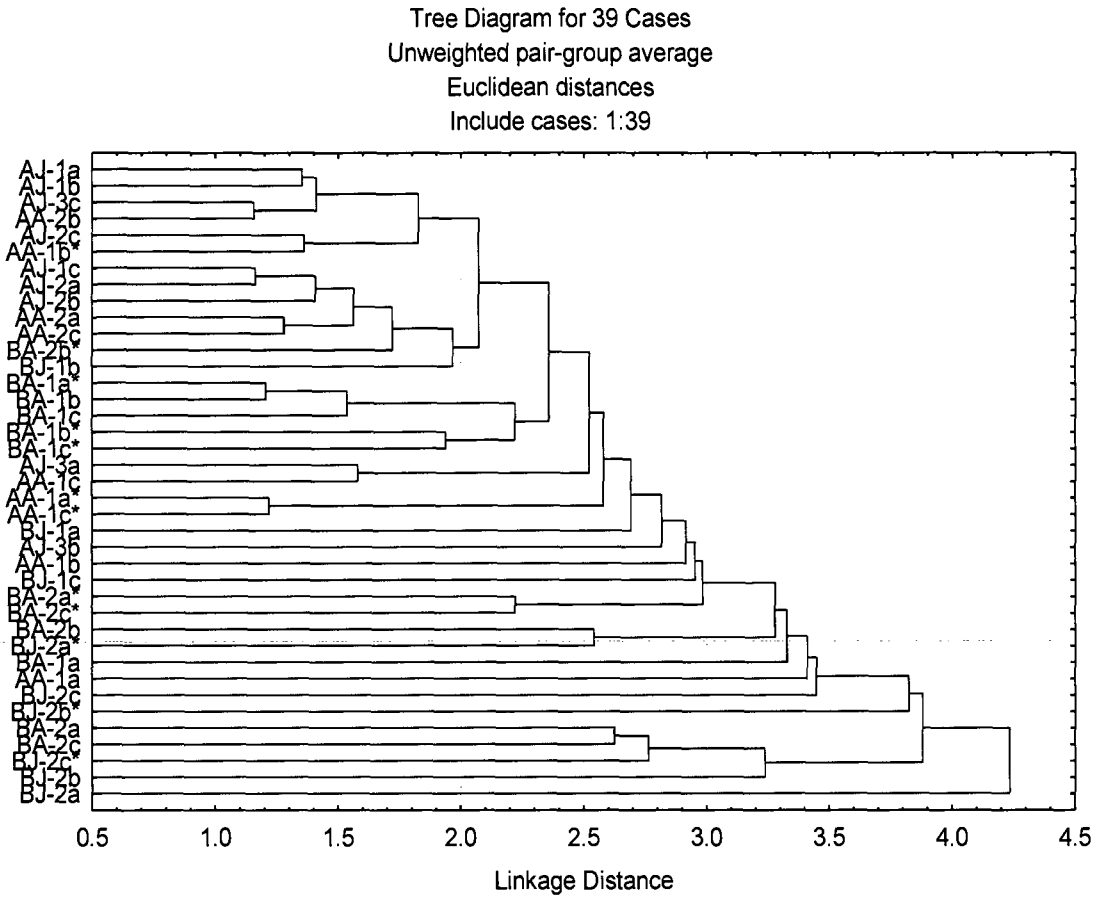


Figure 4.11 UPGMA dendrogram clustering analysis for Taylor transformed BIOLOG™ data comparing oil sands affected (first letter, A) and reference (first letter, B) wetlands. Scale indicates Euclidean distance. Output generated using Statistica 7.1.

4.4 Discussion

4.4.1 Substrate-Related Diversity Indices

Substrate-related diversity indices based on carbon source utilization patterns did not identify consistent differences in functional microbial community profiles between communities from wetlands directly affected by OSPM and those from wetlands unaffected by OSPM. The

effect of oil sands materials on the microbial population may not have been strong enough to be identified using measures of substrate-related diversity. Also, other factors that can impact microbial diversity, and that vary between many of the wetlands sampled, such as soil texture, organic content, vegetation type and density, and concentration of ions present, may have affected the functional microbial community profiles and detracted from the ability of the method to separate oil sands sites from non oil sands sites. Previous studies have found functional diversity parameters (Shannon diversity, richness, evenness) were altered when soil was exposed to different disturbances. Fuller *et al.* (1997) found, for example, that functional diversity parameters decreased in response to trichloroethylene and toluene. More recently, Weber *et al.* (2008) found a distinct shift in the functional potential of the microbial community in a constructed wetland mesocosm when exposed to acid mine drainage. Often, however, studies indentifying a shift in the functional diversity of a microbial population using substrate-related diversity indices are completed with the microbial community being sampled shortly after the disturbance and in which the same study site is examined before and after impact (e.g. Weber *et al.*, 2008). In this study, all of the impacted wetlands examined have been affected by oil sands materials for over 10 years. In addition, it was not possible to examine single sites before and after disturbance; instead, a range of wetlands that have been variably impacted by oil sands materials were examined. Besides impact from OSPM, however, these wetlands likely contain significant variations in structure, size, ecological characteristics, water chemistry, and sediment characteristics. Thus, the lack of clear differences in functional microbial diversity between oil sands-affected and non-oil sands-affected wetlands may reflect processes of microbial adaptation to the disturbance and Shannon diversity indices may not be sensitive enough to detect this gradual shift in microbial community functionality over influences of spatial variability.

Similarly, substrate-related diversity measures could not distinguish between samples from vegetated and non-vegetated areas of Athabasca wetlands when all samples were considered. In contrast, when analysis was constrained to one particular wetland, samples from an

area of the wetland characterized by emergent vegetation consistently displayed higher measures of richness and diversity than samples from an area lacking vegetation. Substrate diversity, or the diversity of potential substrate utilization, can be loosely interpreted as microbial diversity (Weber *et al.*, 2008) and therefore, it can be inferred that microbial communities are relatively more diverse in the vegetated portions of a wetland when compared to the same wetland's non-vegetated counterpart. Because decomposing plants are important sources of organic substrates for bacterial metabolism (e.g. Webster and Benfield, 1986), the presence of plants would increase the diversity of organic substrates, which in turn may be associated with a more diverse assemblage of bacteria. Merkley *et al.* (2004) found, for example, an increase in Shannon diversity in wetlands exposed to cattle grazing, compared to those protected from grazing, and predicted this effect may be partially attributed to the increased diversity of plant species on grazed sites. He *et al.* (2008) recently found higher bacterial metabolic diversity, as measured by the Shannon index, in soil bacterial communities from sites characterized by higher plant diversity. Although it could not be determined from this study, it is possible that the increase in substrate diversity might reflect a more robust microbial community. Weber *et al.* (2008) found that constructed wetland mesocosms planted with *Phragmites australis* were more resistant to changes in microbial ecology after exposure to acid mine drainage than unplanted mesocosms. In order to confirm if microbial communities from vegetated areas of wetland in the Athabasca region are similarly more robust than communities from non-vegetated areas, it would be useful to examine whether communities from areas of vegetation are more resistant to environmental stressors compared to those from non-vegetated areas.

Although CLPP results suggest more diverse and potentially healthier and more robust microbial communities from vegetated areas of wetlands, it must also be considered that the apparent increase in richness and diversity could be biased by the types of carbon sources present on BIOLOG™ EcoPlates. These microplates contain at least nine known carbon sources that are released by plant roots (Campbell *et al.*, 1997). Vegetated wetland environments may select for

microbial communities able to use plant root exudates as a source of carbon giving these communities an advantage in quickly assimilating the plant-associated carbon substrates on EcoPlates.

4.4.2 PCA

A thorough discussion of data transformations in the analysis of BIOLOG™ data is provided by Weber *et al.* (2007) but a brief mention of the transformations used in this study is presented here. Two transformations commonly used in ecological data analysis were employed in this study: a logarithmic transformation and a Taylor power law transformation. These transformations are useful for application to PCA because they improve conformation of the data to the assumptions of parametric analysis (e.g. normality and homoscedasticity) (e.g. Legendre and Legendre, 1998; Taylor, 1961).

The improvements in normality and homoscedasticity seen through formal statistical tests (Table 4.1) were reflected in improvements in PCA plots (Figure 4.5). In accordance with the statistical tests, the Taylor transformed data sets produced the most easily interpretable plot. In the PC ordination of the untransformed data (Figure 4.5a), the presence of outliers (e.g. BA 2a, BA-2c, BJ-2a, BJ-2b, BJ2b*) accounts for much of the variance in the data thereby influencing the rotation of the PCs (Weber *et al.*, 2007). The transformations reduce some of this effect so that other variances within the data can be better evaluated. Aside from employing transformations, outliers can either be removed from the analysis entirely or the weight associated with the underlying variable(s) causing the outliers can be reduced. Since these alternative approaches carry the risk of discarding important information, a transformation is often preferred (Weber *et al.*, 2007).

PC plots of the Taylor transformed data were able to discriminate between CSUPs of microbial communities from oil sands-affected and un-affected wetlands. A clear grouping effect

was seen among OSPM samples, while reference and natural sites were more spread out (Figure 4.5c). In contrast, Hadwin *et al.* (2006) performed clustering analysis using the squared Euclidean distances between extracted rate values from each CSUP curve and found that BIOLOG™ EcoPlates could not consistently distinguish between microbial communities from Athabasca wetlands receiving oil sands process waters and those un-affected by oil sands materials. It is possible that, as mentioned by Hadwin *et al.* (2006), the metabolic fingerprints were affected by factors independent of the receiving process waters being studied by the group. Alternatively, results may have been affected by the cell extraction methodology, or the lack of transformation employed in their data analysis. The age of the wetlands and the time and method of sampling might also explain inconsistencies between the findings of Hadwin *et al.* (2006) and those of the present study.

When only two wetland types were compared, clustering effects were seen with natural sites compared to reference sites (Figure 4.6), OSPM sites compared to natural sites (Figure 4.7), and OSPM sites compared to reference sites (Figure 4.8). Overall, the PC plots suggest that the CSUPs of microbial communities in OSPM sites are the most similar, followed by communities in natural sites, and that the communities in reference sites have variable CSUPs. This suggests that microbial communities affected by oil sands materials are constrained by some effect to a significant degree such that they are distinguishable from natural and reference sites. However, whether the constraint is due to toxic properties present in the oil sands materials, such as NAs or polycyclic aromatic compounds (PACs), or to alternate carbon substrates in these wetlands, cannot be determined from this study. Interestingly, CSUPs of communities from OSPM sites are more similar to CSUPs of communities from natural sites (off mining leases) than reference sites (on mining leases). Given that many of the natural sites are well established, it is expected that microbial communities in these wetlands are well-adapted and, therefore, more stable than communities in reference wetlands.

4.4.3 Cluster Analysis

UPGMA clustering analysis (Figure 4.9; Figure 4.10) was performed on the Taylor-transformed data to determine if the same grouping effect seen with PCA was seen based on Euclidean distances. In general, a similar grouping effect was identified. Clustering analysis identified shorter linkage distances among OSPM wetlands compared to reference wetlands (Figure 4.10) suggesting a higher similarity among microbial CSUPs of oil sands-affected sites. The distinction between natural wetlands was not as clear using this analysis method, although a moderate grouping effect (Figure 4.9) can be seen among natural sites and these sites are similarly linked by shorter measures of Euclidean distance.

Cluster analysis is more subjective than PCA and results may have been influenced by the type of cluster analysis performed and scale used (Fowler *et al.*, 1998). Nevertheless, the same general trends seen with the PC ordinations were seen with the clustering analysis.

4.5 Summary and Conclusions

BIOLOG™ EcoPlates were successfully used to distinguish between microbial communities from wetlands in the Athabasca region that differ in the degree of impact by oil sands materials. When one wetland was considered, substrate-related diversity indices distinguished between samples from vegetated and non-vegetated areas of the same wetland and suggested that communities from vegetated regions are more diverse and potentially more robust than communities from non-vegetated regions. PCA proved useful in discerning between the CSUPs of microbial communities from the three wetland types examined. Communities from oil sands sites were found to be most similar and it is proposed that potential functional diversity may be constrained by oil sands material. Whether the constraint is due to toxic properties associated with the OSPM, other disturbance associated with mining, or because of the alternate carbon substrates present in the material, however, cannot be determined from this study and may

warrant further investigation. Interestingly, a higher similarity in CSUP was seen between oil sands and natural sites although reference sites were expected to represent an intermediary between the two groups with respect to degree of impact from oil sands materials. This raises the question of what an appropriate baseline of comparison will be for studies of this type. The reference sites used in this study, for example, may be too varied in their construction and degree of impact from oil sands materials to be used as an effective basis of comparison for oil sands sites.

This study also supports current literature (e.g. Weber *et al.*, 2007) which suggests that data analysis and transformation must be carefully considered for CLPP studies employing multivariate analysis techniques. In this study, transformations were found to significantly improve the data's suitability for multivariate analysis and the Taylor power law transform, in particular, was effective in producing clear and interpretable PC ordinations.

Although clear differences were noted in the functional microbial community profiles of oil sands-affected and un-affected wetlands, it is difficult to discern the probable causes for these results. As mentioned previously, (Preston-Mafham *et al.*, 2002), the use of BIOLOG™ microplates is therefore best used for comparative purposes and in combination with other alternative characterization methods. In this study, this method was useful to compare the microbial communities between wetlands affected to varying degrees by oil sands materials, and may represent a useful tool for monitoring microbial community structure of oil sands-affected sites as reclamation progresses.

Future research is needed to better elucidate the causes behind the distinct functional microbial community profiles of OSPM-affected and non OSPM-affected wetlands. Wetland mesocosm experiments might provide more controlled environments whereby the effects of contaminants such as NAs, residual bitumen, and PAHs can be tested both individually and in combination for their effects on microbial community functionality. Given that results from this study suggest that vegetation may be an important predictor of microbial health and stability,

efforts should be made to better understand the effects of vegetation presence, quantity, and type on the microbial community. Finally, oil sands researchers should carefully consider the environment type that is best suited as a basis of comparison for the reclamation of OSPM sites.

Chapter 5
Characterization of Bacterial Communities in Wetlands of the Athabasca Oil Sands Region using Denaturing Gradient Gel Electrophoresis

5.1 Introduction

The process required to extract bitumen from the Athabasca oil sands results in the disturbance of land and the production of large amounts of oil sands process-affected materials (OSPM) (Holowenko *et al.*, 2000), and as a consequence, reclamation to a pre-development state is a priority (Pollet and Bendell-Young, 2000). The wet landscape reclamation strategy may represent a viable solution for reclaiming disturbed landscapes while dealing with the large volumes of oil sands-associated waste that is generated through mining and refinery operations (Holowenko *et al.*, 2000). An important component in developing this reclamation strategy will be an effective means through which to monitor the progression of OSPM-impacted ecosystems towards more natural states.

Microbial communities can be valuable indicators for monitoring ecosystem health in disturbed environments. Microorganisms are crucial in maintaining soil productivity through roles in nutrient transformation and cycling, organic matter decomposition, and humus formation (e.g. Azam *et al.*, 1983). They are also capable of degrading many environmental toxicants; in the oil sands region, several studies have demonstrated the biodegradation of naphthenic acids, a primary toxicant in OSPM, by indigenous microorganisms from tailings waters (e.g. Lai *et al.*, 1996; Del Rio *et al.*, 2006) and soil (Biryukova *et al.*, 2007). It follows that microbial community structure might vary with respect to impact from oil sands materials and that microbial community profiles may be useful predictors of ecosystem health as reclamation proceeds.

Despite the potential value in monitoring microbial community structure for reclamation purposes, microbial communities are often neglected as indicators, likely because of difficulties involved with their study (e.g. Torsvik *et al.*, 1998). Recent fingerprinting techniques have been developed that overcome some of the limitations associated with traditional culture-based methods, and which provide a rapid and realistic means to assess microbial community structure. Denaturing gradient gel electrophoresis (DGGE) is an effective molecular profiling technique originally proposed by Muyzer *et al.* (1993) based on sequence variation in ribosomal RNA genes. The 16S rRNA gene is a useful target given its ubiquity in prokaryotes and the presence of both highly conserved and variable regions within the gene (Head *et al.*, 1998). Also, sequences specific for certain microbial groups have been identified within this gene; for example, Nübel *et al.* (1997) used primers to amplify cyanobacterial 16S rDNA. Following amplification of microbial DNA using the polymerase chain reaction (PCR), fragments are separated according to base-pair sequence along a polyacrylamide gel with a linearly increasing gradient of denaturant. Separation is possible due to the decreased mobility of a partially melted DNA molecule compared to the helical form of the molecule (Muyzer *et al.*, 1993). The result is a distinct banding pattern that can be used to compare community structure over time or across environments.

An advantage of DGGE is the possibility of quickly studying multiple, complex samples. DGGE enables taxonomic identification through the excision and sequencing of bands although, resolution to the species level may be difficult since the DNA fragments include only a short region (less than 500 bp) of the 16S rRNA gene (Muyzer *et al.*, 2004; Ogino *et al.*, 2001). Given the technique's reliance on PCR, products can be obtained from very small amounts of DNA but DGGE is subject to PCR biases such as amplification errors, formation of chimeric and heteroduplex molecules, and preferential amplification (Muyzer *et al.*, 2004). Similarly, DGGE is subject to biases induced through the extraction of DNA (see Chapter 3, "Optimization of Methods for the Application of CLPP and DGGE to the Study of Bacterial Communities in

Wetlands of the Athabasca Oil Sands Region"). Other limitations of DGGE include difficulties separating DNA fragments in the denaturant despite sequence variation, complexities associated with sequence heterogeneities in 16S rDNA, and difficulties analyzing habitats containing many microbial populations (Muyzer *et al.*, 2004). In addition, PCR-DGGE may overlook minority members of microbial communities (e.g. Heuer *et al.*, 1997).

