THE FATE OF HAPHTHENIC ACIDS IN WETLAND SEDIMENTS: MICROBIAL METABOLISM AND COMMUNITY STRUCTURE

by

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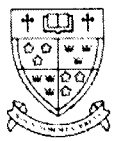

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ABSTRACT

Naphthenic acids (NA) are naturally occurring carboxylic acids released from bitumen during oil sands processing. NA's are acutely toxic to aquatic organisms and are primarily removed by microbial degradation. The purpose of this study was to isolate microorganisms capable of NA degradation and to determine the extent of NA surrogate degradation by microbial communities.

To determine the NA-degrading ability of native microorganisms, microcosms were incubated with ^{14}C-labelled NA surrogates cyclohexane carboxylic acid (CCA), and decahydronaphthoic acid (DHNA), and $^{14}CO_2$ evolution was monitored. All microcosms degraded CCA however, previous exposure to NA's is necessary for DHNA degradation to occur.

Using NA's as the sole carbon source, two bacterial strains identified as *Pseudomonas* species, were isolated from enrichment cultures, and NA degradation was monitored by GC-MS. Co-cultures degraded >99% of NA within 4 weeks. However each bacterium degraded 15% of the total NA mixture.

Keywords: Biodegradation, naphthenic acids, GC-MS, CCA, DHNA.

DEDICATION

This thesis is dedicated to my maternal grandparents Raul Velarde Pareja and Gladys Jochamowitz Leigh.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

BAMES	bacterial acid methyl esters
BDL	below detection limit
CT	nonsegregating tailings known as consolidated tailings at Suncor Inc. and composite tailings at Syncrude Canada Ltd.
CCA	cyclohexane carboxylic acid
DHNA	decahydronaphthoic acid
dpm	disintegrations per minute
FAMES	fatty acid methyl esters
GC	gas chromatograph
GC/MS	gas chromatography/mass spectrometry
LP	lipid phosphate
MFT's	mature fine tailings
NA	naphthenic acids
PLFA	phospholipid fatty acids
TPW	tailings pond water

CHAPTER ONE: INTRODUCTION

1.1 An introduction to the oil sands industry

The earliest documented presence of tar sands in northeastern Alberta was in the 1780's when surveyors noticed oil pitch leaching out of the shores of the Athabasca River (Holowenko, 2000). Since then, tar sands deposits have been found near Athabasca, Cold Lake, Wabasca and Peace River. However, most interest has focused on the largest deposit near the Athabasca Basin. It is estimated that over 1.7 trillion barrels of bitumen are enclosed within the Athabasca Basin thus making it one of the largest reserves of hydrocarbons in the world (approximately 20% of the world's oil reserves) (MacLean 1998). However, only 300 billion barrels of this reserve are recoverable with current technology.

Acknowledging the potential for vast amounts of revenue, an alliance formed between the Alberta government and entrepreneurs began mining near Fort McMurray in the 1920's. However, initial attempts to recover bitumen were unsuccessful and activity in this region decreased between 1949 and the late 1960's, when the Great Canadian Oil Sands Company started its operations. The company processed its first barrel of synthetic crude oil in 1968 and became Suncor Inc. Syncrude Canada Ltd. followed suit in 1978 (MacLean 1998).

The last 30 years have seen the dramatic expansion of the oil sands industry. In addition to Suncor Inc. and Syncrude Canada Ltd., other companies such as Shell are in the process of developing new mines, while at the same time Suncor Inc. and Syncrude Canada Ltd. are preparing for further expansion. At this time, the oil sands industry

generates over 120 million barrels of synthetic crude oil per year and is expected to increase to 400 million barrels per year within the next 10 years (Clemente et al., 2003). Approximately 20 to 25% of Canada's oil supply comes from the oil sands; however this value is projected to increase to over 50% as the more established oil sources continue to be depleted (Holowenko, 2000).

1.1.1 Mining and extraction

The oil sands are located under 10 to 50 meters of muskeg soil and overburden (mainly clays) and are obtained by open-pit mining. Following removal of the soil and overburden, the oil sands are mined using draglines and bucketwheels. The oil sand is then carried from the pit to the extraction plant by hauler trucks or a large network of conveyor belts.

The success of oil sands operations was in part determined by the development of a cost-effective method of extracting the bitumen from the sand. Until the 1920's, hot water flotation processes had been in use. However, modifications made by Dr. Karl Clark of the Alberta Research Council led to the development of the hot water extraction process, which is used to this day (Schramm et al., 2000). In the Clark Hot Water Extraction, the oil sand is sent to large tumblers where it is processed and conditioned with a mixture of hot water, sodium hydroxide (NaOH), and steam. The use of NaOH, leads to an increase in the pH of the resulting tailings to between 8 and 9. In the resulting slurry, the bitumen separates from the sand. This mixture is then aerated to create a lather to separate most of the bitumen from the sand and clay. The slurry is then pumped into large separation vessels, and diluted in hot water. The lather containing the bitumen floats to the surface of the separation vessels as the sand settles out and is removed.

Meanwhile, the slurry is further processed in order to recover any residual oil, which did not separate with the lather. Following the removal of water and fine solids from the lather, the bitumen undergoes various refining stages where it is converted to synthetic crude oil, which is then transported to Edmonton via pipeline. This process is summarized in figure 1.1 (MacKinnon, 1989).

1.1.2 Tailings disposal

Bitumen extraction from the oil sands requires up to 3 m^3 of water for every m^3 of oil sand and produces an average 4 m^3 of waste. This waste is composed mainly of solids, process-affected water, organics and residual bitumen (Gulley and MacKinnon, 1993). Approximately 3 m^3 of this waste consists of water and fine tailings (91.67 and 8.33 % respectively), while the remaining 1 m^3 is tailings sand, which settles out and is then used to create dykes and beaches (List and Lord, 1997). Due to the Alberta government's "zero discharge" policy, Syncrude Canada Ltd. and Suncor Inc., do not release any waste products from their leases. As a result, all fine tailings are contained on site, predominantly in large tailings ponds. Suncor Inc. has five tailings ponds, covering a total of 16 km^2 and containing over 100 x 10^6 m^3 of fine tailings (Holowenko, 2000). Syncrude Canada Ltd. has several settling basins containing an excess of 300 x 10^6 m^3 of fine tailings (Holowenko, 2000). In the ponds, the fine tailings go through a slow process of sedimentation and consolidation. This results in an upper layer of clear water, which is recycled back to the extraction plant (Figure 1.1.) thereby providing 75 % of the water needed for extraction operations (MacKinnon, 1989). Located under the water layer is a transition zone containing a suspension of fine tailings slurry. As depth increases, the