It is possible to overcome some of the limitations associated with using DGGE to study diverse microbial communities by using group-specific primers. Kowalchuk *et al.* (1997), for example, designed DGGE primers specific for ammonia-oxidizing populations in the β-Proteobacteria subdivision and were able to identify changes in this bacterial group between coastal dune sample sites. More recently, group-specific primers are applied in a nested-PCR approach prior to DGGE analysis. This involves an initial PCR using a group-specific primer set to target a subset of the total microbial population followed by a second PCR in which the initial product is re-amplified with general DGGE primers to target a shorter span of the gene within the first product. Figure 5.1 illustrates the total bacterial approach and group-specific (nested PCR) DGGE approaches taken in this study. Boon *et al.* (2001) used this group specific DGGE strategy to analyze methanotrophic members of the α- and γ-Proteobacteria, ammonia-oxidising bacteria, and *Acidobacterium* in activated sludge systems of wastewater treatment plants. Dar *et al.* (2005) applied a three-step nested-PCR-DGGE approach to detect rare members of the sulphate-reducing bacteria (SRB) in complex microbial communities from industrial bioreactors. A nested PCR strategy eliminates the need to design novel DGGE primers and establish optimal conditions for those primers (Dar *et al.*, 2005). Additionally, as shown by Dar *et al.* (2005), a nested-PCR-DGGE may assist in detecting less abundant members of the microbial community although, diverse bacterial subgroups might still only allow for the visualization of dominant species (Boon *et al.*, 2001). Inherent in the group-specific method, however, is the requirement for more than one PCR reaction which carries the risk of even greater PCR bias due to preferential amplification (e.g. Suzuki and Giovannoni, 1996). In addition, primers available for group-specific analyses

depend on the availability of known sequences within the 16S rDNA database and this database may not accurately reflect the diversity of 16S rRNA genes in the environment (Boon *et al.*, 2001). As a result, it is important that as databases are updated, PCR primers are routinely re-evaluated for their specificity to target organisms and their range of sequence matches (Baker *et al.*, 2003).

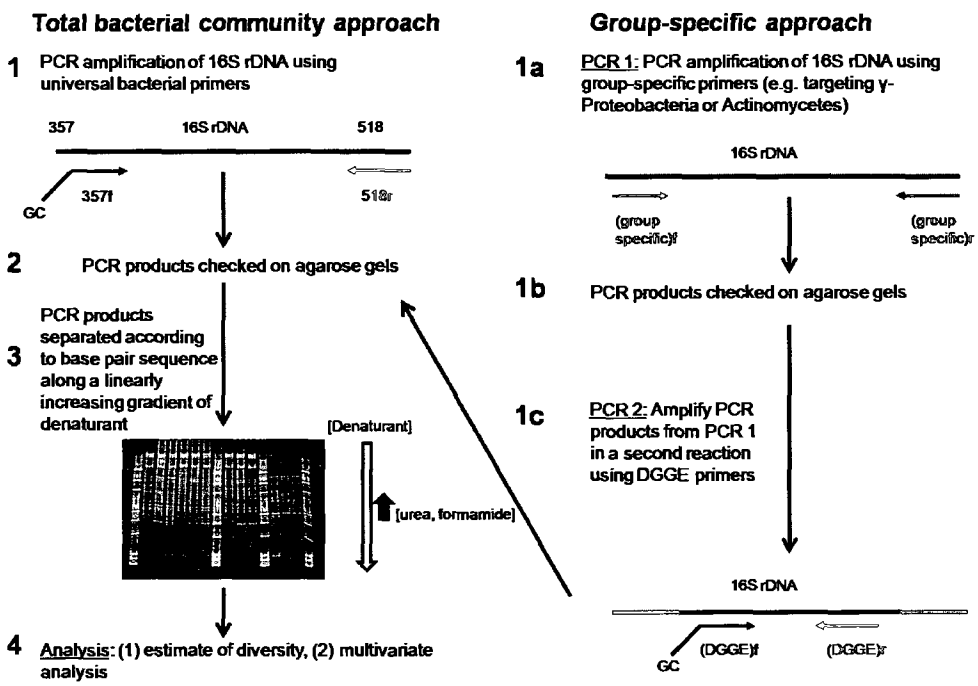


Figure 5.1 Schematic overview of the DGGE strategies used to assess bacterial communities in wetlands of the Athabasca oil sands region. Two group-specific DGGE approaches were applied using nested PCR to evaluate γ-Proteobacteria and Actinomycetes bacterial populations.

DGGE has been used to characterize microbial communities from a range of pristine and disturbed environmental habitats including soils (e.g. Leckie *et al.*, 2004; Rønn *et al.*, 2002), sediment (e.g. Qu *et al.*, 2008), biofilm (e.g. Massieux *et al.*, 2004), wetlands (Nicomrat *et al.*, 2006) and marine environments (e.g. Ogino *et al.*, 2001). Previously, Hadwin *et al.* (2006)

applied DGGE to study sediment samples from wetlands in the Athabasca oil sands region that were variably impacted by oil sands-associated materials. They found that impact from oil sands process waters had a strong effect on the microbial community structure and that DGGE could consistently distinguish off-site wetlands from impacted ones. The identifiable differences between site types supports the idea that microbial community structure assessed through DGGE might be valuable in monitoring ecosystem reclamation as the wet landscape strategy is applied.

The objective of this study was to apply DGGE to analyze the bacterial community structure in wetlands of the Athabasca oil sands region that have been impacted to varying degrees by OSPM. A similar approach to that of Hadwin *et al.* (2006) was taken to analyze the total bacterial community in order to confirm their previous finding that DGGE could distinguish between impacted and non-impacted sites. As a novel addition, group-specific PCR-DGGE was performed to obtain increased resolution into the bacterial populations in these systems. Specifically, the following bacterial groups were evaluated by this approach: (1) γ-Proteobacteria, a metabolically diverse group of Gram negative Bacteria, and (2) Actinomycetes, a group of high GC, Gram positive organisms with filamentous structures (Tortora *et al.*, 2004). This study served as a preliminary step in assessing the suitability of DGGE for monitoring bacterial populations in Athabasca wetland samples throughout the reclamation process.

5.2 Materials and Methods

5.2.1 Wetland Sediment Samples

Samples used in this study were the same as those used in the study entitled "CLPP of Bacterial Communities in Wetlands of the Athabasca Oil Sands Region" described in chapter four except additional samples collected in June 2009 were included in part of this study. Sampling sites used in this study are summarized in Chapter 2 ("Site Description and Sampling

Methodology") in Tables 2.2 (2008 samples) and Table 2.3 (2009 samples). All samples were stored at 4 °C until processed for DNA extraction.

5.2.2 Extraction of Genomic DNA

DNA was extracted from the sediment samples using the Powersoil™ DNA Isolation Kit (Medicorp Inc., Montreal, Canada) following the manufacturer's instructions for the wet soil protocol. All DNA extractions were performed within three days of receipt of samples with the following exceptions: DNA extractions were performed on 2008 samples MP-1_1, MP-1_2, MP-1_3, SW-1_1, SW-1_2, SW-2_2, 4M-1_1, 4M-1_2, and 4M-1_3, at approximately three months following receipt of samples. Due to the need to include these samples in the analysis, an analysis of the reproducibility of banding patterns on DGGE gels of samples extracted at different times and from different locations of the sampling containers, was performed (see below). Extracted DNA was stored at -20 °C.

5.2.3 PCR Amplification of 16S rDNA

Five primer pairs were used to amplify different regions of the 16S rRNA gene (Table 5.1). The Bio-Rad iCycler IQ was used for all polymerase chain reactions.

5.2.3.1 Total Bacterial Community Analysis

The primer pair, 357f-GC/518r, targets the V3 region of the gene and was used to amplify the 16S rDNA of the general bacterial population. Originally, PCR products were generated with the forward primer 63f-GC following Leckie *et al.* (2004) but poor resolution of DGGE bands were obtained with this primer. PCR with primer pair, 357f-GC/518r, was performed according to Zwart *et al.* (1998) with some modifications. Reactions were completed in 50 μL volumes

consisting of 5 μL of template DNA, 500 nM of each of the forward and reverse primers, 10 μL 5X PCR buffer (Promega Fischer Scientific, Whitby, Canada), 3 mM $MgCl_2$, 200 μM each dNTP, and 1.25U *Taq* DNA polymerase. A touchdown PCR protocol was used with an initial denaturation step at 94 °C for 5 minutes. This was followed by 20 cycles consisting of a 1-minute denaturation step at 94 °C, a 1-minute primer-annealing step at A_T, and a 1-minute extension step at 72 °C. The annealing temperature (A_T) was decreased by 1 °C every two cycles from an initial temperature of 65 °C to a temperature of 56 °C in the 20th cycle. Ten additional cycles followed with the same conditions except the A_T was held constant at 55 °C. Finally, a 7-minute extension step was used to ensure PCR products were fully extended.

The primer pair, 357f-GC/518r, was also used to evaluate the reproducibility of DGGE banding patterns. Eight samples from four wetlands were used in this analysis. Replicate DNA extractions from the same sample were PCR amplified and separated on DGGE gels to evaluate the consistency among banding patterns. DNA extractions were performed on samples taken from various areas of the sampling container (left, middle, right) as well as at different time intervals over a period of six months in which time samples were stored at 4 °C. Table C.1 in Appendix C details this experimental setup.

5.2.3.2 Group-Specific Analysis

The remaining primer pairs were used in nested-PCRs to amplify the rDNA of specific bacterial groups. Group-specific DGGE was completed for 2008 samples only. The PCR protocol with the γ-Proteobacteria primer pair was adopted from Mühling *et al.* (2008) but some modifications were necessary in order to obtain specific amplification and to avoid contamination of the blanks. The initial reaction with primers Gamma395f and Gamma871r consisted of 50 nM of each primer, 200 μM each dNTP, 2 mM $MgCl_2$, 10 μL 5X buffer (Promega Fischer Scientific, Whitby, Canada), and 1.25U *Taq* DNA polymerase in a total volume of 25 μL. The PCR cycle

included an initial denaturation step at 96 °C for 4 minutes followed by 25 cycles of a denaturation step at 96 °C for 1 minute, 54 °C for 1 minute, and 74 °C for 1 minute. Finally, a 10-minute extension step at 74 °C was completed. PCR products generated from this initial PCR were then re-amplified using general bacterial primers (518f-GC/785r) appropriate for DGGE. Conditions for the re-PCR were identical to the initial PCR with the following exceptions: 1 μL of the PCR product from the first round was used as the template, reactions were carried out in 50 μL volumes, 30 cycles were completed instead of 25, and the annealing temperature was 56 °C.

Amplification of 16S rDNA of Actinomycetes followed Boon *et al.* (2001) but modifications were required. The following were combined in 25 μL reaction volumes: 200 nM of each of the forward (243f) and reverse (1378r) primers, 200 μM each dNTP, 1.5 mM $MgCl_2$, 10 μL 5X PCR buffer (Promega Fischer Scientific, Whitby, Canada), and 1.25U/50 μL of *Taq* DNA polymerase. PCR was initiated with a 10-minute denaturation at 95 °C followed by 30 cycles of a 1-minute denaturation step at 95 °C, a 1-minute primer-annealing step at 63 °C, and a 2-minute elongation step at 72 °C. A final elongation step was included for 12 minutes at 72 °C. In the re-PCR, 1 μL of the template DNA from the first PCR was re-amplified using the general bacterial primers 338f-GC and 518r. PCR conditions were identical to those of the first reaction with primer pair 243f/1378r except for a modified annealing temperature, completion of the reaction in 50 μL volumes, and 25 cycles in the PCR reaction.

Note that a protocol from Mühling *et al.* (2008) was attempted to amplify bacterial members of the Firmicutes phylum but specific amplification was never obtained and as a result, DGGE was not applied to this group.

In all reactions, blanks were included which contained the appropriate ingredients described above but with water in the place of DNA template. In nested-PCRs, 1 μL of the blank from the initial PCR was used as the template in the second reaction; thus, two blanks were included in all re-PCRs. Positive and negative controls were included in group-specific PCRs

where possible. All products were run on 1% or 1.5% agarose gels to confirm that a single band of the appropriate size was present and that no amplification occurred in the blank(s).

Table 5.1 Description of primers used for DGGE profiling of sediment bacterial communities

Primer[a]	Target group	Target site[c]	Sequence (5' to 3')	Reference
357f-GC[b]	Bacteria	341-357	CCTACGGGAGGC AGCAG	Muyzer *et al.*, 1993
518r	Universal	518-534	ATTACCGCGGCT GCTGG	Muyzer *et al.*, 1993
Gamma395f	γ-Proteobacteria	395-412	CMATGCCGCGTG TGTGAA	Mühling *et al.*, 2008
Gamma871r	γ-Proteobacteria	871-891	ACTCCCCAGGCG GTCDACTTA	Mühling *et al.*, 2008
518f-GC	Universal	518-534	CCAGCAGCCGCG GTAAT	Muyzer *et al.*, 1993
785r	Bacteria	785-803	CTACCAGGGTAT CTAATCC	Lee *et al.*, 1993
243f	Actinomycetes	226-243	GGATGAGCCCGC GGCCTA	Boon *et al.*, 2001; Liu *et al.*, 2007; Heuer *et al.*, 1997
1378r	Actinomycetes	1378-1401	CGGTGTGTACAA GGCCCGGGAACG	Boon *et al.*, 2001; Liu *et al.*, 2007; Heuer *et al.*, 1997
338f-GC	Bacteria	338-358	GACTCCTACGGG AGGCAGCAG	Ovreas *et al.*, 1997

[a] f (forward) and r (reverse) indicate the primer orientation with respect to the 16S RNA gene sequence
[b] GC indicates a 40-bp GC-rich sequence attached to the 5' end of the primer
GC for 357f: 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'
GC for 338f, and 518f: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'
[c] *E. coli* numbering of the 16S gene

Table 5.2 PCR approach with DGGE primers

Target group	Primers used for group-specific PCR	Primers used for re-PCR for DGGE	A_T of nested re-PCR (°C)	Denaturing gradient used for DGGE (%)[a]
Bacteria	-	357f-GC/518r	-	40-65
γ-Proteobacteria	395f/871r	518f-GC/785r	56	45-60
Actinomycetes	243f/1378r	338f-GC/518r	53	50-65

[a] 100% denaturant contains 7 M urea and 40% formamide

5.2.4 DGGE of Bacterial Communities

DGGE was performed using the CBS Scientific system (VWR, Mississauga, Canada) following a protocol by Deslippe *et al.* (2008; personal communication) with modifications. PCR products from reactions with the general bacterial primers were loaded onto 8% (w/v) polyacrylamide gels in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with denaturing gradients generally ranging from 40 to 65%, although the precise gradient was dependent on the primer set (Table 5.2). The electrophoresis was run at 60 °C for 16 h at 75 V (bacteria, γ-Proteobacteria, Actinomycetes). Following electrophoresis, gels were stained for 1 hour in SYBR® Gold nucleic acid gel stain (Invitrogen, Burlington, Canada) and imaged on a UV transillumination system equipped with a SYBR® photographic filter (Invitrogen, Burlington, Canada). DNA ladders used in DGGE analyses were constructed with equal-volume mixtures of PCR products from 16S rDNA fragments of varying GC content (16S gene fragments courtesy of J. Neufeld).

5.2.5 Analysis of DGGE Patterns

DGGE fingerprints were analyzed using Gel Compar II (Applied Maths, Texas, USA). Contingency tables were generated with the "Chart and Statistics Tools" package to illustrate relationships between the number of bands counted and sample types. Fingerprints were analyzed using an unweighted pair group method with arithmetic means (UPGMA) cluster analyses (using the Jaccard correlation coefficient) as well as with Principle Component Analysis (PCA). PCA was found to be more reliable than UPGMA cluster analyses and, therefore, is predominantly shown here. PCA plots were constructed on the basis of both quantitative and non-quantitative parameters but only non-quantitative (presence/absence of bands) PCA plots are shown.

5.3 Results

5.3.1 DGGE Profiles of the Total Bacterial Community

5.3.1.1 Reproducibility of Banding Patterns in Total Bacterial Community Profiles

When replicate extractions from the same sample were PCR amplified with primers 357f-GC and 518r and subsequently run on DGGE gels, a high similarity among samples from the same wetland was noted. Also, it was determined that using sediment from different locations of the sampling container (e.g. left, middle, right) in the DNA extraction had a minimal effect on the ultimate DGGE banding pattern. Banding patterns generated from repeated extractions of the same sample over time were also similar, although some changes in the presence of bands and intensity of bands were noted. See Appendix C for a representative gel image used in this analysis.

Although small changes in banding patterns between samples were recognized visually, replicate samples from the same wetland were more similar to each other than to other samples as

shown by clustering in the UPGMA dendrogram analysis (Figure 5.1) as well as PCA (Figure 5.2) As shown in both figures, four distinct clusters were generated based on the wetland that samples had originated from, despite differences in the DNA extraction as a function of time or space.

Although not specifically addressed, this analysis suggested the potential of a gel-effect. When the same PCR product was run on separate gels, the two banding patterns often did not cluster as closely as would be expected (e.g. Figure 5.1). This potential gel-effect was evident in a number of UPGMA dendrograms generated for other analyses and as a result, PCA results (in which gel effects were minimal or absent) were predominantly used for interpretation.

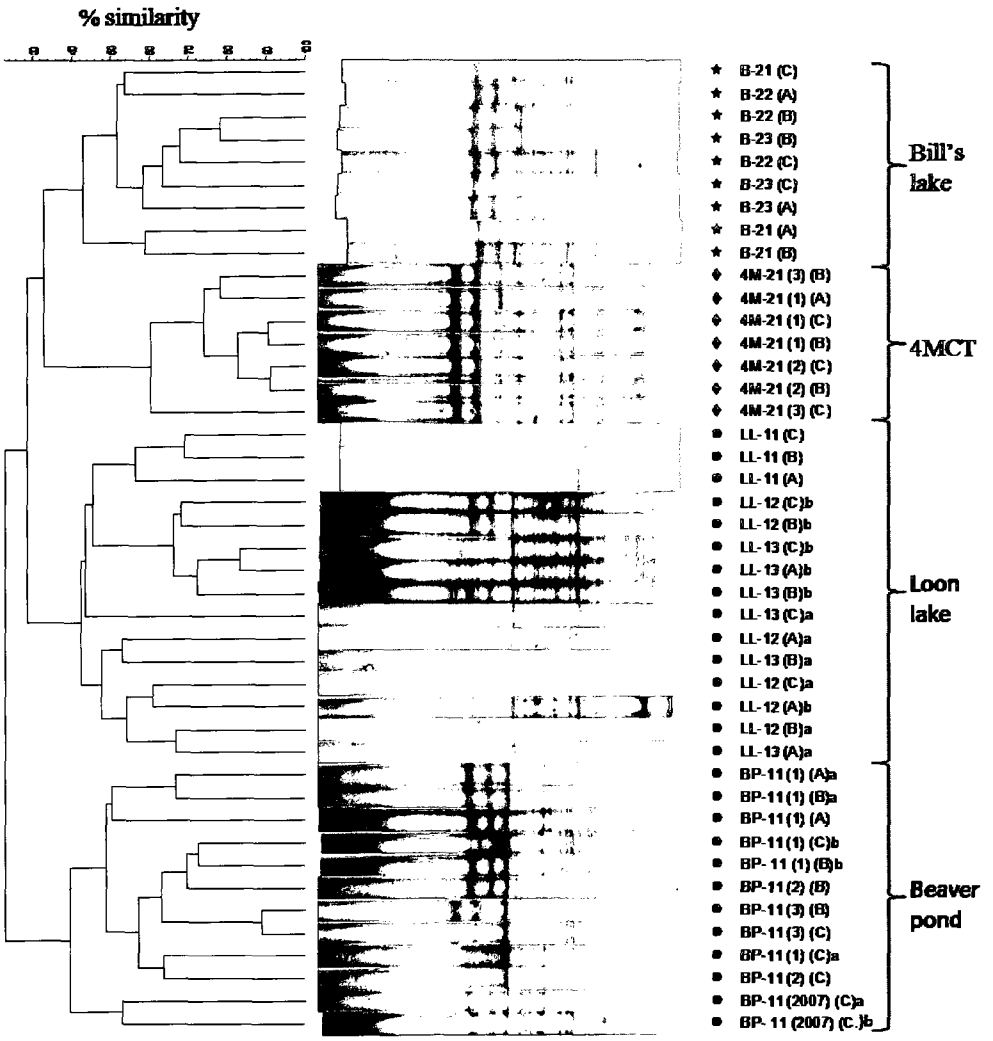


Figure 5.2 DGGE gels produced for samples B, BP, LL, and 4M and corresponding UPGMA cluster analysis generated using the Jaccard correlation coefficient. This analysis was completed to evaluate the reproducibility of banding patterns among replicate DNA extractions that varied with respect to time and/or space. The sample name is indicated followed by a number in brackets if applicable (referring to the location of the sampling container the sample was taken from) and/or a letter in brackets (referring to the time of sampling). A lower case letter "a" or "b" is indicated when the same PCA product was run on two separate DGGE gels. See Appendix C for a complete description of the samples used in this analysis.

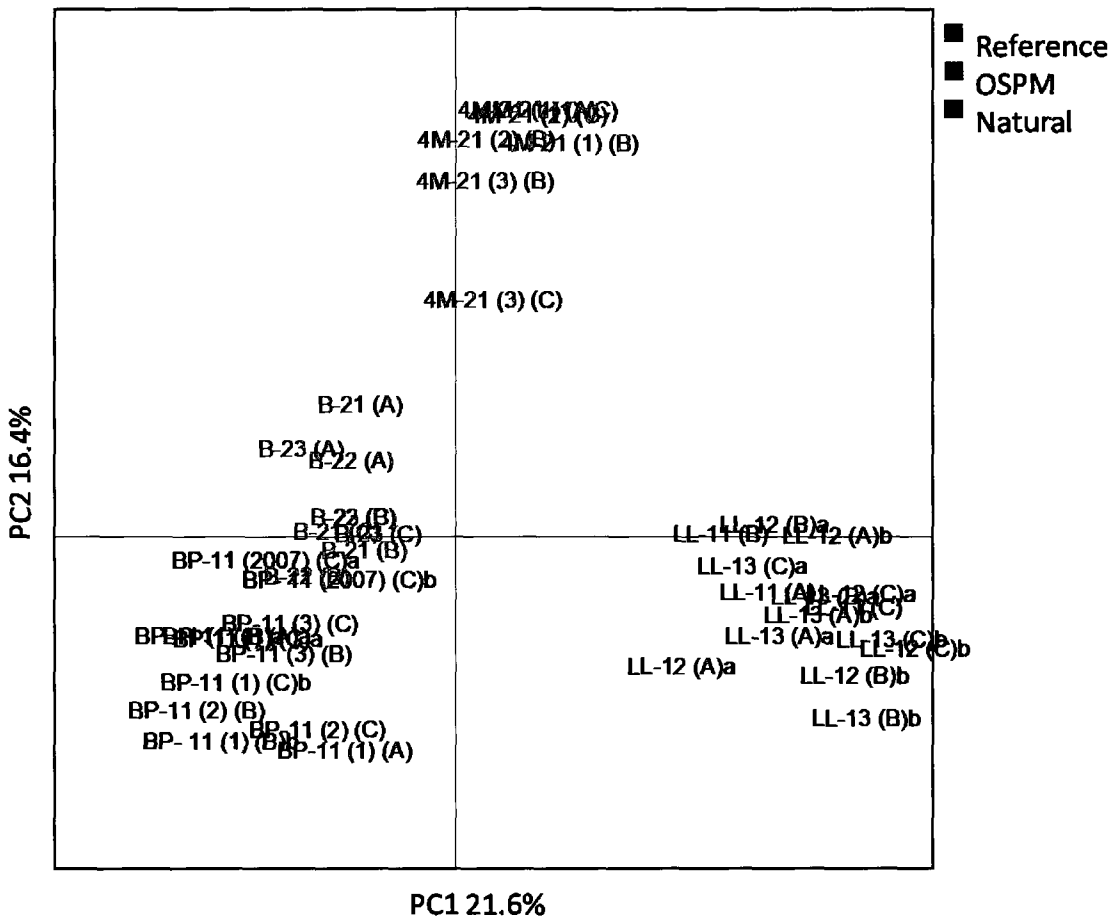


Figure 5.3 PCA plot of the presence/absence of DGGE bands in samples B, BP, LL, and 4M. PC 1 accounts for 21.6% of the variance and PC 2 accounts for 16.4%. This analysis was completed to evaluate the reproducibility of banding patterns among replicate DNA extractions that varied with respect to time and/or space. The sample name is indicated followed by a number in brackets if applicable (referring to the location of the sampling container the sample was taken from) and/or a letter in brackets (referring to the time of sampling). A lower case letter "a" or "b" is indicated when the same PCA product was run on two separate DGGE gels. See Appendix C for a complete description of the samples used in this analysis.

5.3.1.2 Visual Observations in Total Bacterial Community DGGE Profiles

Visual observations of 2008 and 2009 DGGE profiles suggested differences between samples from different wetlands and similarities among replicate samples from the same wetland. Also, smearing patterns in which bands were not clearly discernable were often noted (Figure 5.3).

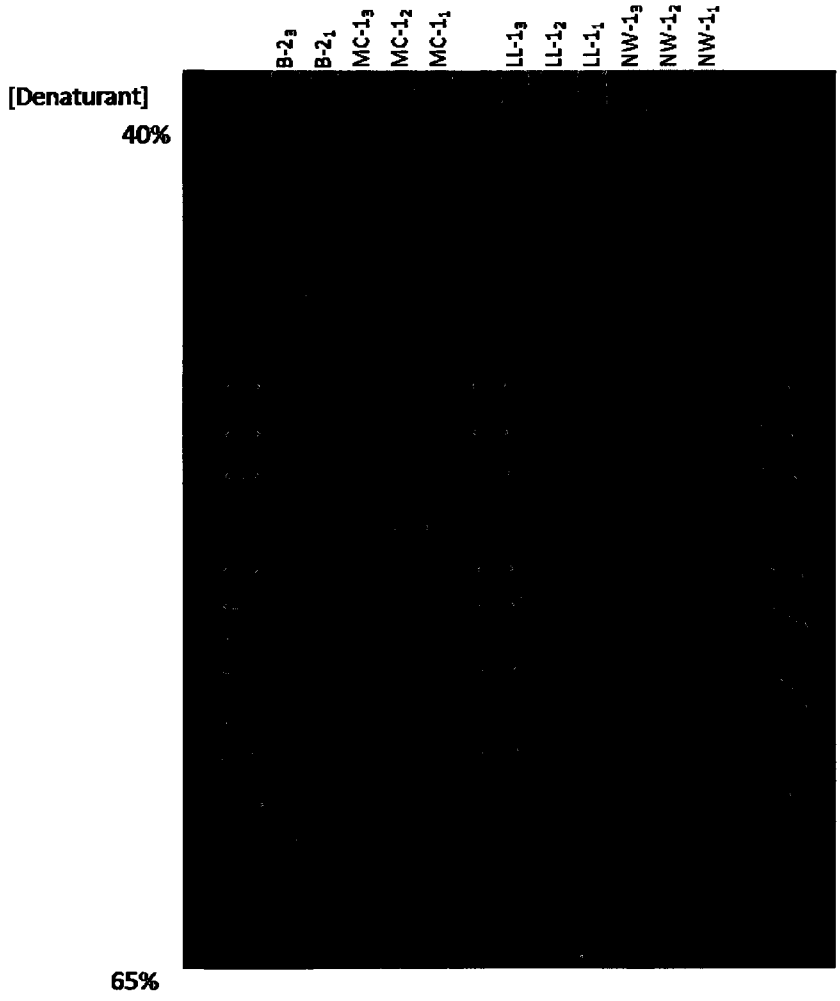


Figure 5.4 Representative 8% polyacrylamide DGGE gel with a urea/formamide denaturant of 40-65% to separate 2008 samples amplified with primers 357f-GC and 518r. Reference samples are unlabeled.

5.3.1.3 Number of Bands Counted in Total Bacterial Community DGGE Profiles

Differences were noted in the number of bands counted as a function of wetland type. In both the 2008 and 2009 samples analyzed, on average, a higher number of bands were counted in OSPM samples than natural or reference samples (Figures 5.4 and 5.5). For example, the majority of 2008 samples from OSPM sites contained at least 23 bands, whereas the majority of natural sites contained less than 17 and the majority of reference sites were counted to have less than 15 bands. Of the 2009 samples, the majority categorized as OSPM contained ≥21 bands while the majority of natural and reference samples contained ≤21 and ≤19 bands respectively. Within the OSPM group, a comparatively high number of bands were identified in samples taken from Mike's Pond (MP) in both 2008 and 2009. In the 2008 samples where a comparison based on vegetation was possible, no consistent differences were identified in the number of bands in vegetated versus non-vegetated samples (data not shown). Similarly, no trends in band number were identified with respect to the available physicochemical data (e.g. pH, dissolved oxygen, dry weight, water depth) or other sample variables (e.g. age of the wetland, location of the wetland on Syncrude or Suncor leases, date the sample was taken, date of the DNA extraction, date of PCR, date of the DGGE gel) with the exception of conductivity which was seen to follow the same general trend as seen with wetland type (see Appendix A for a description of the physicochemical data and other sample variables).

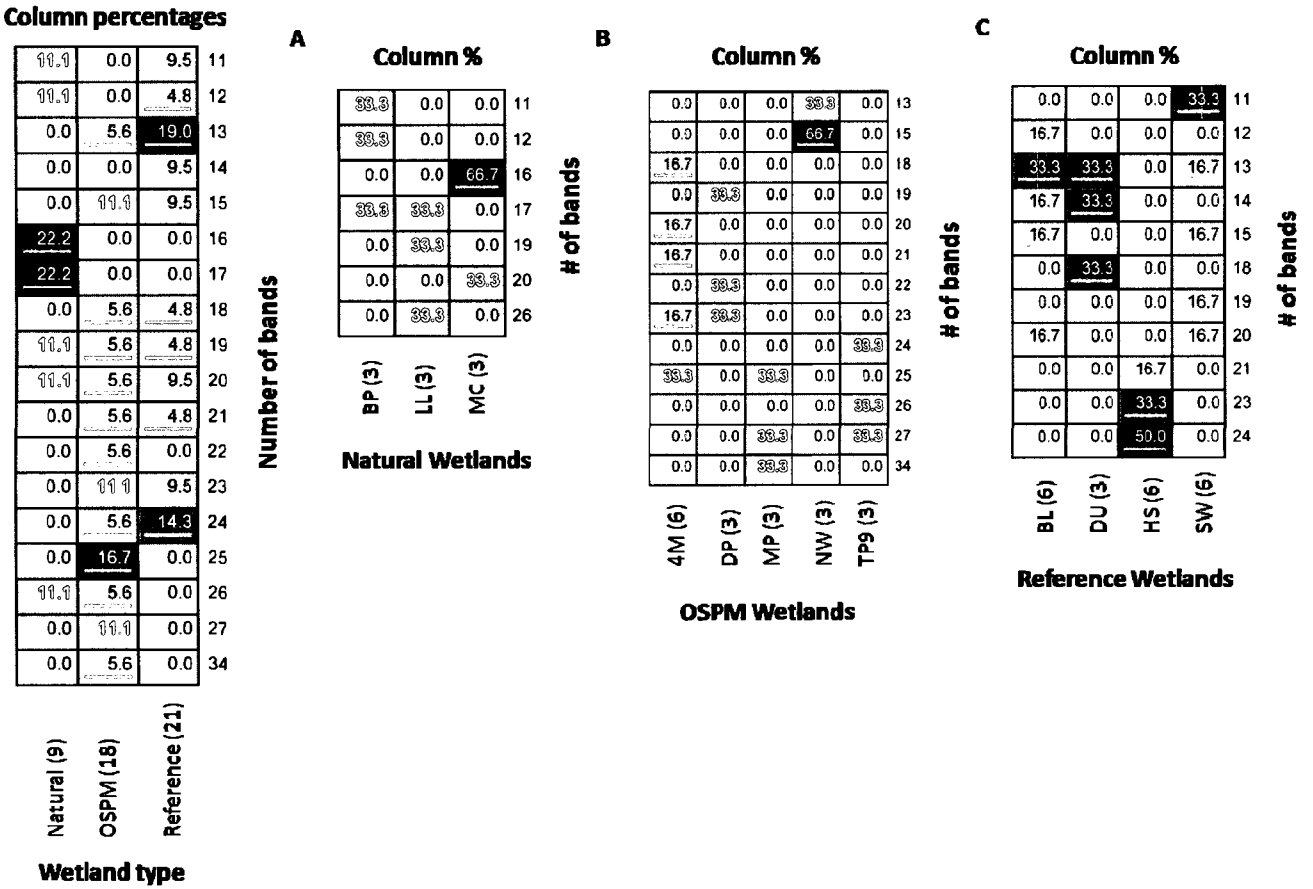


Figure 5.5 Contingency tables illustrating the number of bands counted in lanes of DGGE gels as part of 2008 DGGE gel analysis and as a function of wetland type and wetland. The table on the left illustrates the percent of samples in each category (wetland type: natural, OSPM, reference) containing "y" number of bands. Tables A, B, and C illustrate the percent of samples in each category (wetland) for (A) natural, (B) OSPM, and (C) reference sites respectively. The number of samples (n) in each category is recorded in brackets.

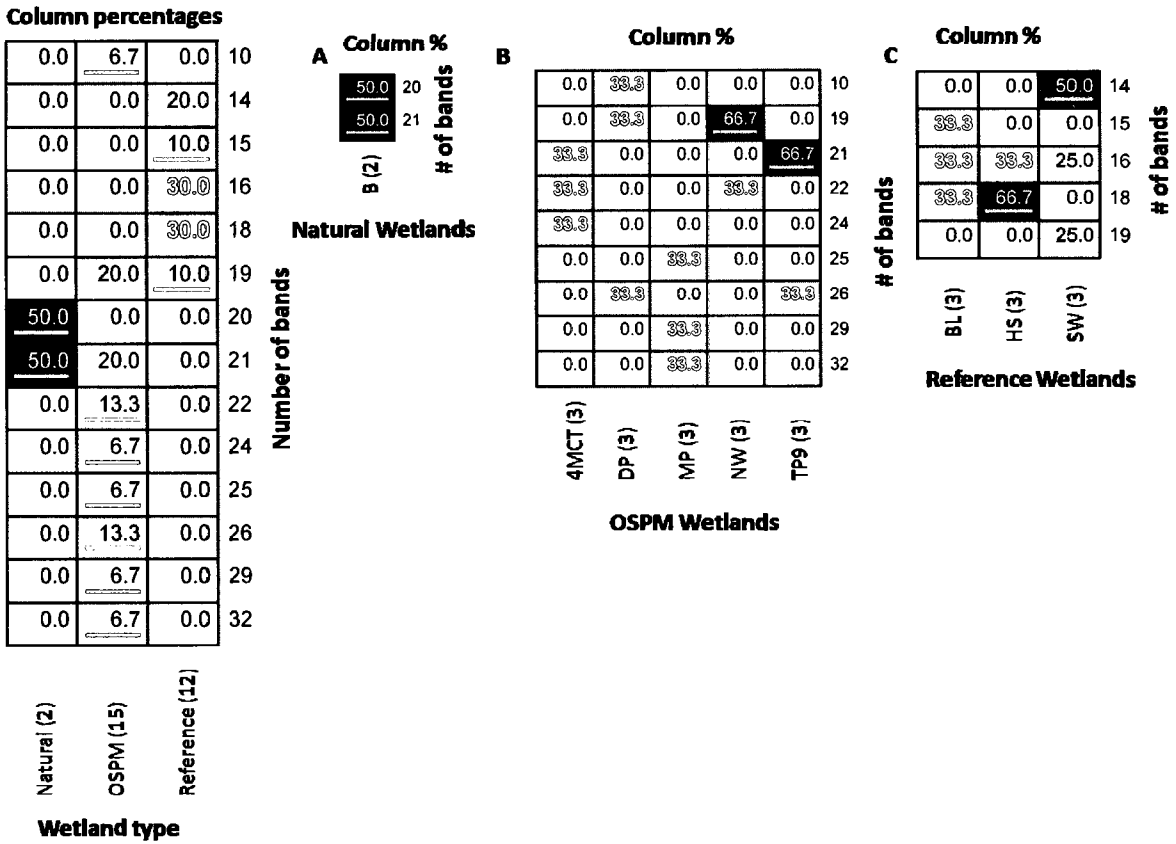


Figure 5.6 Contingency tables illustrating the number of bands counted in lanes of DGGE gels as part of 2009 analysis and as a function of wetland type and wetland. The table on the left illustrates the percent of samples in each category (wetland type: natural, OSPM, reference) containing "y" number of bands. Tables A, B, and C illustrate the percent of samples in each category (wetland) for (A) natural, (B) OSPM, and (C) reference sites respectively. The number of samples (n) in each category is recorded in brackets.