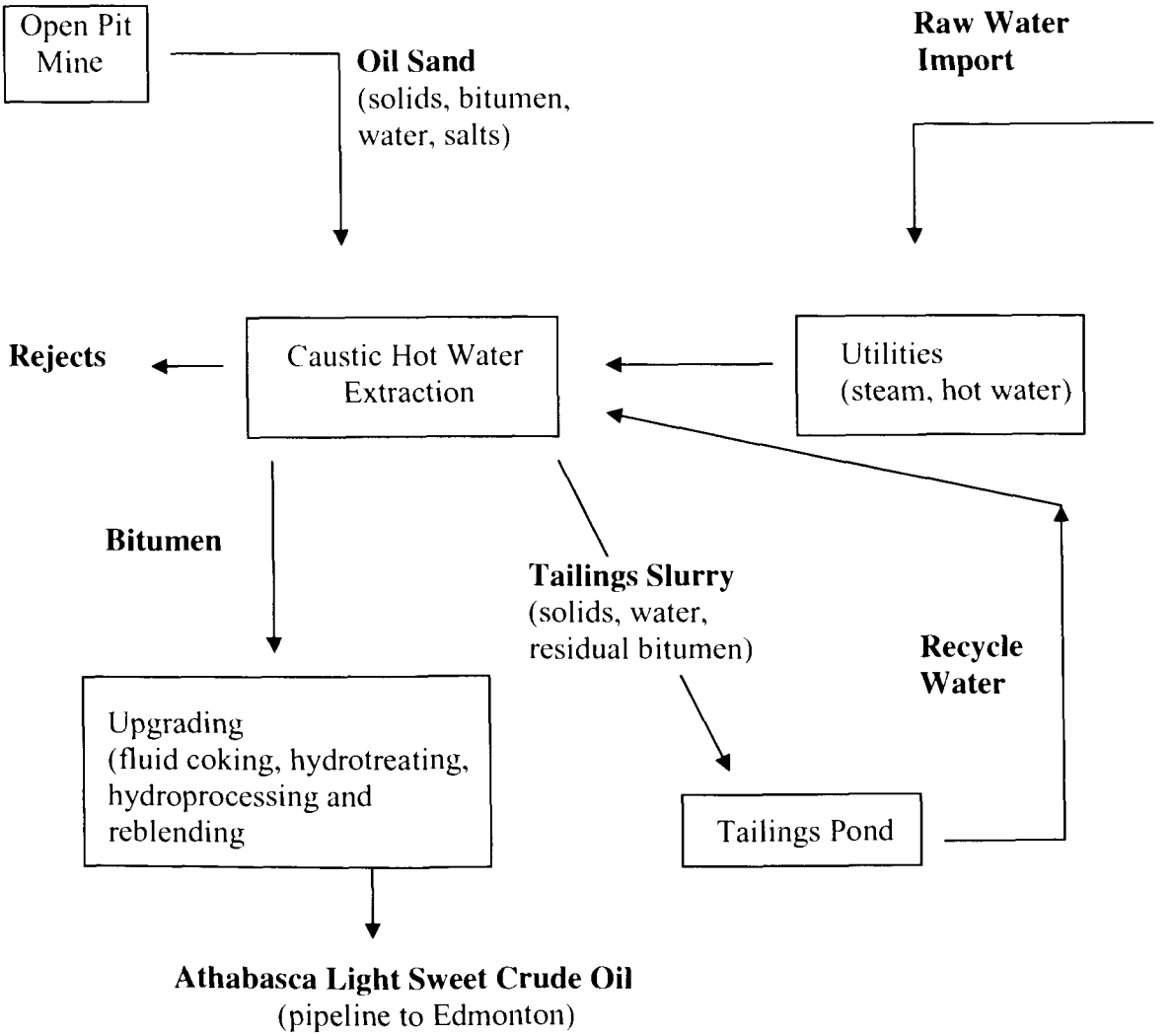


Figure 1.1. General Scheme for the Clark process of oil extraction from the oil sands deposits (Adapted from Holowenko, 1999).

fine tailings develop into a denser and thicker gel-like structure, identified as mature fine tailings (MFT's) (MacKinnon, 1989).

Oil sands tailings pond water (TPW) is acutely toxic to aquatic organisms, with LC_{50}[1] values in 96-hour fish bioassays <10 % and EC_{50}[2] values in the Microtox assay of less than 30%. The toxicity of TPW is highly influenced by the amount of solids and residual bitumen in the pond. In addition, these waters possess high chemical and biological oxygen demand, resulting in low levels of dissolved oxygen (Mackinnon and Boerger, 1986).

The primary compounds responsible for this toxicity are a group of organic acids collectively known as the naphthenic acids (NA's) (McKinnon and Boerger, 1986, Farwell and Dixon, 2000). In addition, Rogers et al. (2002) have demonstrated that NA's cause hepatotoxicity in rodents receiving high oral doses. NA's are a native component of bitumen and are concentrated and liberated into the wastewater during the process of extraction. NA concentrations in the tailings pond surface water range between 80 to 120 mg/L. Not much is known about the chemical composition of these compounds except that they are a complex mixture of cyclic and acyclic alkanes possessing a carboxylated side chain (Figure 1.2) (Lower, 1987). The number of rings in the structure can vary as can the length of the carboxylated side chain as denoted by the subscript m. Little is known about the R group, except that it is thought to be aliphatic. NA's have the general formula $C_nH_{2n+Z}O_2$ (Z = 0, -2, -4...-12) and are categorized into Z groups or families, corresponding to the number of hydrogen atoms lost as their structure becomes denser.

[1] Defined as the concentration at which 50% of the test organisms are observed to exhibit a lethal response.
[2] Defined as the concentration at which 50% of the test organisms are observed to exhibit a specific non-lethal response.