5.3.1.4 PCA of Total Bacterial Community DGGE Profiles

PCA plots generated for 2008 samples amplified with universal bacterial primers revealed potential grouping effects as a function of wetland type although clusters were not distinct. Samples clustered strongly with respect to the particular wetland they were collected from (Figure 5.6). PCA of the 2009 samples suggested clustering as a function of wetland type with OSPM samples clustering quite distinctly from natural and reference sites (Figure 5.7).Within the OSPM group, TP9 and 4M were seen to cluster most strongly for both the 2008 and 2009 samples while MP, NW, and DP clustered on their own or in smaller groups. Within reference samples, HS was relatively distinct. No clear clustering of natural sites was noted in 2008, and analysis of 2009 natural sites is difficult because only one natural wetland was sampled.

In contrast to the UPGMA dendrograms, minimal gel effects were observed in the produced PCA plots. No clustering effect was seen in either of the 2008 or 2009 samples as a function of sampling date, location of the wetland on Syncrude or Suncor leases, age of the wetland, or date of the DNA extraction or PCR amplification. Physicochemical and vegetation data was not available for samples collected in 2009 but for 2008 samples, dissolved oxygen and vegetation were similarly not found to influence clustering. For the 2008 data, conductivity, pH, and water depth were seen to potentially influence clustering on the PCA plots but this effect was not clear (data not shown). In the 2008 and 2009 PCAs, the first two PCs accounted for similar amounts of variance within the data set (25% and 27.7% respectively).

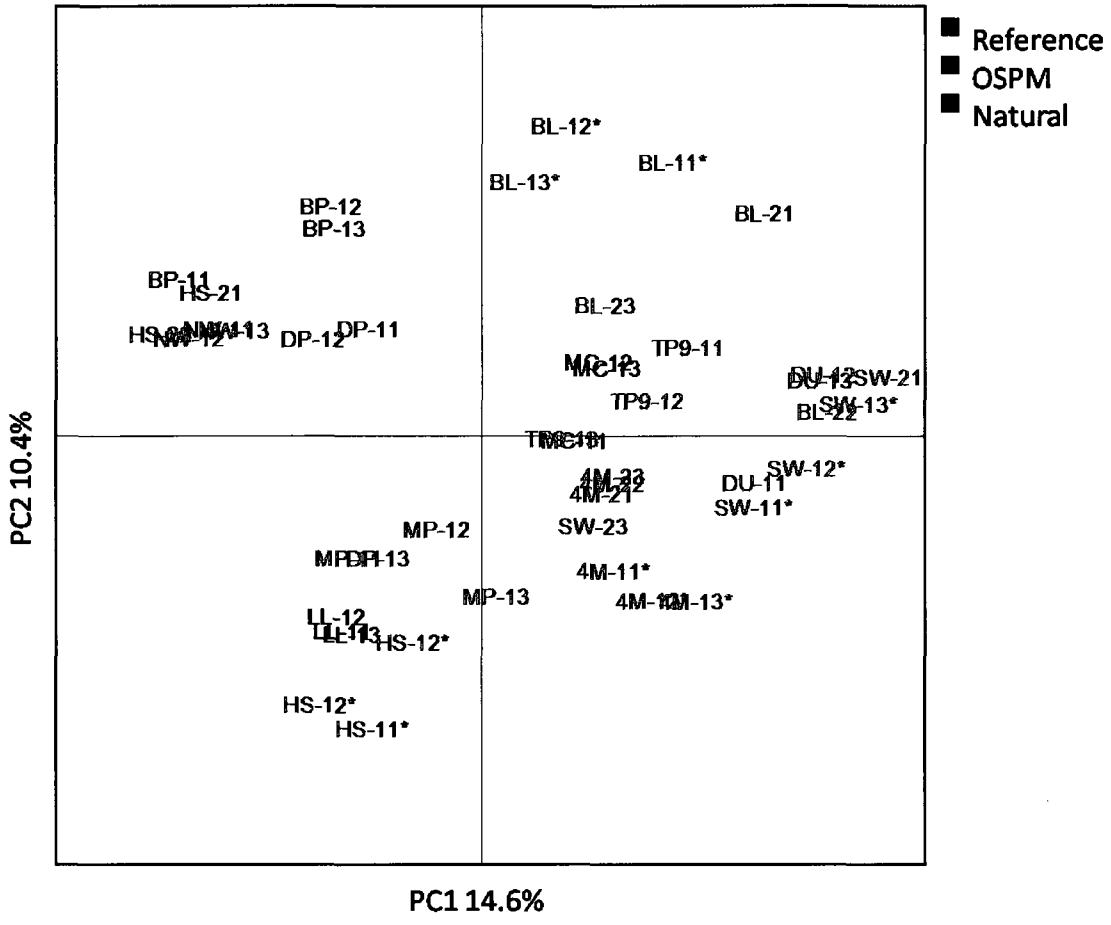


Figure 5.7 PCA plot of the presence/absence of DGGE bands in the total bacterial analysis of 2008 wetland samples. An asterisk (*) indicates the sample is from a vegetated area of the wetland. PC 1 accounts for 14.6% of the variance and PC 2 accounts for 10.4%.

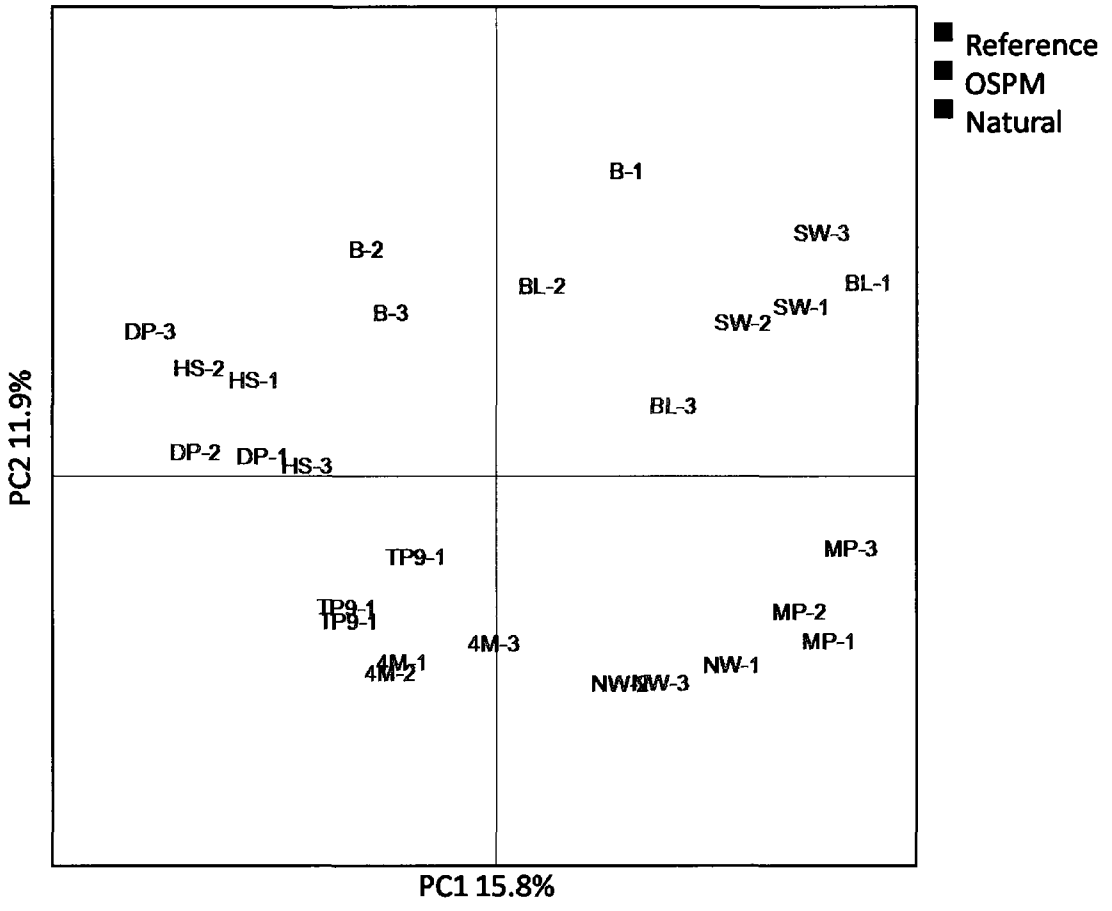


Figure 5.8 PCA plot of the presence/absence of DGGE bands in the total bacterial analysis of 2009 wetland samples. PC 1 accounts for 15.8% of the variance and PC 2 accounts for 11.9%.

5.3.2 DGGE Profiles of the γ-Proteobacteria Subdivison

5.3.2.1 Visual Observations of γ-Proteobacteria DGGE Profiles

Visualization of γ-Proteobacteria DGGE profiles revealed clear banding patterns within a narrow range of the gradient. A high similarity was noted between samples from the same wetland while differences occurred between samples from different wetlands. Also, a low-GC

band that denatured earlier than the fragment in the ladder containing the least GC content was noted in every sample (Figure 5.8).

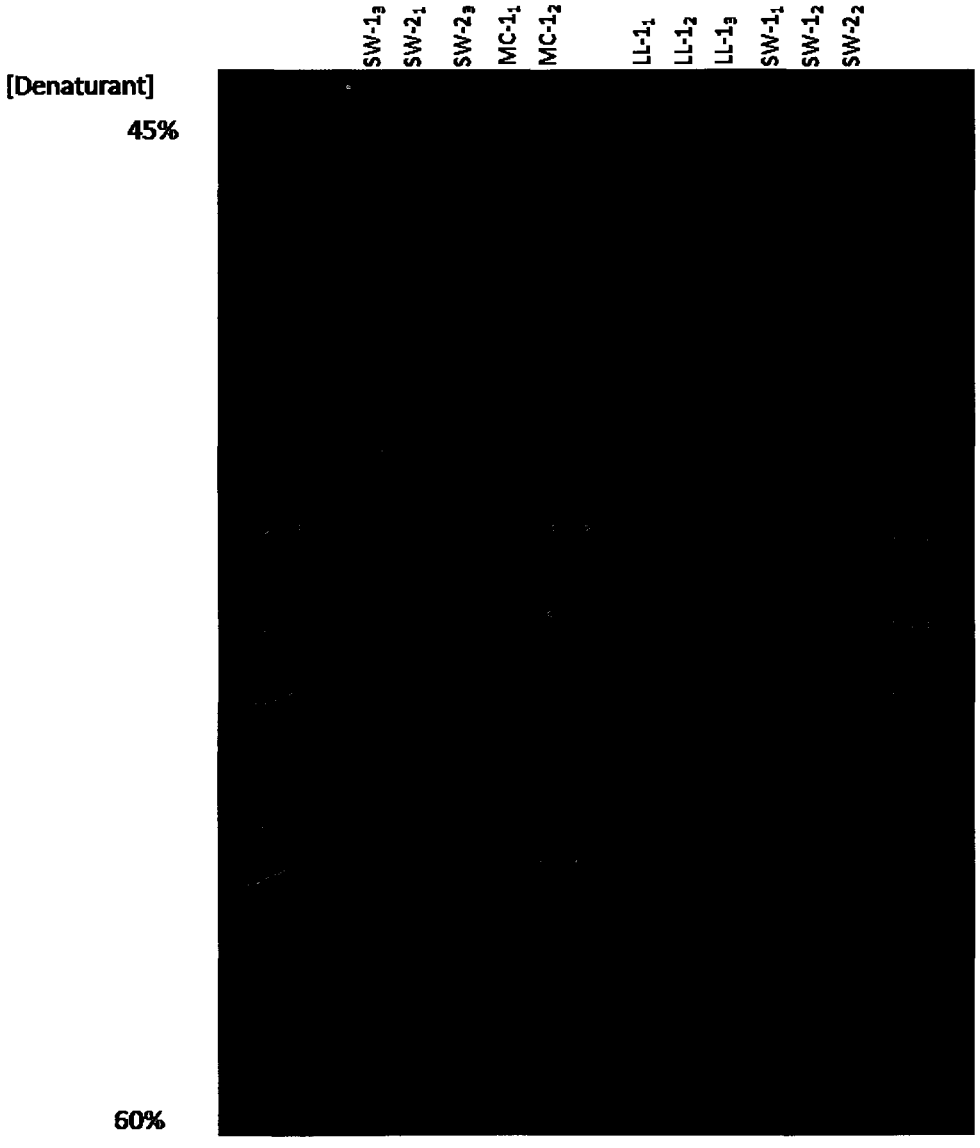


Figure 5.9 Representative banding patterns of 2008 samples amplified with primers specific for the class, γ-Proteobacteria, and separated along an 8% polyacrylamide DGGE with a urea/formamide denaturant of 45-60%. Reference lanes are unlabeled.

5.3.2.2 Number of Bands Counted in γ-Proteobacteria DGGE Profiles

Overall, reference samples contained the greatest range in number of bands while OSPM samples contained the smallest range. Reference samples (specifically, BL and SW) contained the highest number of bands, however, the average number of bands counted in all three wetland types (natural, OSPM, reference) was comparable (8.6±2.2 for natural, 10.6±1.5 for OSPM, and 10.2±4.1 for reference samples). Unlike in the total bacterial analysis, MP contained the smallest number of bands of all OSPM samples (Figure 5.9). No clear trend was identified with respect to the number of bands present and the included physicochemical (e.g. pH, dissolved oxygen) and non-physicochemical (e.g. sampling variables) data.

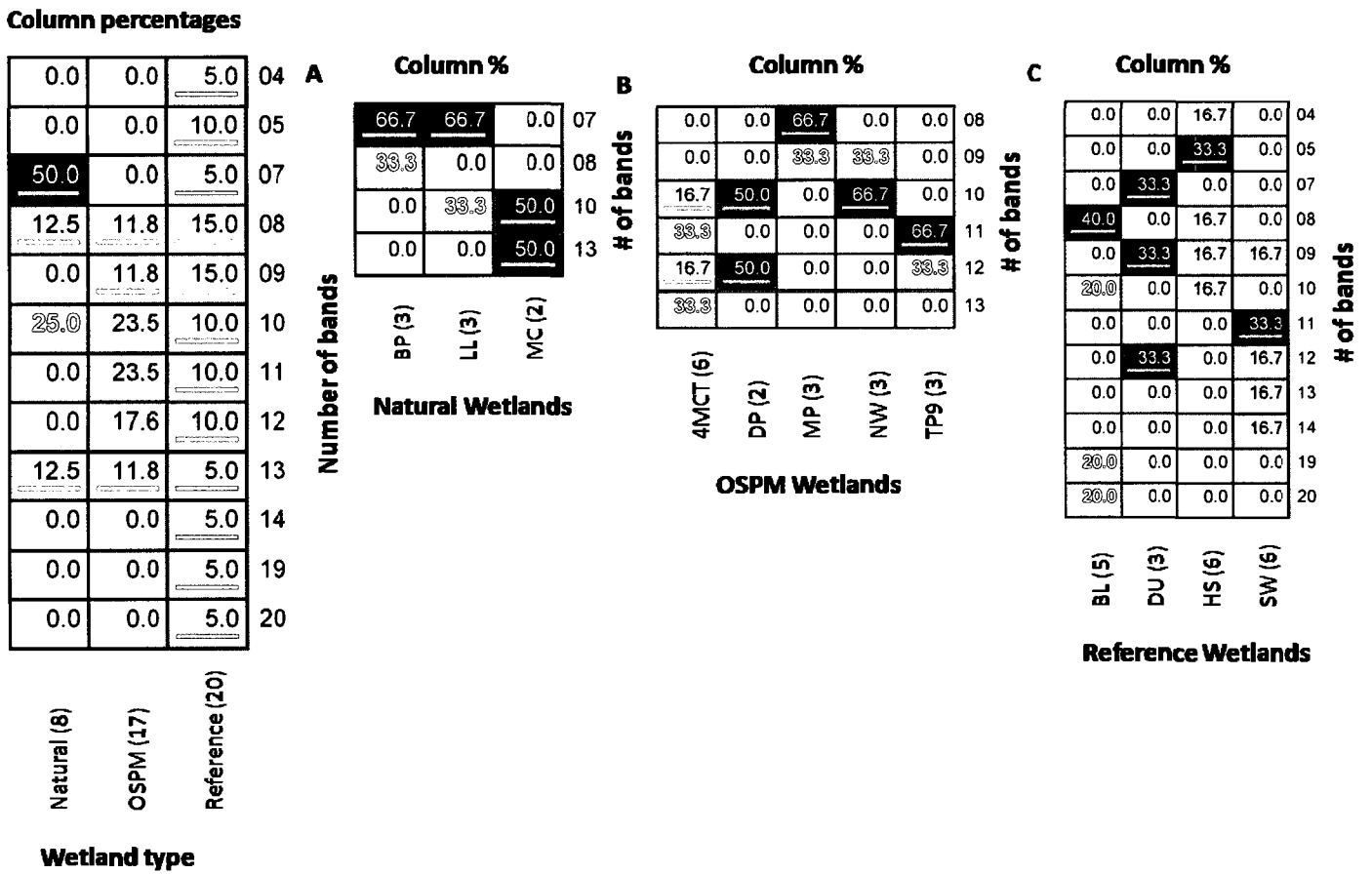


Figure 5.10 Contingency tables illustrating the number of bands counted in lanes of DGGE gels part of γ-Proteobacteria analysis and as a function of wetland type. The table on the left illustrates the percent of samples in each category (wetland type: natural, OSPM, reference) containing "y" number of bands. Tables A, B, and C illustrate the percent of samples in each category (wetland) for (A) natural, (B) OSPM, and (C) reference sites respectively. The number of samples (n) in each category is recorded in brackets.

5.3.2.3 PCA of γ-Proteobacteria DGGE Profiles

An analysis of the γ-Proteobacteria DGGE banding patterns through PCA revealed grouping effects on the basis of wetland type. Results suggested that members of the γ-Proteobacteria subdivision of Bacteria in natural and reference sites were similar to each other, and distinguishable from populations in OSPM wetlands. No gel-effect was observed to influence this PCA. Of the available physicochemical and non-physicochemical (e.g. sampling variable) data, conductivity, dissolved oxygen, and water depth were observed to potentially influence the observed PCA plot. The first two PCs explain 31.8% of the variance in the data (Figure 5.10).

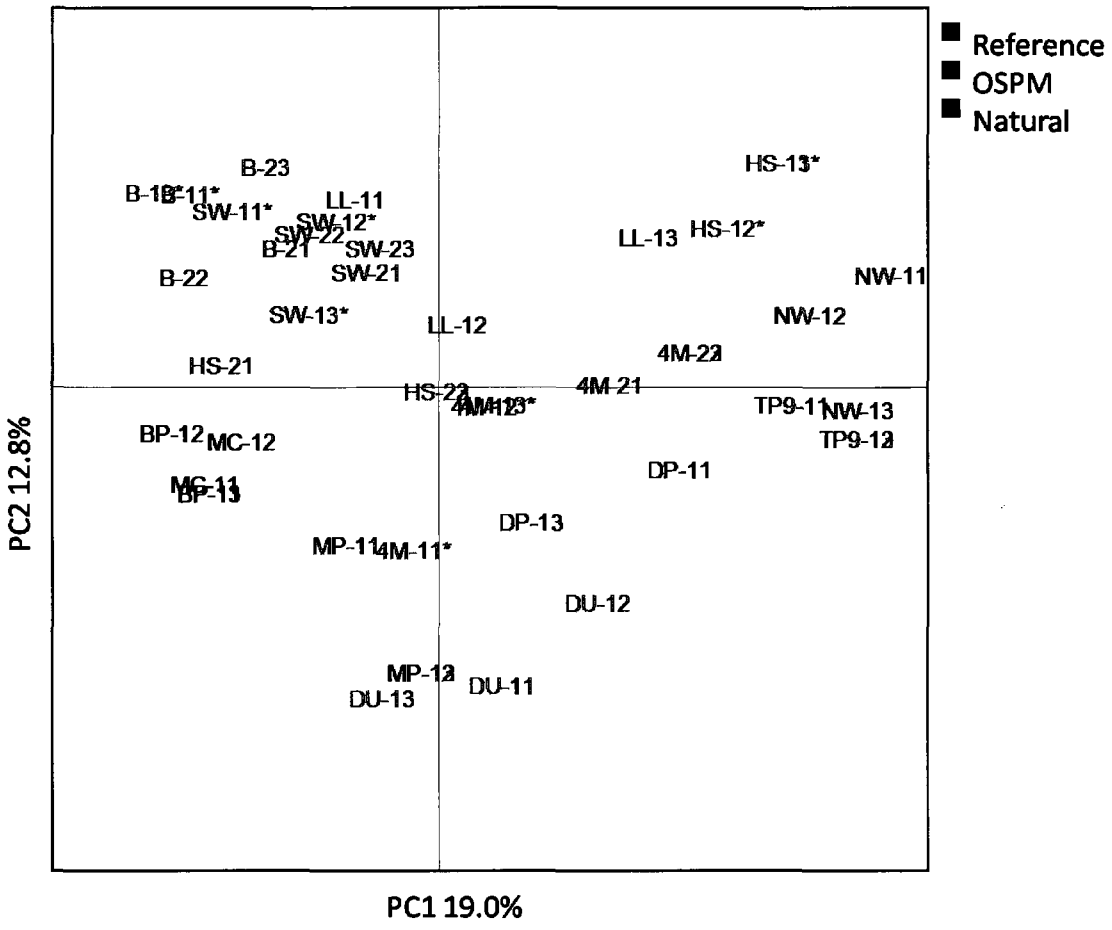


Figure 5.11 PCA plot of the presence/absence of DGGE bands in the analysis of the γ-Proteobacteria subdivision of bacterial communities in 2008 wetland samples. An asterisk (*) indicates the sample is from a vegetated area of the wetland. PC 1 accounts for 19.0% of the variance and PC 2 accounts for 12.8%.

5.3.3 DGGE Profiles of the Actinomycetes Subdivision

5.3.3.1 Visual Observations of Actinomycetes DGGE Profiles

The Actinomycete group-specific PCR-DGGE produced DGGE gels with complex banding patterns over short denaturing conditions. A large number of bands were visualized, and

like previous banding patterns, samples from the same wetland produced highly similar banding patterns while samples from different wetlands were often distinguishable (Figure 5.11).

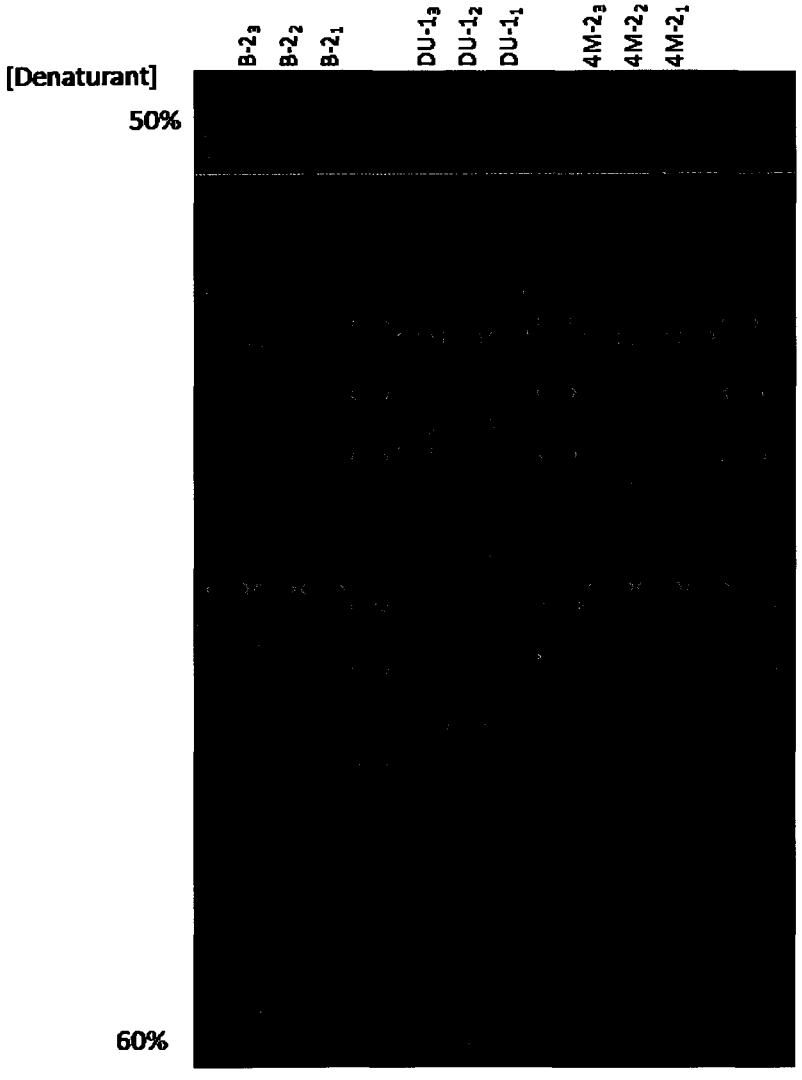


Figure 5.12 Representative banding patterns of 2008 samples amplified with primers specific for the bacterial group, Actinomycetes, and separated along an 8% polyacrylamide DGGE with a urea/formamide denaturant of 50-60%. Reference lanes are unlabeled.

5.3.3.2 Number of Bands Counted in Actinomycetes DGGE Profiles

An analysis of the number of bands confirmed the visual observation of a high number of bands within this bacterial subdivision. Reference samples contained the most variability in the number of bands but there were no consistent differences identified between the number of bands across different sample types (natural, OSPM, reference); samples obtained from natural wetlands contained on average 20.1±3.9 bands, those from OSPM wetlands contained 20±2.7 bands, and those from reference sites contained 21.1±4.7 bands (Figure 5.12). Within particular wetlands, however, samples from vegetated areas of the wetland resulted in banding patterns with a higher average number of bands than samples from non-vegetated areas although much variation was noted (data not shown). No other trends were identified in the various biological and physicochemical data or sampling variables to explain the observed variations in band numbers.

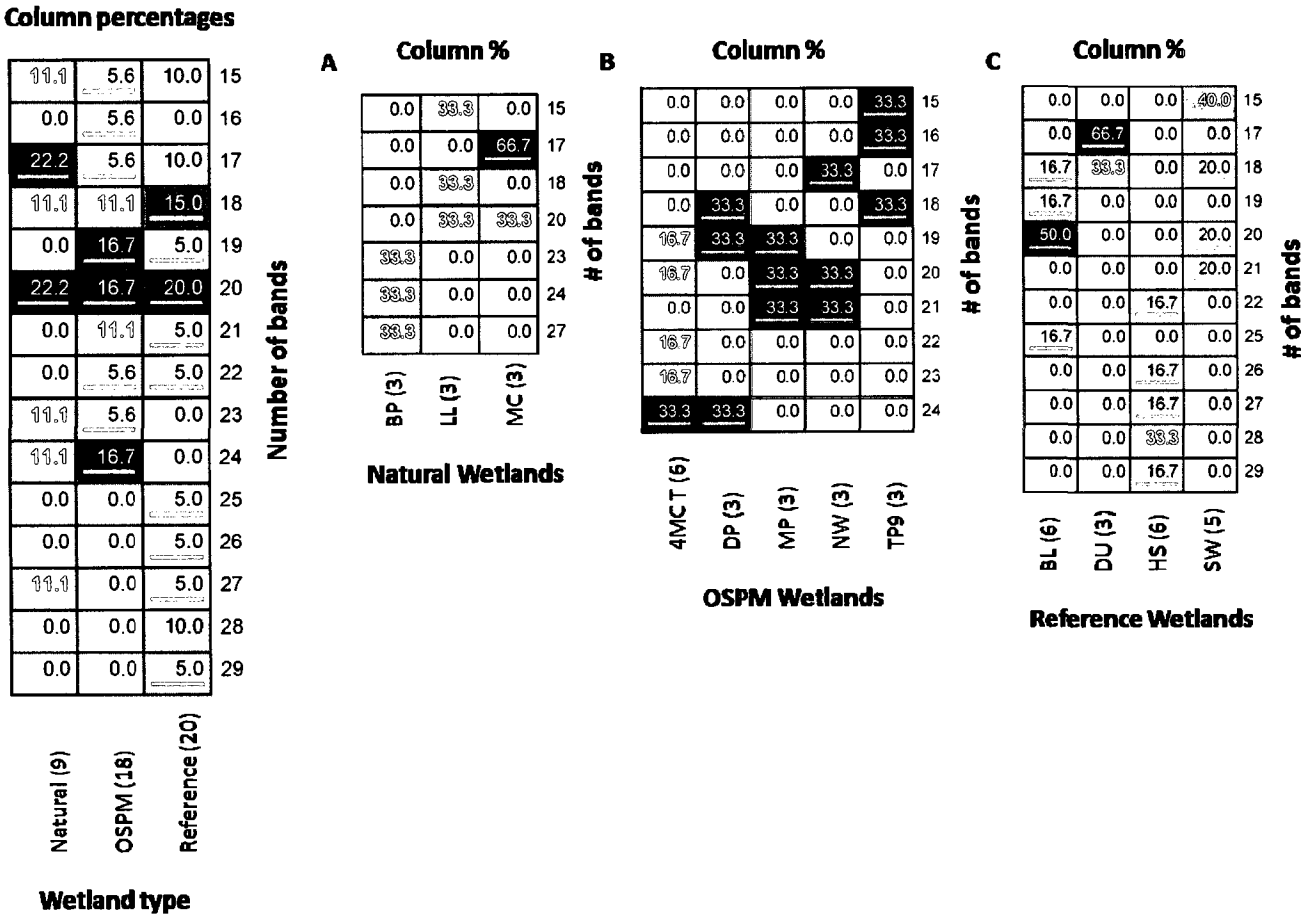


Figure 5.13 Contingency tables illustrating the number of bands counted in lanes of DGGE gels as part of Actinomycetes analysis and as a function of wetland type. The table on the left illustrates the percent of samples in each category (wetland type: natural, OSPM, reference) containing "y" number of bands. Tables A, B, and C illustrate the percent of samples in each category (wetland) for (A) natural, (B) OSPM, and (C) reference sites respectively. The number of samples (n) in each category is recorded in brackets.

5.3.3.3 PCA of Actinomycetes DGGE Profiles

As shown in Figure 5.13, PCA of Actinomycetes DGGE banding profiles revealed grouping effects on the basis of impact from oil sands materials. Actinomycetes populations from OSPM sites grouped strongly while reference and natural sites were more variable. Populations from OSPM and less impacted sites were quite distinct although some overlap between OSPM, reference, and natural sites was evident (e.g. DP, MP, LL). When samples were considered in terms of the available physicochemical and non-physicochemical data, only conductivity and water depth were identified as potential factors influencing the observed clusters. In total PC 1 and PC 2 accounted for 26.5% of the variance in the data set.

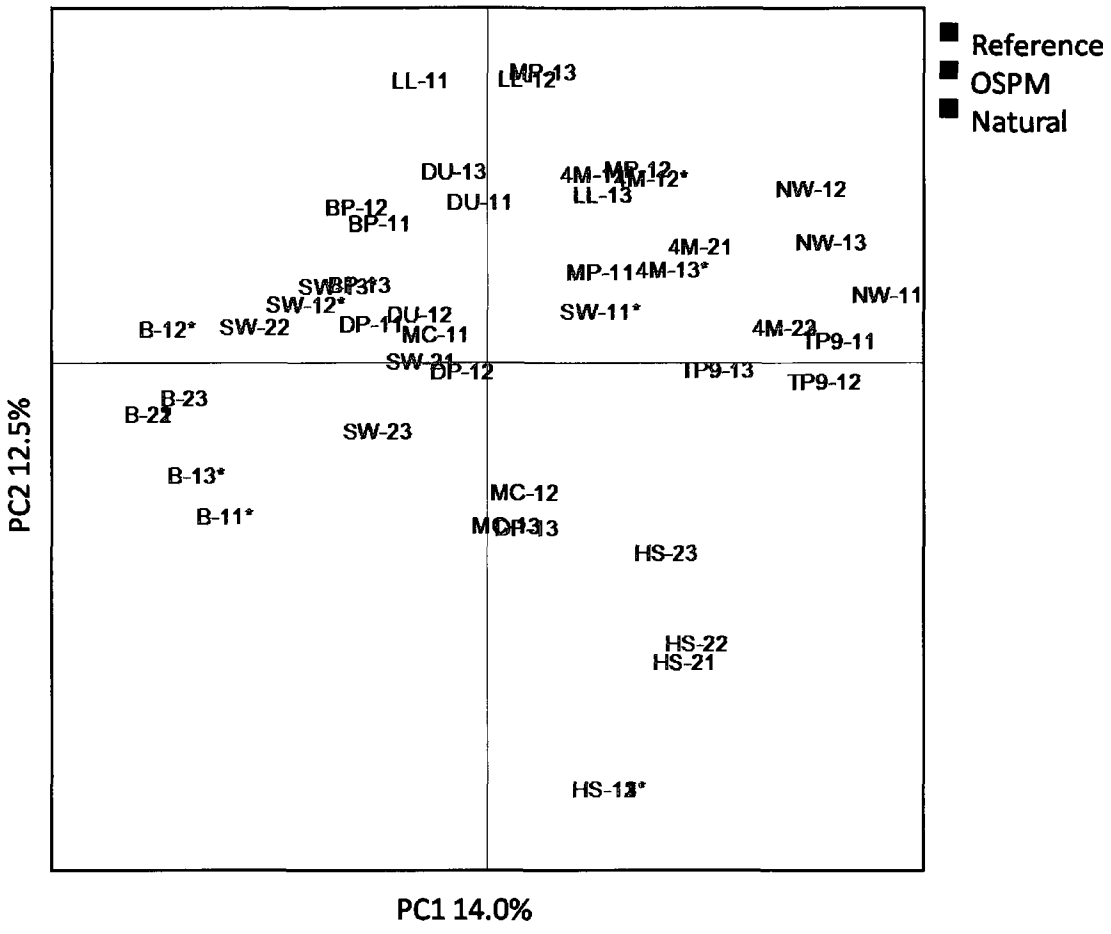


Figure 5.14 PCA plot of the presence/absence of DGGE bands in the analysis of the Actinomycetes subdivision of bacterial communities in 2008 wetland samples. An asterisk (*) indicates the sample is from a vegetated area of the wetland. PC 1 accounts for 14.0% of the variance and PC 2 accounts for 12.5%.

5.4 Discussion

5.4.1 Bacterial Genetic Diversity

It is possible to use DGGE banding patterns to obtain a rough estimate of microbial diversity. Asakawa *et al.* (2008), calculated the richness, diversity, evenness, and stability of bacterial communities in paddy field ecosystems based on the number and relative intensity of DGGE bands. Another group calculated the number of operational taxonomic units (OTU), or different band positions, within various sample types (Leflaive *et al.*, 2008). In this study, a simplified approach was taken whereby the total number of bands within each banding pattern was counted. Every band can loosely be defined as a unique OTU, or phylotype, within that lane. A summary of the findings regarding the number of OTUs in DGGE analyses is included in Table 5.3.