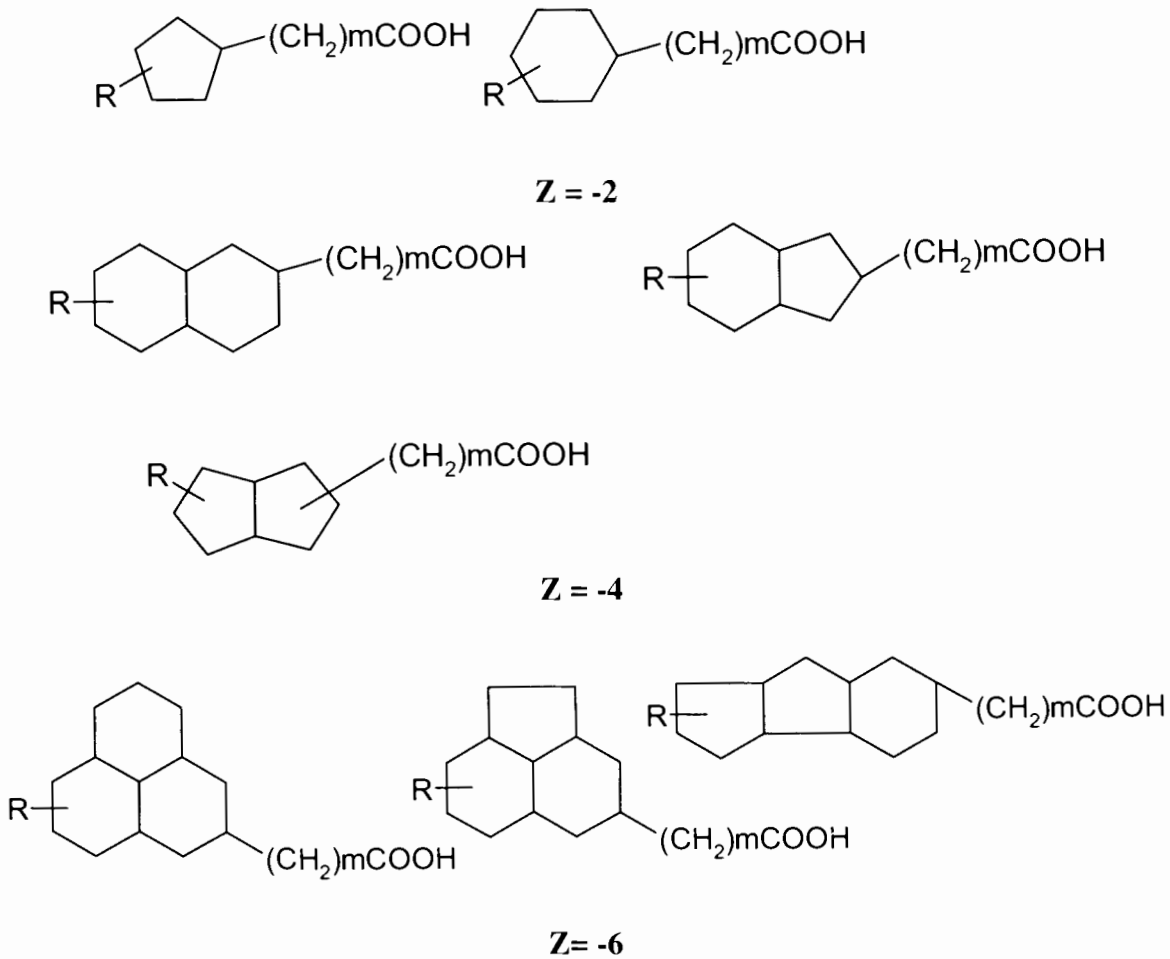


Figure 1.2. Naphthenic acid structures and Z families (adapted from Morales et al, 1993). The letter m is greater than or equal to one.

NA's in the Z = 0 group lack a ring structure and are believed to be highly branched rather than straight-chained fatty acids (Clemente et al., 2003, Cason and Graham, 1965). NA's with one ring belong to the Z = -2 family, while bicyclic NA's belong to the Z= -4 group and so on. Analysis of tailings ponds extract by fast atom bombardment mass spectrometry (FABMS) and gas chromatography-mass spectrometry (GC/MS) has resulted in the identification of ions corresponding to NA's with 0, 1, 2 and 3 rings corresponding to Z values of 0, -2, -4 and -6, respectively (Morales et al, 1993, Holowenko et al, 2001).

1.1.3 Reclamation of the fine tailings

There are several options being investigated for the remediation of fine tailings. These include a dry landscape approach, a wet landscape by means of either natural or constructed wetlands, and more recently, composite or consolidated tailings (List and Lord, 1997). These options must meet the following requirements: There must be a restriction in the direct contact or release of contaminants into the environment as well as in their off-site transport by seepage. There must also be a restriction on the hydrological impacts of the fine tailings deposits. Finally, the landscape must be stable as well as productive and self-sustaining (Gulley and McKinnon, 1993).

The wet landscape approach has been the most commonly used method for the remediation of the large volumes of fine tailings waste (Gulley and McKinnon, 1993). There are two types of wet landscapes termed surface flow and subsurface flow wetlands (Hamilton et al., 1993). In the surface flow wetland approach, process-affected waters are transferred from tailings ponds and enter natural or constructed wetlands over which a layer of water can be placed, thus creating a water-capped lake (Boerger et al, 1992).

The water cap must be deep enough to isolate the fine tailings and prevent sediment suspension thus creating a self-sustaining ecosystem (Lawrence et al, 1991). In this lake ecosystem, the fine tailings would form the sediment and be covered with detritus. This would minimize the mixing of the fine tailings with the water cap thus creating a biologically active zone. In addition, over time, chemical degradation and microorganisms in the lake would degrade any organic compounds moving up the water column from the fine tailings layer thereby detoxifying the process-affected water (Gulley and McKinnon, 1993). Nonetheless, for this approach to work, it is of the utmost importance that the tailings be mature, that is they must have low permeability, high density, high yielding strength and high viscosity. These characteristics would help ensure that a well-stratified lake ecosystem would develop (FTFC, 1995).

In the subsurface flow wetland approach, water flows below the surface through a gravel, crushed rock or soil bed that has been penetrated by the roots of aquatic vegetation. In this type of wetland, the media is saturated but there is no surface water, instead water flows through the vegetation mat and the underlying substrate. Although the water flow is through a largely anaerobic zone, some oxygen is received through the roots of certain aquatic plants. The relative performance of either option in removing pollutants from wastewater is dependant on the nature of the waste and local environmental conditions. However, an advantage of the subsurface flow approach is the fact that odour and mosquito problems are limited (Reed, 1988).

The major drawbacks to the wet landscape approach arise from the fact that the lakes ecosystems formed possess three environments, which can be classified as aerobic (water layer), anoxic (water:fine tailings interface) and anaerobic (fine tailings layer) with

the anaerobic layer being the largest (Holowenko, 2000). A study by Sobolewski (1999), found that a significant amount of the anaerobic microorganisms in an artificial wetland constructed by Syncrude Canada Ltd. were methane-producing microorganisms also known as methanogens. It is quite possible that methane production is detrimental to the reclamation of the fine tailings because methane released from the sediments may affect the densification rate of the fine tailings, gas released from the MFT layer may resuspend the fine tailings and move toxic compounds into the water layer, and methane in the water layer could be consumed along with O_2 by methanotrophs (aerobic methane-utilizing bacteria) (Gulley and MacKinnon, 1993). This could lead to anoxic condition thereby preventing the establishment of an ecosystem with higher forms of life. Finally methane is a greenhouse gas and as such its release into the environment is undesirable.

In the consolidated or composite tailings approach, TPW is treated with gypsum ($CaSO_4 \cdot 2H_2O$). This results in the precipitation of the fine tailings into a nonsegregating slurry known as consolidated tailings (Suncor) and composite tailings (Syncrude). Settling of the slurry is followed by the quick release of water. This process allows a dry landscape approach to tailings reclamation in which the CT's are buried under a layer of overburden followed by a layer of soil (List and Lord, 1997).

1.2 Microbial degradation of naphthenic acids

Previous research has shown that NA's are primarily removed by sediment microorganisms (Nix et al., 1993) although the precise mechanism of degradation is unknown. Morales et al. (1993) have shown that in samples obtained from oil sands process-affected waters, the majority of NA's belong to the Z= -4 family, and that the rate of biodegradation is inversely proportional to the number of carbons (especially >17)

and the degree of cyclization. A study by Herman et al. (1994) showed that an enrichment culture composed of *Pseudomonas stutzeri* and *Alcaligenes denitrificans*, which was isolated from oil sands tailings using a commercial mixture of NA's was able to convert over 50% of the organic carbon into CO_2 and resulted in the complete absence of detectable toxicity as measured by the MICROTOX assay following the biodegradation of NA's. Moreover incubation of the same enrichment culture with organic acids extracted from the oil sands tailings resulted in mineralization of approximately 20% of the organic carbon as well as a reduction of acute toxicity to approximately one half of the original level. In addition, it was found that the NA mixtures were degraded by the mixed culture only (Herman et al., 1994).