Table 5.3 Summary of DGGE diversity measures for the total bacterial community analysis, and the group-specific analyses of γ-Proteobacteria and Actinomycetes

		Average # OTU[a]	Range # OTU	Patterns of Diversity (# OTU)
Total bacterial community (2008)	**Overall**	**19.3±5.1**	**11-34**	• OSPM > reference or natural • LL highest # OTU among natural • MP highest # OTU among OSPM • HS highest # OTU among reference
	Natural	17.1±4.4	11-26	
	OSPM	22±4.5	13-34	
	Reference	17.7±4.9	11-24	
Total bacterial community (2009)	**Overall**	**20.0±4.8**	**10-32**	• OSPM > reference or natural • MP highest # OTU among OSPM
	Natural	20.5±0.7	20-21	
	OSPM	22.4±5.1	10-32	
	Reference	16.4±1.8	14-19	
γ-Proteobacteria	**Overall**	**10.1±3.1**	**4-20**	• similar # OTU in OSPM, reference, natural • MP lowest # OTU among OSPM • HS lowest # OTU among reference
	Natural	8.6±2.2	7-13	
	OSPM	10.6±1.5	8-13	
	Reference	10.2±4.1	5-20	
Actinomycetes	**Overall**	**20.5±3.8**	**15-29**	• similar # OTU in OSPM, reference, natural • 4MCT highest # OTU among OSPM • HS highest # OTU among reference
	Natural	20.1±3.9	15-27	
	OSPM	20.0±2.7	15-24	
	Reference	21.1±4.7	15-29	

[a] (average ± standard deviation)

PCR-DGGE of the total bacterial community of 2008 and 2009 samples revealed, on average, a greater number of OTUs in samples classified as OSPM than in those classified as reference or natural. A number of explanations might account for this result. A lack of predators in more highly contaminated sites might result in an increase in the number of bacteria. It is well known, for example, that grazing by bacteriophagous microfauna reduces bacterial cell numbers (e.g. Rønn *et al.*, 2002). Additional support for this hypothesis comes from a recent thesis in which A. Legg found that testate amoebae, a group of protozoa known to prey upon bacteria, are less prevalent in OSPM sites (Legg, 2009). It should also be considered that the observed increase in the number of OTUs in OSPM sites may not reflect the true bacterial diversity of the

community. It is generally accepted that PCR-DGGE allows for the resolution of only dominant (>1%) members of the microbial community (e.g. Muyzer *et al.*, 1993), and that dominant species can create intense bands that hinder the visualization of bands of less dominant organisms (Nicomrat *et al.*, 2006). Further, the co-migration of DNA from closely related species (Nicomrat *et al.*, 2006) and the presence of multiple rRNA gene operons in the some bacteria (Amann *et al.*, 1995) complicates our ability to reliably interpret banding patterns as a function of true diversity.

Another finding consistent in the 2008 and 2009 sample analysis is that samples from a wetland referred to as Mike's Pond (MP) were found to contain the highest number of OTUs when compared to other OSPM sites. This is interesting because MP is affected by CT process waters (Table A.2) and was characterized by low amounts of vegetation in 2007 and very high measures of conductivity (Appendix A). Further, in a thesis by A. Legg, MP is recorded as containing 0% emergent vegetation in both the 2007 and 2008 field seasons and this site is noted to contain depressed levels of organic content (Legg, 2009). These ecological and physicochemical parameters tend to suggest that MP may be a highly impacted site; for example, the measures of conductivity might reflect the presence of organics such as NA, or salt, which are known to be elevated in CT and process waters (e.g. Fedorak *et al.*, 2003; Leung *et al.*, 2003), and the lack of vegetation could be due to an inability for diverse assemblages of plants to survive on this site. Additional toxicological and physicochemical data would be beneficial in interpreting the apparently high bacterial diversity in this wetland. Based on the available data for this study, however, it is tentatively concluded that diversity assessed using a total bacterial community analysis with DGGE may not be appropriate for predicting wetland health and the originally proposed hypothesis that an increase in bacterial diversity may be an indication of a stable and healthy wetland ecosystem, may not be accurate.

When a group-specific approach was applied, a high number of bands were identified relative to the number of bands counted when a total bacterial approach was taken. In the bacterial class, γ-Proteobacteria, banding patterns revealed a maximum of 20 OTUs (counted in reference sample, SW) (Figure 5.9; Table 5.3) and in the Actinomycete group-specific analysis, DGGE revealed banding patterns containing up to 29 OTUs (Figure 5.12; Table 5.3). In total, this implies there may be at least 49 OTUs that can be attributed to these two bacterial groups although a maximum of 34 bands were identified when a total bacterial approach was used (Table 5.3). Clearly, many of the bands that were revealed when a group-specific approach was used were not counted in profiles obtained from the total bacterial analysis. This may reflect the presence of minority members of the population that went undetected when a total bacterial approach was taken. Group-specific PCR-DGGE approaches, utilizing multiple PCR amplification rounds and more phylogenetically specific primer sets, allow for the detection of less abundant bacterial groups in complex communities (e.g. Dar *et al.*, 2005). Phyllips *et al.* (2000), for example, were able to detect ammonia oxidizers making up a small fraction (≤0.01%) of the total soil bacterial community using a nested PCR approach with specific primers. Further, the surprisingly high number of bands within Actinomycetes profiles suggests that this may be an important bacterial group in wetlands of the Athabasca oil sands region that deserves further attention. Within the Actinomycetes group, a higher number of bands in vegetated samples compared to non-vegetated samples was identified in the same wetland. This trend is similar to that seen when CLPP was applied (Chapter 4) and therefore, provides some support that Actinomycetes could be functionally important members of the bacterial population.

In contrast to when a total bacterial approach was applied, group-specific analyses revealed a similar number of OTUs in OSPM samples compared to reference and natural samples.

This finding could similarly reflect limitations of the total bacterial approach for detecting minority members of the microbial community. It is possible that OSPM samples contain more dominant bacterial members than reference or natural sites thereby accounting for the high diversity of bands seen in the total bacterial DGGE approach. Evidence from the group specific DGGE analyses, however, suggests that an interpretation that OSPM samples contain a higher overall diversity of bacteria than reference or natural sites may not be accurate; if this were true, a higher number of bands might have been expected in the γ-Proteobacteria and Actinomycetes groups as well.

Consistent with the relatively high number of OTUs identified in the three DGGE analyses, visual observations of banding patterns on DGGE gels tended to suggest the presence of complex and diverse bacterial communities in the wetland sediment samples. The visualization of smearing patterns on many of the gels (e.g. Figure 5.3) may be a result of the high diversity present in the samples. This has previously been noted to cause smearing or poorly resolved banding patterns (e.g. Boon *et al.*, 2001). Banding patterns produced with group-specific primer sets seemed to exhibit improved resolution of bands suggesting that, in diverse environments like soil or sediment, group-specific analyses may provide more manageable population sizes to evaluate through this method.

5.4.2 Homogeneity among Replicates

In all PCR-DGGE analyses, clear visual similarities in the banding patterns of samples from the same wetland were noted. Consistent with this observation, triplicate samples from the same wetland nearly always clustered together in UPGMA clustering analyses as well as when

assessed through PCA (e.g. Figure 5.6). This implies that the bacterial communities present in wetlands in the Athabasca region are quite homogenous over a distance of a few meters. This finding is consistent with that reported by Hadwin *et al.* (2006) who found that replicate samples from the same Athabasca wetland, sampled at 3 meters distance, were highly similar in DGGE banding patterns. This group noted that banding patterns of triplicate samples were identical in band presence and position in 19 of the 24 wetlands sampled. Other literature, however, is variable with regards to the homogeneity of bacterial communities over short distances. As cited by Hadwin *et al.* (2006), for example, one group using T-RFLP to study denitrifying microbial communities in marine sediments found variability even within samples from the same centimeter-wide sampling core (Scala and Kerkhof, 2000). Other researchers have been unable to detect differences in bacterial communities between sample types using DGGE, but could detect differences using other methods such as phospholipid fatty acid analysis (PLFA) (e.g. Leckie *et al.*, 2004). Therefore, it is possible that more variability may be present within common areas in Athabasca wetland sediments than is detectable with this method.

Where six samples were taken from the same wetland, but from different sites in that wetland (one site with emergent vegetation and one lacking emergent vegetation), often the three replicates from each of the two sites (vegetated versus non-vegetated) grouped separately (e.g. Figure 5.6). This suggests that vegetation may influence the genetic microbial community structure. Differences in the functional microbial community profiles of vegetated versus non-vegetated communities were also noted in Chapter 4 (CLPP of Bacterial Communities in Wetlands of the Athabasca Oil Sands Region). This is not surprising since plants provide important sources of organic substrates for bacterial metabolism (e.g. Webster and Benfield, 1986) and may therefore, encourage the growth of different bacterial species.

The reproducibility of banding patterns was also assessed when samples were taken for DNA extraction from different locations in the sampling container and/or at different time intervals over a period of months. Banding patterns remained highly similar despite variations in the DNA extraction over time or space (Figure 5.1, 5.2). In addition to supporting the abovementioned discussion that bacterial communities appear highly similar over short distances within common areas of the same wetland, this study revealed that the bacterial communities in these samples are fairly stable even when subject to prolonged storage at 4 °C. However, some changes, particularly in the intensity of bands, were visualized in samples as DNA extractions were delayed between refrigerator storage. Changes in the bacterial communities that have been stored over time might reflect the gradual shift towards loss of cold-sensitive organisms and dominance of cold-tolerant ones.

Although the reliability of inter-gel comparisons was not specifically evaluated due to time constraints, it was found to be potentially important. In a number of analyses completed on separate days, when replicate PCR products were run on different gels, the samples did not cluster as closely as expected (see Figure 5.1). This is likely a result of imperfect gel normalization in the analysis and variability in the gradients formed in different gels, as discussed previously by others (e.g. Neufeld and Mohn, 2005). Some researchers have addressed the issue of using multiple gels in DGGE analyses and proposed solutions. Neufeld and Mohn (2005), for example, found that the use of fluorophore-labeled primers as intra-lane standards improved normalization both within and between gels. Unfortunately, as noted by this group, this requires an expensive laser-scanning instrument that may limit its application. It is apparent that a consensus is needed for the most effective way to deal with multiple gel comparisons since often, as in this study, multiple gels cannot be avoided.

5.4.3 Clustering Effects observed through PCA

Using PCA, samples were compared as a function of various characteristics including wetland type (OSPM, reference, natural), and various physicochemical (e.g. vegetation, pH, etc.) and non-physicochemical (e.g. sampling date, date of DGGE gel, etc.) parameters. A summary of many of the visualized clustering effects that will be discussed below are included in Table 5.4.

Table 5.4 Summary of the observed clustering effects seen when DGGE data for total bacterial community analysis, and group-specific analyses of γ-Proteobacteria and Actinomycetes, were analyzed using PCA

DGGE Analysis	PCA clustering effects
Total bacterial community (2008)	• moderate clustering effect on the basis of wetland type but overlap between groups is noted • TP9 and 4M samples group well within OSPM cluster • HS samples somewhat distinct from reference cluster • no clear clustering of natural sites • conductivity and water depth may influence clusters
Total bacterial community (2009)	• more pronounced clustering effect on the basis of wetland type than 2008 • some overlap between reference and OSPM sites • TP9 and 4M samples group well within OSPM cluster • HS somewhat distinct from reference cluster
γ-Proteobacteria	• clustering on the basis of wetland type but overlap between groups is noted • MP and 4M-11* somewhat distinct from OSPM cluster • HS and DU samples somewhat distinct from reference/natural cluster • reference/natural sites cluster strongly while OSPM samples are more diverse • conductivity, dissolved oxygen, and water depth may influence clusters
Actinomycetes	• clustering on the basis of wetland type but overlap between groups is noted • DP somewhat distinct from OSPM cluster • HS samples distinct from reference/natural cluster • OSPM sites cluster more strongly than reference/natural sites • conductivity and water depth may influence clusters

Clustering on the basis of wetland type was observed when PCA was used to analyze the banding patterns of the total bacterial community (for 2008 and 2009 samples), and γ-Proteobacteria and Actinomycetes subsets of the community. These results strongly suggest that there are genetic differences in the bacterial communities in impacted and less, or non-impacted wetlands. It is expected that the cause for these differences might be attributable to oil sands-associated materials but it is difficult to speculate the precise causes for the observed clusters. These results support the findings of Hadwin *et al.* (2006) who similarly found that DGGE could distinguish impacted from non-impacted sites.

The finding that there are genetically distinguishable γ-Proteobacteria populations in impacted and non-impacted sites is not surprising. It is expected that there are selective pressures within impacted wetlands that encourage the growth of bacteria that are capable of degrading environmental toxicants present in OSPM and members of this group are well known to play key roles in the degradation of contaminants. *Pseudomonas* species which fall within the γ-Proteobacteria subdivision, for example, can synthesize an unusually large number of enzymes and metabolize a variety of substrates and, as a result, are important in decomposing uncommon chemicals (Tortora *et al.*, 2004). In the oil sands region, *Pseudomonas* spp. have been found to be important in the degradation of NA; for example, *Pseudomonas putida* and *Pseudomonas fluorescens* were isolated from an active tailings pond using NA as the sole carbon source and this co-culture was found to remove >95% of commercial NAs (Del Rio *et al.*, 2006).

No studies to date have specifically addressed Actinomycetes within wetlands of the Athabasca region which makes it difficult to speculate why PCA applied to the DGGE profiles of this group produced the most clearly discernable grouping effects on the basis of wetland type (Figure 5.13). Actinomycetes are believed to be the most abundant group of bacteria in soils and

key players in biodegradation and the decomposition of organic materials (Liu *et al.*, 2006). They have filamentous structures with hydrophobic cell surfaces that are thought to support adherence and stabilization of interfaces; for example, they promote sludge flocculation in activated wastewater treatment plants (e.g. Lemmer *et al.*, 2000, as cited by Boon *et al.*, 2001). It is possible that the high diversity of this group, and the clear differences observed in this group in impacted and non-impacted sites, reflects the capabilities of members of this group to carry out, as of yet, unidentified processes involving unique carbon and energy sources present in impacted wetlands. Alternatively, perhaps Actinomycete species play a role similar to that documented in water treatment plants (Lemmer *et al.*, 2000, as cited by Boon *et al.*, 2001) by promoting the stabilization and separation of oil sands material from overlying fresh water, an important step in the ultimate reclamation of these sites. Although the precise role of Actinomycetes in Athabasca wetlands is unknown, it is interesting to note that using phospholipid fatty acid analysis (PLFA) Hadwin *et al.* (2006) found that fatty acid signatures characteristic of Actinomycetes increased in impacted sites in August of 2000 at the same time that an increase in stationary-phase microorganisms and a decrease in microbial NA metabolism was detected. Interpretation on the basis of this finding is difficult because this group monitored a relative change in the number of Actinomycetes which was not possible in this study. Nevertheless, the finding that Actinomycetes were identified as potentially important groups in both this study and that of Hadwin *et al.* (2006) is interesting given the almost 10-year age difference in wetlands between these studies. This might imply that Actinomycetes is an important and reliable group that could be useful for monitoring purposes.

Overall, despite similarity in sediment bacterial communities in impacted wetlands, it is obvious that communities were not identical. Samples from wetlands Mike's Pond (MP), and

Demo Pond (DP), for example, were often removed from the central cluster of oil sands-impacted wetland samples. Variability within OSPM bacterial communities was also observed by Hadwin *et al.* (2006) and this group suggested that the vegetation type, the size of the wetland, and the order in which colonizing microbes arrive in the wetland could all influence the observed variation within impacted sites. These factors may be important contributors to the observed variability seen in this study as well. In addition, location of wetlands with respect to mining activities and roads, and sediment characteristics, were not addressed in this study and could be important. It would be useful to compare clustering more precisely as a function of the degree of impact by OSPM rather than simply the presence or absence of impact. Data regarding NA or PAH concentration may provide further insights into the causes for the observed clusters and to the variation within wetland categories. For example, it may help to determine whether the degree of impact, or type of impact (e.g. process waters or CT) could predict clustering effects seen within the OSPM group. Also, the question of whether certain reference sites have a tendency to cluster with OSPM sites because of increased impact from oil sands materials (e.g. through dyke seepages), could be better addressed. The lack of complete distinction between OSPM-affected and less, or non-impacted sites could also reflect the age of the wetlands studied; the reference and OSPM sites ranged in age from 11 to 24 years in 2008. Over the 11 or more years since direct impact by OSPM, microbial communities may have adapted to the oil sands materials and populations with the ability to degrade components of OSPM (e.g. NA) may have altered wetlands so they were more conducive to microbial community establishment. In support of this idea, the same general trends (Table 5.3-5.4) were observed for both 2008 and 2009 samples suggesting that bacterial communities within these sites may have now reached a relatively stable state. It is expected that more distinct clustering effects on the basis of site type would have been

observed in wetlands more recently exposed to OSPM. The observed variability both within impacted sites as well as within reference/natural sites in which samples from HS, for example, were often seen as distinct, also raises the issue of choosing appropriate study sites to serve as a basis for comparison.

In this study, visual analysis of PCA plots revealed that certain physicochemical parameters, in particular, conductivity, dissolved oxygen and water depth, may influence some of the variation in the ultimate bacterial community structure. Conductivity in particular was consistently identified as a potentially important parameter. For example, on close examination of the PCA plot of Actinomycetes samples (Figure 5.13), it is clear that samples from wetlands characterized by a high conductivity (>1000) (see Appendix A) cluster to the right of the vertical axis while those from wetlands with a lower conductivity (<1000) cluster to the left of the axis. Further, within reference samples, bacterial communities in the samples from wetland HS often appear distinct from the typical reference and natural clusters. HS is characterized by the highest measure of conductivity of all reference sites, a high measure of dissolved oxygen (Table A.2), and it is potentially impacted by oil sands materials through dyke seepages (Table A.3). These unique variables might help to explain why communities in these samples are seen to group more closely with some OSPM samples compared to reference or natural ones. Additional data analysis would be needed to determine if these visual observations are statistically valid; a canonical correspondence analysis (CCA) could be used to explain the structure of DGGE profiles in terms of quantitative environmental descriptors (Fromin *et al.*, 2002). This analysis was not performed in this case because of time limitations and because the appropriate software was not immediately available. The suggestion that conductivity may be an important physicochemical parameter in shaping the genetic structure of bacterial communities, however, tends to be supported by Legg

(2009) who found conductivity to have a highly significant impact on testate amoebae assemblages in Athabasca wetlands. Also, conductivity in general, seems to be related to impact by oil sands materials; OSPM samples obtained in 2008 were recorded as having significantly higher measures of conductivity than most natural and reference sites. The association between high conductivity and oil sands impact makes sense when the presence of organics such NA, sulfate, and salt, which are all known to be significantly elevated in impacted sites is considered (e.g. Fedorak *et al.*, 2003; Leung *et al.*, 2003; Del Rio *et al.*, 2006).