A microcosm study by Lai et al. (1996) measured the effect of phosphate addition on the degradation rates of radiolabelled surrogate NA's and toxicity of tailings pond water (TPW). The addition of phosphate to TPW led to a twofold increase in the rate of surrogate degradation as well as an increase in the rate of oxygen consumption. These results are in agreement with those of an earlier study by Herman et al. (1993) in which they measured the degradation of several carboxylated cycloalkanes by microbes indigenous to the oil sands tailings. In this study, the researchers showed that compounds with methyl substituents on the cycloalkane ring were more recalcitrant than the unsubstituted compounds. They also showed that microbial activity was both nitrogen- and phosphorus-limited (Herman et al., 1993).

Lai et al. (1996) also showed that phosphate addition resulted in a significant decrease in the acute toxicity of TPW to fathead minnows and that decreases in dissolved oxygen concentration resulted in a decrease in the rate of surrogate NA degradation, thus

indicating that NA degradation is an aerobic process. Further evidence that the majority of NA's removal is via aerobic metabolism comes from a study by Holowenko et al. (2001). In this study, the effects of mono and bicyclic surrogate NA's, commercially available NA's, and organic acids extracted from the oil sands tailings on methanogenesis were examined in anaerobic microcosms composed of either oil sands fine tailings or domestic sewage sludge. The results of this study showed that neither NA mixture was able to stimulate the production of methane in the anaerobic microcosms, nor did the mixtures inhibit methanogenesis. However, they did show that the monocyclic surrogates were used as methanogenic substrates in oil sands fine tailings microcosms. The bicyclic surrogate was not degraded under anaerobic conditions. These results indicate that although a methanogenic consortium may degrade some of the simpler NA's (such as those belonging to the Z= 0 and –2 families), other NA's in the mixture (i.e. those belonging to the Z= -4 and higher families) might inhibit the anaerobic degradation process. Hence NA removal is primarily an aerobic process.

1.3 Introduction to microbial communities and their analysis

Knowledge of microbial community structure is essential to a variety of ecological and environmental studies in seemingly unrelated disciplines such as chemical and environmental engineering, soil microbiology, biodegradation and bioremediation, and marine microbiology (Ogram and, Feng, 1996). A variety of techniques are currently available for analyzing various aspects of community structure, and many of these techniques are easily applied with slight modifications to communities for which they were not originally intended. Although analysis of microbial community structure is of

the utmost importance in environmental microbiology, it is also one of the most technically challenging (Ogram and Feng, 1996).

The majority of microorganisms present in many environments may not be readily cultured by current technologies and therefore are most likely not included in most analyses. Current estimates suggest that less than 1% of microorganisms present in many environments are readily culturable, indicating that techniques based on laboratory cultivation are significantly biased (Torsvik et al. 1990a and b). It is therefore possible that most species in many microbial communities have never been described. Even if all the microorganisms present in a community were culturable, because of time constraints it is not feasible to enumerate all species present except in rather simple communities. Therefore, many approaches either study small, well-defined groups of microorganisms, such as those involved in nitrification or degradation of a particular xenobiotic, or use a broader-spectrum approach and define the relative numbers of individuals capable of utilizing an array of carbon sources (Kennedy, 1994) or possessing characteristic fatty acids (Vestal et al. 1989).

To avoid the difficulties and restrictions associated with laboratory cultivation, many techniques have been developed that do not require cultivation. These techniques are based on the direct extraction of biological markers such as nucleic acids or fatty acids from environmental samples have been developed (Sayler and Layton, 1990 and Vestal et al, 1989). The composition of the extracted biological markers can be analyzed and depending on the nature of the analysis, information regarding the structure, activity and in some cases the nutritional state of the community can be obtained. All of these techniques have their limitations and biases that must be taken into account during the

interpretation of community analysis data. A summary of several currently used techniques is presented below.

1.3.1 Analysis based on single carbon source utilization

To understand the role of microbial communities in different environments, it is essential to possess knowledge of microbial community function as well as functional diversity (Preston-Mafham et al., 2002). Microbial community function refers to the actual catabolic activity expressed whereas functional diversity refers to the ability of the microbial community to adapt their metabolism and their relative composition to a variety of environmental parameters. To obtain information on functional diversity, Garland and Mills (1991) introduced the use of the commercially available BIOLOG MicroPlate™, which is based on the utilization patterns of 95 single carbon sources.

This technique was originally developed for the rapid identification of bacterial isolates by single-carbon-source utilization. The substrates are coupled to a tetrazolium dye. Metabolism of the substrate in a particular well leads to the reduction of the tetrazolium dye resulting in colour change on the plate thereby providing a unique metabolic fingerprint. Several types of BIOLOG MicroPlates containing different substrates are available and are extensively reviewed by Preston-Mafham et al., (2002).

The major advantage of this technique to the analysis of microbial communities is the fact that it is very quick and simple to implement thus allowing the analysis of a large number of samples in a relatively short period of time. There are however several factors that must be taken into consideration. First, collecting environmental samples invariably causes disturbances on the sample's physical, chemical, and biotic components resulting in an altered environment. Thus, time between sample collection and plate inoculation

must be kept to the minimum in order to prevent major shifts in the communities as a result of the altered environment (Pennanen, 2000). Second, in order to overcome the effects of different inoculum densities on colour formation, the plates must be monitored over an extended period of time in order to determine the kinetics of colour development (Preston-Mafham et al., 2002). Third, the substrates may not be able to support growth of all members of the communities thereby providing a biased representation of the functional/metabolic capabilities of the soil community (Bossio and Scow, 1995). Nonetheless, the extensive range of carbon sources results in an array of selection pressures, thus allowing normally unculturable organisms to survive and contribute to colour formation (Smalla et al., 1998). Finally, the substrates used in the plates may not reflect the natural environment from which the samples were collected (Glimm et al., 1997). Because of these and other reasons, analysis of microbial communities by single carbon source utilization provides insight into the functional ability of the communities and is better suited for the comparison of communities rather than community characterization.

1.3.2 Analysis based on direct extraction of biological markers

To avoid the existing limitations and biases associated with laboratory cultivation of microorganisms, a number of alternative methodologies have been developed which are based on the analysis of biological markers extracted directly from environmental samples. The most frequently used biological markers are nucleic acids (DNA and RNA) and phospholipid fatty acids (PLFA). Analysis of these molecules generates different information regarding the structure and activity of microbial communities, and the user should choose the molecule and the method that best suits the specific application.

Nucleic acid based approaches to the analyses of microbial communities

Gene probes

Hybridization of gene probes to nucleic acids extracted directly from environmental samples provides information which is largely dependent on the target nucleic acid. Hybridization to DNA is useful for the rapid identification of a specific characteristic of community structure such as the presence and relative concentrations of genes encoding a specific function. In contrast, RNA hybridization is an indicator of the relative activities of the target groups (Ogram, and Bezdicek, 1994). The activities of certain non-rRNA genes may be estimated by using mRNA as the target, although many mRNA's are too unstable to be detected in this way (Ogram and Feng, 1996).