Although PCA could distinguish OSPM impacted sites from natural and reference sites in all three of the completed analyses, the group specific analyses appeared more effective at producing clear DGGE banding patterns and, in turn, more easily interpretable plots. This is likely attributed to the reduced diversity within subgroups compared to the total bacterial community. While the group-specific approach may be superior to a total bacterial analysis for complex microbial communities in Athabasca wetland, there are a few important considerations. As mentioned by Boon and colleagues (2001), primer F243 (used in this study) may not be ideal for all studies of Actinomycetes; it fails to match the 16S rRNA gene of some Actinomycetes while matching the 16S rDNA of some non-target organisms (Heuer *et al.*, 1997). Also, bands identified in group-specific profiles were not sequenced, and it is unknown whether all bands represent actual members of the targeted groups. In support of the applied primer sets, Boon *et al.* (2001) completed sequence analysis of three randomly chosen bands from Actinomycetes profiles and confirmed that the bands represented members of the Actinomycetes group. Additionally, Mühling *et al.* (2008) found that their proposed γ-Proteobacteria primers were highly specific for the target group. Positive and negative controls appropriate for both groups were also included in

PCR reactions when possible and amplification results were always consistent with the primer set used.

5.5 Summary and Conclusions

DGGE analysis was successfully applied to monitor the bacterial communities in a range of wetlands in the Athabasca region. A high degree of similarity among replicate samples from a few meters distance was noted, suggesting homogeneity of bacterial communities within short distances in these wetlands. Triplicate samples from two different locations (vegetated versus non-vegetated) within a particular wetland, however, did not always cluster suggesting that vegetation may exert a strong influence on the ultimate bacterial genetic profile.

When the number of bands was counted in DGGE banding profiles generated with universal bacterial primers, a higher number of OTUs was detected, on average, in OSPM samples compared to natural or reference sites. A number of explanations may account for this finding including: (1) difficulties associated with using DGGE to detect minority members of the community; (2) a lack of predation in more impacted sites; and (3) the presence of toxicological stressors that result in more dominant members of bacteria within impacted sites. When a group-specific approach was applied, a similar number of OTUs were identified in samples classified as OSPM, reference, and natural providing some support for the first explanation regarding minority members of the microbial community.

PCA analysis of DGGE banding patterns revealed grouping effects as a function of wetland type which strongly suggests that there are genetic differences in the bacterial communities in impacted and less, or non-impacted wetlands. Further, this supports results of

Hadwin *et al.* (2006) who similarly found that DGGE could distinguish between impacted and non-impacted communities. Given its ability to distinguish between site type, DGGE may be a useful method for monitoring the bacterial populations in wetlands of the Athabasca region as reclamation proceeds. Clear banding patterns and PCA plots were more effectively achieved when group-specific approaches were applied. In particular, the OSPM samples used in this study appeared to have a relatively consistent Actinomycetes population while members of the γ-Proteobacteria population were more varied in these systems. Despite clustering effects based on site-type when a total bacterial DGGE approach and group-specific approach were applied, it was clear that groups were not completely distinct. This lack of distinction could be due to the age of the wetlands examined and represent microbial adaptation to oil sands materials.

Future research should consider a group-specific approach for studying microbial communities in sediments from wetlands of the Athabasca region in which the bacterial communities are complex. Studies aimed at identifying the functional role of specific groups identified as potentially important, such as Actinomycetes populations, may be useful. An analysis of younger, more recently impacted wetlands is also strongly advised. Finally, more in-depth comparisons of bacterial community structure in relation to physicochemical parameters (e.g. conductivity), and toxicological parameters (e.g. degree of impact by NA or PAHs) may provide further insights into the causes for the observed clustering effects and the observed variability within wetland types.

Chapter 6
General Discussion

6.1 Summary and Implications of Functional and Genetic Approaches

Bacterial communities were assessed through the use of two complementary yet distinct community profiling methods: CLPP with BIOLOG™ EcoPlates was used to compare the metabolic diversity and function of heterotrophic microbial populations while DGGE was used to assess changes in species composition using the 16S rRNA gene. Using multiple methods in microbial community studies is important given the limitations and potential biases imposed by various approaches (e.g. see Kirk *et al.*, 2004). In this section, results for both approaches will be summarized and followed by an overall interpretation of bacterial communities in Athabasca wetlands as assessed through the synthesis of results from both techniques.

6.1.1 Bacterial Communities in Wetlands of the Athabasca Oil Sands Region

Multivariate analyses of CLPP and DGGE results strongly suggested that there were differences in the bacterial community function and structure in impacted and non-impacted wetlands, although variation within and between groups was noticed. More specifically, CLPP revealed a trend in which microbial communities from OSPM wetlands were most similar to each other followed by communities in natural sites, and that communities from reference wetlands were variable. Substrate-related diversity indices applied to CLPP revealed a vegetation effect whereby microbial communities from vegetated zones of a particular wetland were functionally richer and more diverse than communities from non-vegetated areas of the same wetland. By applying DGGE using universal bacterial primers to target bacterial 16S rDNA, samples from

OSPM wetlands were found to contain, on average, a higher number of operational taxonomic units (OTUs) than reference or natural samples, although this observation was not made when group-specific approaches were applied. DGGE results suggested that bacterial communities are fairly homogenous over a distance of a few meters in wetlands; in almost all cases, replicates from a common area of the same wetland clustered together, but not always when samples were taken from two areas of a wetland that differ in terms of presence or absence of emergent vegetation. By applying PCA to DGGE banding profiles, clear genetic differences were noted between impacted and non-impacted wetlands. Additionally, certain physicochemical variables (e.g. conductivity, water depth, and dissolved oxygen) were identified as potentially important in shaping the bacterial community structure in these sites. Group-specific DGGE approaches were more effective than the total bacterial approach in producing clear banding patterns and more easily interpretable PCA plots. Notably, γ-Proteobacteria populations were found to be varied within OSPM samples while Actinomycetes populations were more similar within impacted sites. A summary of trends related to the relative variability in the three wetland groups, as identified through both CLPP and DGGE, is provided in Table 6.1. In this table, the interpretation of variability is based on cluster analyses (CLPP) and/or multivariate analyses (PCA) (CLPP, DGGE) and refers to how strongly certain groups were seen to cluster relative to other groups.

Although both techniques could discriminate OSPM from natural and reference sites, many observations made through one technique were not clearly complemented by the other. This is not entirely unexpected since the techniques analyze different aspects of the microbial community and each is associated with its own advantages and disadvantages. For example, the application of BIOLOG™ detects only metabolically active heterotrophic bacteria and does not reflect the true metabolic diversity *in situ* (Garland and Mills, 1991) but, results generated

through this approach can reflect minority members of the microbial population (Konopka *et al.*, 1997). DGGE by contrast, does not allow the distinction between metabolically active and non-active populations and many minority members may be overlooked (Muyzer *et al.* 1996). He *et al.* (2008) found a strong disconnect between bacterial taxonomic (DGGE) and metabolic (CLPP) diversity and suggested that the lack of correlation might be attributed to bacteria in dormant growth stages or metabolic redundancy within the bacterial population.

Table 6.1 Summary of the relative variability of the bacterial communities in OSPM, reference, and natural wetlands as measured through CLPP and DGGE. The numbers 1-3 are used to summarize the relative variability observed through PCA and/or cluster dendrograms where 1 symbolizes the lowest variability of the three wetland categories (i.e. strong clustering effect) and 3 the highest variability (i.e. weak clustering effect) of the three wetland categories. Where variability appeared similar between groups the same number is used.

Wetland type / Method		OSPM	Reference	Natural
CLPP	Total bacteria	1	3	2
DGGE	Total bacteria	2	2	1
	γ-Proteobacteria	3	2	2
	Actinomycetes	1	3	3

6.1.2 Implications of Bacterial Functional and Genetic Diversity

The finding that both CLPP and DGGE detected differences in the bacterial communities in impacted and non-impacted wetlands strongly suggests that impact by oil sands materials and/or the process of mining oil sands, has a detectable impact on the resulting bacterial community structure and function. The impact by oil sands material or mining appears sufficiently strong to overcome the variability (e.g. differences in the structure of wetlands, water chemistry, sediment characteristics) that is expected when one examines a range of different

wetlands in a natural environment. Clusters separating OSPM from natural and reference sites, as seen through both methods, however, were not completely distinct. Thus, although impact by oil sands mining and materials appears to be an important factor in shaping the overall microbial community profile, other factors or combinations of factors are likely important when observing more specific variations both within and between wetland groups. Also, the overlap seen between many impacted and less, or non-impacted wetlands leads to a few primary interpretations, for example: (1) that impact by OSPM is not sufficiently severe as to cause a complete alteration from a typical natural community structure, or (2) some wetlands classified as natural or reference may have been exposed to oil sands materials and the resulting community structure in these sites reflects adaptation to the associated stressors. Also, it should be emphasized that the age of the wetlands studied were upwards of 11 years since direct impact by oil sands materials. At this point, it is expected that early microbial communities have degraded and detoxified many components of oil sands materials and allowed for more diverse and co-metabolic communities to colonize and adapt to these sites. It is possible that microbial communities in impacted sites have now reached a stable state; support for this comes from DGGE results in which consistent trends were noted in samples collected in 2008 and 2009. Repeated sampling events and bacterial community analysis would help to confirm this trend.

PCA and UPGMA cluster analysis of CLPP data tended to suggest that microbial communities in OSPM sites were the most functionally similar followed by natural sites, and that reference sites were quite functionally diverse. In the DGGE analyses, this same trend is not clearly present. By DGGE, the distinction with respect to natural and reference clusters was not clear and instead, these sites tended to cluster as one although some outliers (e.g. samples from wetland HS) were noted. Perhaps this inconsistency reflects the importance of minority members

(which may not have been detected by DGGE but detected through CLPP) in shaping the functional profile of the microbial community. In the CLPP analysis, the variation in functional profiles in reference wetlands may have been exaggerated by samples noted to be somewhat removed from other samples within the same group in DGGE analyses (e.g. samples from wetland HS). This highlights the importance of carefully selecting study sites for comparison purposes of this kind. It is also possible that some species are functionally redundant and therefore, that a difference in genetic community structure does not necessarily imply a difference in function. The trend in terms of relative variability within groups seen with CLPP is generally supported by results seen with the PCR-DGGE of the Actinomycetes subset of the bacterial population, although no distinction was made between reference and natural sites in the latter approach. Given that Actinomycetes were also found to be abundant within Athabasca wetland samples with an average of 20.5±3.8 OTUs, it can be hypothesized they are a metabolically important group of organisms within these sites and that Actinomycetes may be significant contributors to the observed functional diversity. It should also be noted, however, that the number of samples within each wetland classification (OSPM, reference, natural) was not equal and although an attempt was made to take this into consideration, some bias may be present in the designation of groups as more or less variable as a result.

While originally, it was hypothesized that a greater bacterial diversity may be detected in healthy, non-impacted wetlands, this was not found to be the case in this study. Using CLPP, the only detectable difference in diversity occurred as a function of presence or absence of vegetation in which samples from vegetated areas of a wetland displayed higher measures of richness and diversity than samples from non-vegetated areas within the same wetland. With this method, no differences in diversity were found as a function of wetland type (OSPM, reference, natural).

Using DGGE to estimate genetic diversity, a higher number of OTUs were counted in impacted sites compared to non-impacted sites when a total bacterial approach was taken. Further, the highest number of bands was detected in what was expected to be one of the most highly impacted sites (Mike's pond). This suggests that increased bacterial diversity in these wetlands, in fact, may not be representative of healthy, sustainable systems. It is expected that limitations of the methods applied in this study prevent accurate diversity estimates. Other factors that may complicate the relationship between diversity and wetland health are a lack of predation, or impact by environmental stressors.

Overall, while the functional and molecular methodologies employed did not produce identical results, both results suggest key differences in microbial communities in impacted and non-impacted sites and support previous research (e.g. Del Rio *et al.*, 2004; Hadwin *et al.*, 2006). Microbial communities in wetlands of the Athabasca oil sands region are complex and their structure and function is likely impacted as a result of oil sands materials, mining processes, or a related parameter or combination of parameters. However, communities are not completely distinct in impacted and non-impacted sites and this lack of distinction may be attributed to microbial adaptation to oil sands materials or could imply that the impact is sufficiently extreme as to prevent the re-establishment of communities to more natural states.

6.1.3 Bacterial Community Structure as an Indicator for Wetland Reclamation

This is the second study of its kind to show that DGGE can distinguish between wetland site types in the Athabasca area (see Hadwin *et al.*, 2006) and the first to show that CLPP with BIOLOG™ EcoPlates can similarly distinguish between wetlands that are impacted, and less impacted. Given that both CLPP and DGGE could distinguish between OSPM-impacted and less

impacted or non-impacted sites, both may be useful as tools to monitor the bacterial population structure and function as wetland reclamation proceeds. CLPP with BIOLOG™ EcoPlates, in particular, is a very simple and efficient technique that may be valuable to the oil sands industry. Additional research is needed, however, to confirm that the findings generated in this study are reproducible over multiple sampling events. While PCR-DGGE of the total bacterial community could often distinguish between wetland type, PCA plots were sometimes difficult to interpret and clusters were not clear. Targeting of smaller subsets of the bacterial population through PCR-DGGE proved more effective in identifying differences in OSPM-affected sites and less impacted ones (reference and natural). Therefore, group-specific DGGE may be a more realistic profiling method for monitoring the relatively complex microbial populations present in these systems. In comparison to CLPP with BIOLOG™ microplates, DGGE is a more complex technique requiring specialized equipment and expertise. While agreement between CLPP and DGGE is not perfect, the same dominant trends were observed in this study and their continued use together as indicator tools may be useful.

6.2 Limitations and Future Directions

This study was limited by the availability of samples and the logistics of sampling events; results typically reflect a single "snapshot" of the bacterial communities in each sampling season. As a result, it is difficult to assess the microbial community structure as a function of OSPM impact because the potential variability in community structure over time within sampling seasons is not known. Future studies would benefit from repeated sampling events over a year or more in order to better assess the variation in bacterial community structure over time. Further, the need to ship samples over a period of a few days from Alberta to Ontario resulted in

uncertainties regarding potential changes in the bacterial community structure from its natural state. While studies of bacterial communities in their natural environments are important, it is impossible to account for the total extent of variation present in different sampling locations and over time. Studies in which the goal is to obtain a more precise model of the bacterial community with respect to specific factors would benefit from the application of controlled experimental setups such as through the use of wetland mesocosms. Also, an assessment of younger OSPM-affected wetlands is strongly recommended in order to better understand the evolution of microbial communities in impacted sites over time. If it is not possible to monitor younger wetlands in the Athabasca region, mesocosms to mimic younger OSPM systems could be constructed. For example, through wetland mesocosms it may possible to determine the time required for microbial communities to become well-developed and similar to communities in less impacted sites, and to determine if there is an upper limit of OSPM within wetlands that prevents the re-establishment of bacterial populations to more natural states. Additional biological, physicochemical, and toxicological data specific for the samples used would also have benefited the analysis of results generated in this study. With additional data, more in-depth statistical techniques could then be applied to determine the effect of physicochemical, biological, and toxicological parameters on the bacterial community structure. The inherent methodological limitations associated with the applied techniques are addressed in the respective chapters. While additional research would be beneficial to confirm findings produced through both CLPP and DGGE, it may be particularly important to repeat CLPP with BIOLOG™ given that results from this study and that published by Hadwin *et al.* (2006) are conflicting. It should be noted that different approaches to sample preparation and data analysis were taken in this study compared to that of Hadwin *et al.* (2006). Additional CLPP studies might also benefit from the use of custom

BIOLOG™ MT plates in which carbon sources can be tailored for the specific application to oil sands-affected wetlands. I also recommend that oil sands researchers discuss appropriate baselines of comparison for use in studies such as this given the variability observed both within and between wetland groups.

6.3 Integrative Nature of this Project

Given that this thesis is towards a degree in Integrative Biology, a final discussion of the integrative nature of this project is provided here. Integrative biology is generally defined as a multidisciplinary and transdisciplinary approach to scientific questions, although variations in this definition exist (Wake, 2008). Important principles in integrative biology include: collaboration among experts with diverse expertise, exploring issues from a hierarchical approach (e.g. observation, experimentation, modeling), broadening analyses to multiple levels of biological organization and various taxa, applying multiple techniques, and facilitating integration through research, education, and outreach (Wake, 2003; Wake, 2008). Wake (2008) also notes that integrative biology is most effectively applied to problems and questions that are inherently multidisciplinary which, I believe, is the case in this study where the overall issue is that of wetland reclamation in the oil sands region.

Integration should be evident in this thesis through the use of multiple techniques (CLPP with BIOLOG™ Ecoplates and DGGE) to study bacterial communities from the diverse yet complementary perspectives of both function and genetics. Through DGGE, bacteria as a genetic whole were examined in addition to specific subgroups within this domain. Also, an attempt was made to link information at the level of bacterial communities to other microbial populations and the function of wetland ecosystems as a whole. Based on new information generated through this

study, ideas were ultimately proposed that could assist in the solution of a real environmental issue. Truly thorough integration, however, is difficult to achieve in a short time period. Therefore, I believe the exceptionally integrative nature of this project will result from the application of the results discussed herein to other areas of ongoing research. In particular, this project was completed as part of a collaborative effort to monitor various groups of microbial communities in wetlands of the Athabasca oil sands region with the ultimate goal of developing a useful indicator for application to reclamation. Results from this work will be linked with work regarding testate amoebae and fungi in order to better understand the microbial community structure as a whole in light of wetland health. Finally, communication and collaboration will continue by applying the novel findings produced through this larger-scale project to make recommendations to oil sands developers regarding the re-establishment of wetlands following oil sands impact.