The greatest advantage of the application of gene probes to microbial community analysis is the fact that it can be a very specific means of detecting and enumerating individual genotypes. Its greatest limitation is the lack of knowledge concerning the molecular genetics of microbial communities in their environment. In the event that more than one gene is responsible for a given function, hybridization may underestimate the total concentration of target organisms. On the other hand, if regions of the probe hybridize with non-target genes in the sample, overestimation of the target gene may occur. It is for these reasons, that gene probes should be relatively short (preferably no longer than 500 bp) segments of an internal region of a well-characterized gene and the hybridization should be carried out under highly stringent conditions (Ogram and Feng, 1996). Alternatively, Vourdouw et al. (1993) have developed reverse sample genome probing method in which the sample DNA is labelled and used as a probe against a set of known genes of interest thus generating a community fingerprint.

Comparisons of communities by percent G + C profiles

Holben and Harris, (1991) have compared the distributions of G + C content in the community DNA to analyze shifts in microbial community structure over time or following an environmental disturbance. The rationale behind this method is that bacterial chromosomes have G + C contents that are characteristic of their taxonomic groups. Therefore, it is believed that the relative proportions of G + C contents in DNA extracted from a community are consequently characteristic of the relative proportions of specific taxonomic groups within the community. In that study, they were able to show that changes in the G + C content of the microbial community DNA arose as a result of organic amendments, chloroform fumigation and aerobic versus anaerobic conditions.

Analysis of rRNA and rDNA

Analysis of the diversity of rRNA and rDNA is of great value in characterizing community structures and the relative activities of phylogenetic groups of interest. There are a wide variety of strategies that can be used such as; sequencing and phylogenetic analysis (Giovannoni et al. 1990, Liesack and Stackebrandt, 1992), hybridization with group specific probes (Amman et al, 1995), classification by RFLP patterns for the placement into different operational taxonomic units (Moyer et al. 1994), and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993). Because the sample DNA includes DNA from all members of a community, total community structure analyses are conducted on rDNA rather than rRNA molecules. However, when the interests of the study lie in the analysis of the metabolically active members of the community, rRNA should be analyzed rather than rDNA.

The simplest approach to analysis of rDNA is hybridization with phylogenetic probes, using procedures similar to those described above. Amman et al. (1995) have listed a variety of probes that have been designed to hybridize exclusively to specific phylogenetic groups. Although this approach is very useful in the characterization of active groups within a community, its major limitation is the fact that it cannot be used for the identification of specific members of the community, since probes are directed toward broad phylogenetic groups (Ogram and Feng, 1996).

The DGGE method of analysis is based on the separation of DNA fragments of identical or nearly identical length but differing in sequence. This method was first developed for the detection of single-base changes in genes for the diagnosis of genetic diseases and in genetic linkage studies (Myers et al. 1987). More recently, DGGE has been extended to characterize microbial populations in environmental samples by separating PCR amplification products generated by using primers flanking conserved genes such as the 16S rRNA gene (Muyzer et al. 1993). Separation is based on changes in the electrophoretic mobility of similar-sized DNA fragments migrating in a gel containing an increasing gradient of denaturing agents such as urea and formamide. The changes in electrophoretic mobility are related to the denaturation of double stranded DNA in certain regions referred to as the melting domains (Stahl, 1996). Since the temperature of the gel is held constant, the melting temperature for each melting domain varies according to the concentration of denaturant and therefore by its position in the gel. When the DNA enters a region of the gel containing sufficient denaturant, partial denaturation occurs resulting in an arrest of electrophoretic mobility. Sequence variation within the melting domains affects their denaturing behaviour, and sequence variants of

the different amplification products stop migrating at different positions in the denaturing gel (Lerman et al. 1984).

DGGE analysis of PCR-amplified 16S rDNA fragments provides with a relatively rapid method for the characterization of community structure in a wide variety of environments including microbial mats and deep-sea hydrothermal vents (Ferris et al. 1995, and Teske et al. 1996). More specific community structure information can be obtained by further analysis of the DGGE banding pattern via sequencing or hybridization (Muyzer et al. 1993)

In spite of its usefulness, DGGE analysis has some limitations. Firstly, complete separation of the wide variety of fragments amplified from a highly diverse bacterial community may not be possible with current technology (Stahl, 1996). The phylogenetic information obtained from sequencing individual bands is limited by the fact that only fragments up to 500 bp can be separated. PCR amplification may introduce bias via the preferential amplification of a subgroup of sequences within the total population (Reysenback et al. 1992). Finally, the nucleotide sequence of rRNA operons of a single species can vary significantly, and as such it is possible for a single species to contribute to multiple bands on a DGGE gel (Stahl, 1996).

Individual DNA fragments may be cloned into vectors designed for this purpose (Liesack and Stackebrandt 1992). Once the clone library has been generated, the number of clones to be analyzed may be reduced by comparison of similar RFLP patterns. A disadvantage to this reduction in the number of clones for analysis by RFLP grouping is the possibility of overestimation of community diversity as a result of divergent rRNA operons within some strains (Ogram and Feng, 1996).

The most detailed information concerning the phylogenetic affiliations of the individual members of the community is obtained by sequencing each of the individual clones. This is being increasingly done, however cost is quite prohibitive for many laboratories therefore many of these studies are only conducted on communities with low species diversities, such as those found in hot springs, and in bioreactors.

Finally, Venter et al. (2004) have adapted the whole genome shotgun sequencing method, which was previously used for the rapid sequencing of individual organisms'genomes to the study of microbial communities in the Sargasso Sea. That study resulted in the discovery of 150 new bacterial species as well as the identification of over 1.2 million new genes thus demonstrating a previously unseen level of microbial diversity in seawater. However although this method is still prohibitively expensive for routine use.

Phospholipid fatty acid analysis

PLFA's extracted from environmental samples have been used to study microbial community structures and metabolic states and to compare similarities and differences among different microbial communities (Bobbie and White, 1978, and Frostegard et al. 1993).

The relative concentrations of different kinds of polar lipids in each membrane are characteristic for the type of membrane, cell type, and the species; however, the fatty acid components of the individual membrane lipids are not fixed and may vary with nutritional state and environmental conditions in order to maintain membrane fluidity (Kieft et al. 1994). By studying the changes of fatty acid profiles, especially the presence of certain PLFA as biomarkers for certain microbial types, an indication of the metabolic

activity of the microbial community may be obtained (Frostegard et al. 1993, and Guckert et al. 1986).

PLFA patterns derived from environmental samples provide quantitative analysis, however the elucidation in terms of precise components may not be possible due to overlapping composition between constituents (White et al. 1996). That is, quantitative comparisons of total community PLFA patterns accurately mirror shifts in community composition but may not provide definitive analysis of shifts in specific microorganisms. Examples of signature PLFA's are shown in Table 1.1.

In PLFA analysis it is of the utmost importance to consider the environment from which the sample was obtained when interpreting the results. For example, terminally branched saturated PLFA are common to Gram-positive aerobes as well as to some Gram-negative anaerobes such as the sulphate-reducing bacteria (White et al. 1996). Monoenoic PLFA's are found in all Gram-negative organisms and many types of microeukaryotes. Polyenoic PLFA are indicative of microeukaryotes as are saturated PLFA longer than 20 carbons. Bacteria usually contain greater amounts of 16 carbon fatty acids whereas eukaryotes contain greater amounts of 18 carbon fatty acids (White et al. 1996).

As mentioned above, PLFA analysis can be used to obtain insight into the nutritional and physiological status of the microbial community. Starvation and stationary-phase growth lead to the conversion of monoenoic PLFA to cyclopropyl PLFA. Increases in cyclopropyl PLFA formation have also been associated with a shift toward anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies (White et al. 1996). Starvation or environmental stress often results in a relative increase

Table 1.1. Example of signature PLFA's (adapted from Zelles, 1999)

Abbreviation	Type of Fatty Acid	Indicator for, or isolated from
PLFA	Phospholipid Fatty Acids	Microbial Biomass
EL-SATFA	Ester-linked saturated FA's	Prokaryotes and eukaryotes
Straight chain	Straight-chain FA's	Widespread
Straight 20C saturated	Saturated straight chain longer than 20C atoms	Eukaryotes
Cyclopropyl	Fatty acids containing a cyclopropyl ring	Stationary-phase, starvation
Iso/anteiso	Position of methyl branch is *iso* or *anteiso*	Gram-positive aerobes, Gram negative anaerobes
10Me	Methyl branching on the 10^{th} C atom	Actinomycetes
EL-MUFA	Ester-linked monounsaturated FA's	
ω7	Double bond on the 7^{th} carbon from the methyl end	Gram-negative aerobes some strict anaerobes
ω9	Double bond on the 9^{th} carbon from the methyl end	Gram-positive, widespread
ω8	Double bond on the 8^{th} carbon from the methyl end	Methanotrophs
EL-PUFA	Ester-linked polyunsaturated fatty acids	Eukaryotes, cyanobacteria
EL-HYFA	Ester-linked hydroxyl fatty acids	
Alpha	Hydroxy substitution at position 2 nearest to carboxyl end	*Pseudomonas* spp.
Beta	Hydroxy substitution at position 3 nearest to carboxyl end	*Mycobacterium* spp.

in the *trans*-monoenoic PLFA compared to the *cis* isomers (Guckert et al. 1986). For example, Heipieper et al. (1992) have showed that *Pseudomonas putida* P8 increases its production of *trans*-unsaturated fatty acids as a result of exposure to phenol.

The major limitation of this method is that it is difficult to correlate the changes in PLFA patterns with the dynamics of specific groups of organisms, and more data is often required (Frostegard et al. 1993). This can be complemented with the analysis of other signature lipids such as sterols for fungi, nematode, algae, and protozoa (Nes, 1977 and, White et al. 1980), and by genetic studies such as DGGE.

1.4 Aims of Study

This study formed part of a larger study, the purpose of which was to determine whether there are particular physico-chemical features of wetlands that receive process-affected water, that are associated with high rates of microbial degradation of NA's. Specifically, the rates of degradation of two surrogate NA's by sediment microbial communities was measured, as well as the composition of the microbial communities. Community structure analysis was performed using PLFA analysis.

Specific Aims of the Thesis Research

1. To determine whether as NA concentration correlates with high rates of surrogate NA degradation in a large number of diverse on and offsite wetlands.
2. To characterize the sediment microbial communities in these wetlands by obtaining a "fingerprint" of the sediment microbial communities via PLFA analysis to determine whether particular communities possess enhanced NA degradation.

3. To determine whether NA concentration correlates with high rates of surrogate NA degradation in a large number of diverse on and offsite wetlands.

4. To isolate and identify microorganisms able to grow on NA's as their sole carbon source and to determine the extent of NA degradation *in vitro*.

CHAPTER TWO: MATERIALS AND METHODS

All studies described in this chapter were performed by the author with the exception of the surrogate NA mineralization studies, which were also performed by Ms. Linda Pinto and Ms. Alison Hadwin.

2.1 Description of wetlands visited

Water and sediment samples were obtained for analysis in the summers of 2000 and 2001. For both years, the sites were visited in late June, July and August. The same twelve sites were sampled in each visit. For each site, sediment and water samples were taken approximately three meters apart on a transect; these subsamples are referred to as A, B and C. Of the eleven wetlands, Highway 63, Tower Road, and Fort McKay are all "off site" wetlands and do not receive any process-affected water. The rest of the wetlands are exposed to process-affected waters by dyke seepage or by direct exposure. Pond 5 is an active tailings pond that receives fresh input waters (both regular and consolidated tails) and is not a wetland environment (see Table 2.1 for a general description of each site).

2.2 Sampling methods

Sediments were taken from the top 10 cm at sites A, B and C as noted above. All sediments were immediately passed through a 2 mm metal sieve and samples divided into clean plastic containers. All sediments were stored in cleaned polypropylene jars except for the samples intended for PLFA analysis. These were stored in a 5% formalin solution in sterile 50 ml polypropylene tubes (Falcon) as described by Federle and White (1982).

Table 2.1. The location and description of the twelve sites sampled in this study.

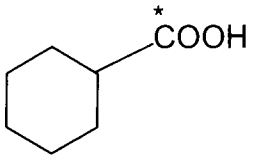
SFU Code	Site (letter abbreviation)	Latitude (N)	Longitude (W)	Observations
1	Natural Wetland (NW)	56^{0}58.837	111^{0}30.618	On-site, large dyke seepage wetland
2	Crane Lake (CL)	56^{0}59.678	111^{0}33.179	On site, reclaimed area, dyke seepage, parkland and bird sanctuary
3	Im CT (ImCT)	56^{0}59.091	111^{0}31.864	Newly formed wetland where consolidated tailings have been covered with muskeg
4	Scirpus Pond (SP)	56^{0}59.448	111^{0}31.826	On site shallow pool created by seepage from dyke around tailings pond 2
5	West Hummock (WH)	56^{0}58.861	111^{0}31.098	Shallow wetland on-site, receives some input
6	Highway 63 (H63)	56^{0}57.245	111^{0}28.382	Offsite, control wetland small shallow lake
7	High Sulphate Pond (HSP)	56^{0}59.841	111^{0}33.197	Large wetland on site in reclaimed area
8	Fort McKay (FMK)	56^{0}08.259	111^{0}36.151	Offsite, control wetland beside Athabasca river
9	Tower Road (TR)	56^{0}44.707	111^{0}29.883	Offsite, control wetland on outskirts of Fort McKay
10	Pond 5 (P5)	56^{0}00.081	111^{0}31.593	Tailings pond receiving fresh CT water, oily patches in water and sediments
11	Mature Fine Tailings Pond (N) (MFTN)	56^{0}59.455	111^{0}32.092	Well established on site pond, no longer receives input
12	Mature Fine Tailings Pond (S) (MFTS)	56^{0}59.455	111^{0}32.092	Well established on site pond, no longer receives input

Free water and sediments were stored at 4^0C until shipped by road to SFU or Syncrude's Edmonton Research Centre for water chemistry analysis (performed by Dr. M. MacKinnon).