6.4 Conclusions

6.4.1 Major Conclusions

- both CLPP with BIOLOG™ EcoPlates and DGGE could distinguish between wetland type (OSPM, reference, natural) when PCA and/or UPGMA cluster analysis was performed suggesting valid differences in the functional and genetic diversity of bacterial communities in these sites
- the functional diversity of microbial communities in OSPM-affected wetlands is reduced when compared to natural, and especially reference sites, and is expected to be constrained by OSPM-related materials

- vegetation may be an important factor in reclaiming productive ecosystems as seen by higher measures of functional richness and diversity in samples from vegetated areas as measured through CLPP and differences identified through DGGE
- in contrast to the original hypothesis that increased diversity may reflect wetland health, a greater total bacterial diversity was found in impacted wetlands compared to reference or natural sites when DGGE was used; this could be explained by limitations of the approach to obtain a true measurement of diversity, that bacteria may be near the top of the food chain in impacted wetlands, or more dominant bacteria are present in impacted sites
- γ-Proteobacteria populations are different from populations in most reference/natural wetlands and may reflect the selection for degraders (e.g. NA degraders) in impacted sites
- Actinomycetes populations are similar in OSPM wetlands and different from communities in reference/natural wetlands and may play an important role at the sediment-water interface in impacted sites
- CLPP with BIOLOG™ EcoPlates may represent a viable option for easily and efficiently profiling microbial communities in Athabasca wetlands as reclamation proceeds
- a group-specific DGGE approach may be more effective than a total bacterial approach at profiling the 16S rDNA of the relatively complex bacterial communities in Athabasca wetlands and may be a useful tool to monitor microbial populations as reclamation proceeds

6.4.2 Minor Conclusions

- vortex mixing in the presence of sodium pyrophosphate provided an effective means to extract bacterial cells from the wetland sediment samples

- the Powersoil™ DNA Isolation Kit was effective at extracting similar amounts of DNA from Athabasca wetland sediment samples
- the proper treatment of data for interpretation of CLPP results is crucial; data transformations (in particular, the Taylor Power Law transformation) were useful in this study to improve multivariate analysis
- bacterial communities are quite genetically homogenous over a distance of a few meters within wetlands
- some physicochemical parameters (e.g. conductivity, water depth, and dissolved oxygen) were seen as potentially important physicochemical parameters to influence genetic bacterial community structure
- given the variability both within and between wetland groups as measured through CLPP and DGGE, it is clear that a consensus is needed regarding appropriate sites to use as bases of comparison in studies of this kind

Appendix A

Table A.1 Additional Description of Wetlands Sampled in July and August of 2007

Wetland (sample)	Water Depth (cm), State	Identity and Percent Vegetation Cover	Date Sampled (mo/day/ year)	Visual Description
Mike's Pond (MP-1)	16cm	*Potomogeton* 30, bare sediment 70	07/28/07	- light coloured, 1 cm water layer
4 Meter Consolidated Tailings (4MCT-1)	30cm	Water 95, *Scirpus* 5	07/27/07	- wet, 1 cm water layer
Test Pond 9 (TP9-1)	50cm, turbid	*Typha latifolia* 10	07/26/07	- wet
Natural Wetland (NW-1)	6cm	*Potamogeton filiformis* 80, bare sediment 20	07/27/07	- wet with twigs
Demo Pond (DP-1)	50cm, turbid	*Potomogeton* (not filiformis) 90, bare sediment 10	07/26/07	- moist, no water on surface, twigs
Beaver Pond (BP-1)	23cm	*Chara* 40, bare sediment 55	07/28/07	- wet, 1 cm water layer
Bill's Lake (BL-1)	28cm	*Lemna* 10, *Walfin* 50, *Chara* 25, *Typha* 15	07/27/07	- wet with twigs
High Sulfate (HS-1)	26cm	*Chara* 75, Open sediment 25	07/27/07	- frozen when received, wet with vegetation
Shallow Wetland (SW-1)	7cm, clear	*Typha latifolia* 40, *Scirpus*, *Hippuris* 3, *Utricularia* <1, *Lemna* <1, open water 10, *Ceratophyllum* 70	07/26/07	- moist sediment, a lot of vegetation, water layer
(SW-3)	42cm, clear	*Hippuris* 10, *Utricularia* <1, *Potamogeton filiformis* 15, *Sparganium* 29	07/26/07	- dry sediment
South Ditch (SD-1)	48 cm, clear	*Charales* 80, *Potomogeton filiformis*, bare sediment	07/26/07	- mostly water, some sediment
South West Corner Waste Area 11 (SWC-1)	22cm	*Chara/Nitella, Potomogeton, Utricularia*	07/27/07	- dry with vegetation
Small Beaver (SB-1)	33cm	*Carex rostrata* 20, water 70, *Lemna* 4	07/28/07	- mostly water with vegetation

[a] information regarding water depth/state and vegetation was provided by A. Legg

Table A.2 Description of Sediment Samples Collected in Summer of 2008

Sample #	Date Sampled[c] (mo/day/year)	Vegetation[a]	Water Depth (cm)[a]	Conductivity (µs/cm)[a]	pH[a]	% dO[a]	Turbidity[ab]	% dry weight	Visual Description
TP9-1_1	07/10/08	Non	60	1345	6.92	94.4	3	81.05	- very wet, clay-texture, twigs
TP9-1_2								84.11	- same as above
TP9-1_3								80.25	- same as above, small insect
NW-1_1	07/16/08	Non	10	1330	7.8	78	2	77.60	- very wet, sticky, clay-texture
NW-1_2								82.50	- very sticky, clay (?)
NW-1_3								89.14	- dryer than other 2 samples, sticky
DU-1_1	07/16/08	Non	39	971	7.4	97.7	1	75.10	- smell, dark brown, very liquid
DU-1_2								77.68	- same as above
DU-1_3								79.64	- same as above
LL-1_1	07/16/08	Veg	11	835	7.64	104.8	1	74.99	- light brown, very liquid
LL-1_2								79.52	- grey-brown
LL-1_3								76.61	- grey-brown, debris
MC-1_1	07/16/08	Veg	9	433	7.05	96	1	84.29	- small worm alive in sample
MC-1_2								89.25	- chocolate brown, debris
MC-1_3								87.26	- very liquid, debris
SW-1_1	07/10/08	Veg	12	877	7.5	103.9	1	87.82	- lots of vegetation (green), brown
SW-1_2								84.56	- same as above
SW-1_3								80.04	- same as above
SW-2_1	07/10/08	Non	40	877	7.5	103.9	1	74.77	- rust coloured debris, dark brown
SW-2_2								83.77	- light brown, debris
SW-2_3								80.97	- vegetation (?), rust coloured debris
MP-1_1	07/10/08	Non	22	4689	7.97	114	2	89.35	- thick, light brown, debris, smooth
MP-1_2								83.80	- same as above
MP-1_3								87.43	- same as above
4M-1_1	08/10/08	Veg						79.55	- visible oil (<DP), smell, rust coloured debris
4M-1_2								79.58	- same as above
4M- 1_3								74.57	- same as above

4M-2_1	08/10/08	Non	15	2290	7.97	123	3	80.78	- dark brown, very liquid, visible oil
4M-2_2								78.47	- same as above
4M-2_3								80.00	- same as above
DP-1_1	08/10/08	Non	60	2233	7.27	80.4	3	86.30	- visible oil, sticky (clay?)
DP-1_2								80.25	- same as above
DP-1_3								89.45	- same as above
BP-1_1	07/10/08	Non	32	2244	7.3	97.8	2	77.18	- dark brown, rust coloured debris, thick
BP-1_2									
BP-1_3								79.95	- same as above
								80.92	- same as above
BL-1_1	08/10/08	Veg	35	883	6.95	105	3	80.50	- black bottom, liquid top, rust coloured debris
BL-1_2									
BL-1_3								82.48	- same as above
								77.24	- same as above
BL-2_1	08/10/08	Non	65	883	6.95	105	3	83.52	- dark brown, thick, rust coloured debris
BL-2_2									
BL-2_3								81.79	- same as above
								82.04	- same as above
HS-1_1	08/10/08	Veg	32	2998	7.71	160.6	2	75.40	- dark brown, thick, a lot of debris
HS-1_2								79.32	- same as above
HS-1_3								73.63	- same as above
HS-2_1	08/10/08	Non	7	2998	7.71	160.6	2	80.65	- very thick, debris, dark brown
HS-2_2								78.04	- same as above
HS-2_3								80.32	- same as above

[a] information regarding vegetation, water depth, conductivity, pH, dissolved oxygen (dO), and turbidity was provided by A. Legg
[b] turbidity is based on a visual estimate where 1 is the least turbid and 3 is the most turbid
blank= unknown

Table A.3 Description of Wetland sites and Amendments

Wetland	Description/Amendment	Status
Loon Lake	• large pond surrounded by roads; area was disturbed and possibly excavated but never mined; roads were built around the pond in the late 60s; filled with water from precipitation and runoff	Control
MacLean Creek	• formed opportunistically in 2000 when a dam was constructed adjacent to a depression in the boreal peatland; water levels, fed by surface runoff, rose to establish this wetland	Control
Beaver Pond	• natural wetland was (temporarily) affected in 2004 by removal of other upstream beaver dams threatening nearby access road	Control
Duck Pond	• formed opportunistically after the mid-berm road was constructed; was water pooling in Duck Pond in the early 1980s, although the footprint retreated over the years as a result of reclamation activities; reclamation placed top soil and planted trees nearby in 1990	Reference
Shallow Wetland	• one of six Large Scale Test Ponds (LSTP) established in 1993; constructed wetland that ranges between 0.5 to 0.75m deep; originally, a single wetland was constructed to store non-process-affected waters diverted from the West Interceptor Ditch but subsequently, a dividing berm was constructed to take advantage of the very different topography; water levels are supplemented from surface waters collected in and pumped from a sump located along the north berm	Reference
Bill's Lake	• opportunistic wetland, occurred due to the break in slope and depression between the two reclamation sites; this lake is part of the capping depth study and was formed in 1996; 20 to 50 cm of saline overburden at the bottom of the watershed	Reference
High Sulphate	• reclaimed by adding 15 cm of muskeg soil; this wetland developed naturally in a depression in this reclaimed area in 1987; water levels rose in the depression and were fed by precipitation, runoff and possibly seepage from overburden	Reference
Demonstration Pond	• a Large Scale Test Pond constructed in 1993; nine meters of fine tailings overlain by 2.5 meters of diverted local surface stream flow	OSPM
Mike's Pond	• surface area approximately 4ha, contents – CT Release water from 1997 CT Prototype	OSPM
Natural Wetland	• tailings sand w/ 15cm peat-mineral mix cap; opportunistic wetland, fed by runoff (~25%) and dyke seepage (~75%; CT process water was added to the west end in 1996 and 1997	OSPM
Test Pond 9	• one of six LSTP; defined as the Tailings Pond Water Detoxification and consists of 6000m^3 tailings pond surface water that was transferred from the Mildred Lake Settling Basin (MLSB)	OSPM
4MCT	• 4 m, uncapped CT, constructed over overburden, two areas capped w/ 20cm of peat-mineral reclamation mix, CT process water circulates through this wetland at 75L/min	OSPM

Table A.4 Additional description of 2009 wetland sediment samples[a]

Wetland code	Sample	% dry weight[b]	Visual description[b]
MP	MP-1	90.71	-thick sediment, light brown colour, smooth
	MP-2	90.72	- same as above
	MP-3	89.32	-same as above
4M	4M-1	79.14	- dark colour, very liquid, smell, can see oil
	4M-2	79.26	- same as above, alot of debris
	4M-3	87.15	- same as 4M-3
TP9	TP9-1	84.45	- grey-brown colour, alot of debris, smooth
	TP9-2	83.42	- same as above but very thick
	TP9-3	81.79	- same as TP9-1 but more liquid
NW	NW-1	90.28	- very dark in colour, thick
	NW-2	93.54	- thick, dark brown, alot of debris
	NW-3	86.88	- very thick, dark (almost black)
DP	DP-1	87.86	- grey-brown in colour, pieces of twigs
	DP-2	89.98	- lighter brown, sooth, thick
	DP-3	85.59	- same as DP-2
BP	BP-1	91.54	- dark in colour, thick, twigs/roots present
	BP-2	91.11	- medium brown, lots of debris, thicker
	BP-3	90.24	- same as BP-1 but much thicker
BL	BL-1	89.80	- very thick, lots of debris (twigs), brown-grey
	BL-2	90.50	- same as above
	BL-3	90.39	- same as above
HS	HS-1	89.00	- alot of liquid with a sediment layer, dark brown
	HS-2	86.95	- same as HS-1
	HS-3	90.51	- thick, rocks/stones present, dark brown, twigs
SW	SW-1	82.52	- alot of liquid, lighter brown
	SW-2	88.11	- light brown, alot of liquid, alot of debris
	SW-3	81.84	- brown-grey, alot of liquid, alot of debris

[a] all samples taken in 2009 were from non-vegetated sites

[b] % dry weight, and visual descriptions courtesy of Heather McCormick, 2009

Appendix B

Table B.1. Samples and their corresponding code used for PCA of BIOLOG™ data

Sample	Code for PCA	Sample	Code for PCA
TP9-11	AJ-1a	HS-11	BA-2a*
TP9-12	AJ-1b	HS-12	BA-2b*
TP9-13	AJ-1c	HS-13	BA-2c*
MP-11	AJ-2a	HS-21	BA-2a
MP-12	AJ-2b	HS-22	BA-2b
MP-13	AJ-2c	HS-23	BA-2c
NW-11	AJ-3a	DU-11	BJ-1a
NW-12	AJ-3b	DU-12	BJ-1b
NW-13	AJ-3c	DU-13	BJ-1c
4M-11	AA-1a*	SW-11	BJ-2a*
4M-12	AA-1b*	SW-12	BJ-2b*
4M-13	AA-1c*	SW-13	BJ-2c*
4M-21	AA-1a	SW-21	BJ-2a
4M-22	AA-1b	SW-22	BJ-2b
4M-23	AA-1c	SW-23	BJ-2c
DP-11	AA-2a	LL-11	CJ-1a
DP-12	AA-2b	LL-12	CJ-1b
DP-13	AA-2c	LL-13	CJ-1c
BL-11	BA-1a*	MC-11	CJ-2a
BL-12	BA-1b*	MC-12	CJ-2b
BL-13	BA-1c*	MC-13	CJ-2c
BL-21	BA-1a	BP-11	CA-1a
BL-22	BA-1b	BP-12	CA-1b
BL-23	BA-1c	BP-13	CA-1c

Appendix C

Table C.1. Description of samples used to assess the reproducibility of DGGE banding patterns when samples were taken from different locations and/or at different times

Label[a]	Sample Abbreviation	Date DNA Extracted	Label[a] for Date of Extraction	Location of Sample
B-2_1	B-2_1	August 27, 2008	A	N/A[c]
		November 30, 2008	B	
		February 9, 2009	C	
B-2_2	B-2_2	August 27, 2008	A	N/A
		November 30, 2008	B	
		February 9, 2009	C	
B-2_3	B-2_3	August 27, 2008	A	N/A
		November 30, 2008	B	
		February 9, 2009	C	
LL-1_1	LL-1_1	July 24, 2008	A	N/A
		November 30, 2008	B	
		February 9, 2009	C	
LL-1_2	LL-1_2	July 24, 2008	A	N/A
		November 30, 2008	B	
		February 9, 2009	C	
LL-1_3	LL-1_3	July 24, 2008	A	N/A
		November 30, 2008	B	
		February 9, 2009	C	
BP-1_1 (1)	BP-1_1	August 28, 2008[b]	A	Top left
		November 30, 2008	B	corner
		February 9, 2009	C	
BP-1_1 (2)	BP-1_1	November 30, 2008	B	Center
		February 9, 2009	C	
BP-1_1 (3)	BP-1_1	November 30, 2008	B	Bottom right
		February 9, 2009	C	corner
BP-1 (2007)	BP-1	February 9, 2009	C	N/A
4M-2_1 (1)	4M-2_1	August 28, 2008[b]	A	Top left
		November 30, 2008	B	corner
		February 9, 2009	C	
4M-2_1 (2)	4M-2_1	November 30, 2008	B	Center
		February 9, 2009	C	
4M-2_1 (3)	4M-2_1	November 30, 2008	B	Bottom right
		February 9, 2009	C	corner

[a] label used solely for this experiment
[b] did not distinguish where I took the sample from with respect to the sampling container
[c] N/A is used when no distinction between sampling location within container was made

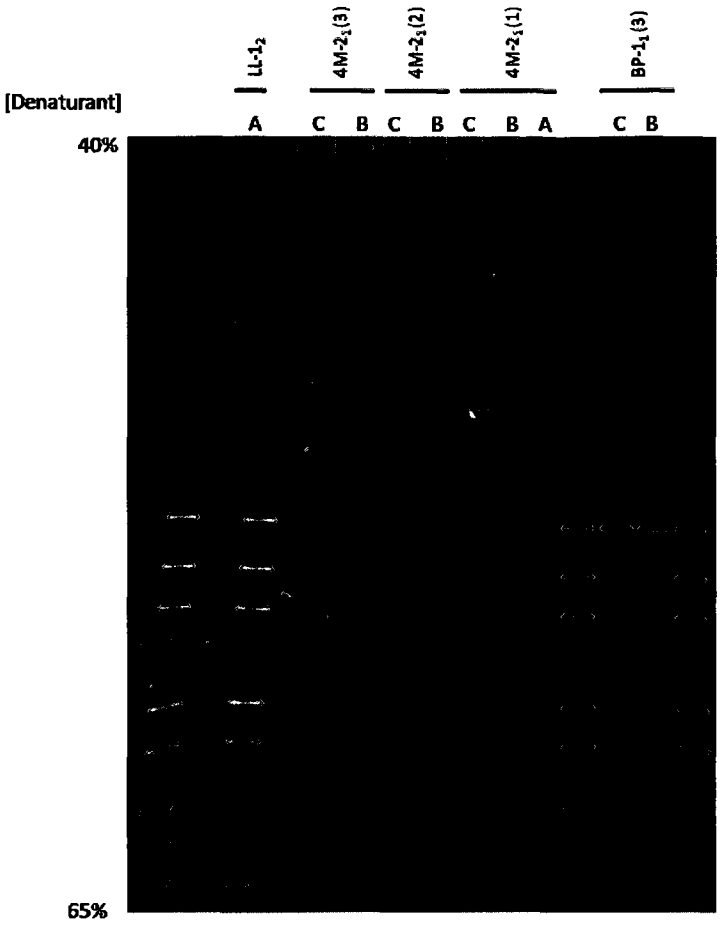


Figure C.1. Image of an 8% DGGE gel with a urea/formamide gradient of 40-65% used to separate DNA fragment amplified with primer pair 357f-GC-518r. This gel was used to assess the reproducibility of banding patterns when replicate DNA extractions were completed with variations in sampling location with respect to the sampling container or in the date of extraction. The letters listed above each banding pattern and corresponding to a certain wetland sample refers to the date of DNA extraction: (A) July or August 2008; (B) November 2008; (C) February 2009. The numbers in brackets refer to where the samples were taken from with respect to the sampling container: (1) top left corner; (2) center; (3) bottom right corner.

Chapter 7
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