2.3 Microbial degradation of surrogate naphthenic acids in microcosms

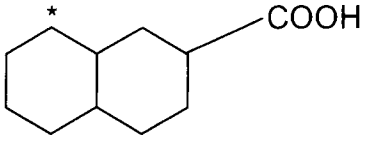
The NA surrogates chosen were ^{14}C-labelled cyclohexane carboxylic acid (CCA) (radiolabel on the carboxyl group) and decahydro-2-naphthoic acid-8-^{14}C (DHNA) (Z = -2 and -4 surrogates respectively) (Figure 2.1). CCA was obtained from American Isotopes. DHNA (98%, 7.5 mCi/mmol) was synthesized for our laboratory by Sigma Radiochemicals and was used as the Z = -4 surrogate (Figure 2.1). Microcosms containing sieved sediments and natural waters were incubated at 20^0C and the headspace gas was sampled for $^{14}CO_2$ evolution as described by Schley et al. (1998). Briefly, sieved sediment was added to sterile 20 ml with open-top caps sealed with Teflon/silicon septa. One-gram samples of water saturated sediment was weighed out for each sediment subsample (A, B and C) and the vials were then incubated with 5 ml of each sediment's appropriate pore water. The amount of radiolabel added to each sample was approximately 320,000 disintegrations per minute (dpm, 1dpm = 1.66 x 10^{-2} Bq). Control samples were also weighed out in triplicate and 5 ml of the appropriate pore water was added. These samples had been autoclaved three times with 24 hours between each autoclaving, and were used as killed controls for the detection of abiotic ^{14}C release. Dry weight values were determined for each sediment and the data was normalized to dry weight.

A



Z = -2

B



Z = -4

Figure 2.1. Chemical structure of the surrogate naphthenic acids used in this study. (A) Cyclohexane carboxylic acid (CCA), and (B) *cis*-Decahydro-2-naphthoic acid (DHNA). * Denotes the position of the radiolabelled carbon.

The headspace gas in each sediment was withdrawn into a 60 cc plastic syringe and bubbled through 5 ml of a $^{14}CO_2$ and ^{14}C-volatiles trapping solution, SCMMEA, which is a mixture of Ready Gel liquid scintillation cocktail (Beckman Instruments Inc., Mississauga, ON, Canada), 40% methanol (reagent-grade, Anachemia Science, Montreal, PQ, Canada) and 10% monoethanolamine (Anachemia Science) (Abbott et al, 1992). The ^{14}C in each scintillation vial was determined by liquid scintillation counting. Counts per minute were converted to dpm using the quench curve and the following equation: dpm = (cpm/%efficiency) x 100, and corrected for background. Background counts were determined from killed control vials. After each sampling for $^{14}CO_2$, each vial was sparged with approximately 15 cc of sterile room air delivered through a 0.2μm filter. Dissolved oxygen and pH values were determined in a set of parallel vials that received no radioactive substrate. DO concentrations were measured on a YSI 58 dissolved oxygen meter (Yellow Spring Instruments Co., Ohio USA).

Vials were incubated at 20^0C for two weeks. Each vial was sampled daily in the first week and three times per week in the second week for a total of 8 readings per sample. The rates were determined as follows: the cumulative counts at the end of the experiment (DHNA) or at the time point were they reached a plateau (CCA) were divided by the number of days required to reach that point by each replicate. The three rates were then averaged and the standard deviation determined. The nine replicate dry weight determinations were also averaged and standard deviations obtained. The average percent dpm/day was then divided by the average dry weight and the new standard deviation calculated. In order to simplify the analysis, the samples were combined into the following three categories: Offsite (NA levels < 5 mg/L and comprising H63, FMK,

and TR), Low impact (NA levels between 5 and 10 mg/L and comprising CL and HS) and high impact (NA levels > 10 mg/L and comprising NW, 1mCT, SP, WH, P5, MFTS and MFTN) and new standard deviations were calculated by combining the previously obtained standard deviations.

2.4 Microbial community and biomass analysis

2.4.1 Lipid extraction

All solvents used were of analytical grade. Glassware was washed in Micro phosphate-free detergent, rinsed ten times with double distilled water, and five times with water purified with a four-cartridge nanopure system (Barnstead), and then baked at 250^0C for 48 hours. Preserved sediments were centrifuged at 2500 g in order to remove excess formalin. The supernatants were then removed and the sediments lyophilized for twenty-four hours. The lyophilized sediments were either combined into composites of the three samples A, B, and C, prior to extraction (for PLFA analysis) or extracted directly (for biomass analysis). The lyophilized sediments were extracted by a modification of the method of Bligh and Dyer (1959). Briefly, the sediments were extracted overnight in a one-phase mixture composed of chloroform, methanol and phosphate-buffered saline (PBS) (1:2:0.8, v/v/v) except when the extracted lipids were to be used in lipid-phosphate determination. In that case, water was used instead of PBS. The extraction sample was then centrifuged at 9000 g and the supernatants transferred to new centrifuge tubes. The sediment residue was resuspended in a mixture of chloroform and methanol (2:1, v/v), reextracted for 30 minutes and recentrifuged at 7000 rpm. The supernatants were pooled and the monophasic system was separated with the addition of equal volumes of chloroform and PBS (5ml). The lipid-containing phase was

concentrated *in vacuo* at 37 ^{0}C to a volume of approximately 2ml and transferred to glass vials. The lipids were then dried under a stream of N_2 and stored at –20 ^{0}C until use.

2.4.2 Lipid fractionation

Lipids were fractionated in order of increasing polarity by a modification of the method developed by Zelles and Bai (1993). Briefly, the lipid extract was resuspended in a minimal amount of chloroform and transferred to a silica gel solid phase extraction cartridge (Supelco, Oakville, ON), which had been previously conditioned with 5 ml methanol followed by 5 ml of chloroform. The lipids were then fractionated into neutral, glyco- and phospholipid-containing polar lipids with 5 ml of chloroform, 10 ml of acetone and 5 ml of methanol respectively. The phospholipid-containing polar lipid was fraction retained and dried at 40 ^{0}C under a stream of N_2.

2.4.3 Phospholipid fatty acid analysis

2.4.3.1. Mild alkaline methanolysis

Prior to methanolysis, known amounts of methyl tridecanoate (13:0) and methyl nonadecanoate (19:0) (Aldrich, Oakville, ON) were added to the polar lipid mixture. The phospholipids were then subjected to a mild alkaline methanolysis by the method of Dowling et al, (1986). Polar lipids were dissolved in 1 ml methanol/toluene (1:1, v/v), 1 ml of 0.2 M KOH in dry methanol was added and the mixtures were heated at 37 ^{0}C for 15 minutes. Sequential additions of 2 ml hexane, 0.3 ml 1 M acetic acid and 2 ml water were made. The resulting biphasic mixture was vortexed, and the hexane (upper) layer containing the fatty acid methyl esters (FAMES), was removed. The aqueous phase was

reextracted twice more with 2 ml hexane. The hexane fractions were combined, dried under a stream of N_2 and stored at –20 ^{0}C.

2.4.3.2. Fatty acid methyl ester purification

Crude FAMES were loaded onto a thin layer silica gel (Whatman KG, 0.25mm, 20 x 20 cm) plate. Methyl nonadecanoate was spotted on the end lanes of each plate. The TLC plates were then developed in hexane/diethyl ether (1:1, v/v). After development the end lanes were cut out and sprayed with 0.01% (w/v) rhodamine to detect the standard. The end lanes were then aligned with the rest of the TLC plate and areas at R_f values corresponding to the standards were then scraped off from the plate and the FAMES were eluted from the silica gel with 6 ml of hexane:diethyl ether (9:1, v/v). The solvent was dried under a stream of N_2 and stored at –20 ^{0}C.

2.4.3.3. Gas chromatography and gas chromatography mass spectrometry

The purified FAMES were dissolved in 0.1 ml hexane and analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). The column used was a 30m x 0.25mm id DB-5 capillary column (phenylmethyl silicone, J & W Scientific, Folsom CA). Hydrogen was used as the carrier gas and injections were made in splitless mode. The temperature program was as follows: initial temperature of 80 ^{0}C for 1 minute, increasing at 20 ^{0}C min^{-1} to 160 ^{0}C, then increasing at 5 ^{0}C min^{-1} to the final temperature of 270 ^{0}C, which was held for 10 minutes. Relative retention times of FAMES were compared with those of bacterial fatty acid methyl ester (BAME) standards (Supelco, Oakville, ON) (Appendix 2).

Gas chromatography/mass spectrometry analyses were carried out with a Varian 3800 gas chromatograph coupled to a Varian Saturn 2000 MS detector in EI mode. The

GC conditions were identical to those described above, but helium was used as the carrier gas and the GC oven was held at 80 ^{0}C for 2 minutes. Energy in the electron impact was 70 eV.

2.4.3.4 Fatty acid nomenclature

Fatty acids were named according to the convention X:YωZ, where "X" is the number of carbon atoms in the chain, "Y" is the number of double bonds, and "Z" is the number of carbon atoms from the methyl end of the molecule to where the first double bond is encountered. Prefixes are as follows: "i" = iso-branched (methyl branch on the second carbon from the methyl end), "a" = anteiso-branched (methyl branch on the third carbon from the methyl end), "10Me" = methyl branch on the tenth carbon from the carboxyl end, and "cy" = cyclopropyl, 2-OH and 3-OH indicate hydroxyl substituents on the second and third carbon from the carboxyl end, respectively. The suffixes "c" and "t" stand for the *cis* and *trans* geometric isomers of the double bond, respectively.

2.4.4 Microbial biomass determinations

The microbial biomass was determined for the sediment samples collected in 2001 by analysis of the lipid-phosphate content after digestion of extracted lipids with potassium persulfate (Findlay et al., 1989). Briefly, the dried lipid samples were incubated overnight at 95^{0}C with 0.45 ml of a saturated potassium persulfate solution (5% w/v) (Aldrich). Phosphate released by digestion was determined by the method of Van Veldhoven and Mannaerts (1987). A solution of 0.1 ml of ammonium molybdate [2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 5.72 N H_2SO_4] was added to the digested samples, and the mixture was allowed to stand for 10 minutes. This was followed by the addition of 0.45 ml of a solution containing 0.111% polyvinyl alcohol (100% hydrolyzed, average

molecular weight 14,000) and 0.011% malachite green in water (prepared by dissolving the polyvinyl alcohol at 80^0C, allowing to cool and adding the malachite green). The solution was then allowed to stand for 30 minutes and the A_{610} was then measured on a Pharmacia LKB Ultrospec III spectrophotometer (zero set with a cuvette filled with deionized water). Amounts of phosphate were calculated by using the regression line from a standard curve prepared by digesting 5, 10, 15 and 20 nmol glycerol phosphate as described above after subtracting a reference blank.

2.5 Microbial degradation of naphthenic acids in by mixed and pure cultures

2.5.1 Naphthenic acid-degrading enrichment cultures

A sample of wetland sediment (Natural Wetland, 1.0g, wet weight) was suspended into 25 ml of mineral salts medium containing 0.04% (w/v) of commercially available NA mixture (Kodak Chemicals, Rochester NY) as the sole carbon source. NA's were dissolved in 0.1 M NaOH to a stock concentration of 20 mg/ml and filter sterilized through a Whatman 0.45 μm filter. The mineral salts medium used throughout this study was that of Focht adjusted to pH 8.2 (1994) (see table 2.1 for a description of the components). Successive transfers (4% v/v) of the enrichment culture into fresh NA containing medium were made on a twice-weekly basis, and incubated at 28^0C on a rotary shaker (100 rpm). After four successive transfers, 0.2 ml aliquots of the enrichment culture were spread-plated on R2A (Difco) plates and incubated at 28^0C for 48 hours.

2.5.2 Identification of Naphthenic acid degrading microorganisms

2.5.2.1 Physiological identification of naphthenic acid degrading organisms

Individual colonies were removed from the R2A plates, streaked onto fresh plates to ensure purity, and then subjected to taxonomic evaluation. Phenotypic identification was performed using API 20 NE identification strips (Biomerieux, Lyons, France). Inoculation of the strips was performed according to the manufacturer's instructions and identification was done using the API 20 telephone database.

Table 2.2. Components of Focht's mineral salts medium

Stock solutions	Additions (ml)	Final concentration (mM)
K_2HPO_4, 1 M	10	10
NaH_2PO_4, 1 M	3	3
$(NH_4)_2SO_4$, 1 M	10	10
$MgSO_4$, 1 M	1	1
$Ca(NO_3)_2$, 1 M	0.1	0.1
$Fe(NO_3)_2$, 1 M	0.01	0.01
Trace Minerals (below)	1	
$MnSO_4$		0.001
$ZnSO_4$		0.001
$CuSO_4$		0.001
$NiSO_4$		0.0001
$CoSO_4$		0.0001
Na_2MoO_4		0.0001

2.5.2.1 Molecular identification of naphthenic acid degrading organisms

Individual colony types from the R2A plates were streaked onto fresh plates to ensure purity. Subsequently, single colonies were used to inoculate 5 ml of tryptic soy broth (TSB) (BDH), which was then incubated overnight at 28^0C on a rotary shaker at 100 rpm. These cultures were then transferred to 15 ml polypropylene centrifuge tubes (Falcon), centrifuged for 15 minutes at 2500 g. To remove residual medium, the pellets were resuspended in sterile PBS and centrifuged twice more as described above.

DNA was extracted using a modification of the method of Dewettinck et al, (2001). Briefly, the entire bacterial suspension was transferred to a 15 ml polypropylene centrifuge tube and 3 ml of 0.1 M sodium phosphate buffer (pH 8.0) was added. An additional 11 ml of a solution containing 100 mM NaCl, 500 mM tris-HCl and 10% SDS (pH 8.0) was then added. The mixture was boiled for 10 minutes after which time it was cooled on ice and vortexed. A 5 ml aliquot was removed and 2 ml NH_4OAc were added. The sample was then cooled at 4^0C for 5 minutes and 400 µl were aliquoted to sterile 1.5 ml centrifuge tubes. DNA was then precipitated by the method of Sambrook et al. (1989). Briefly, 800 µl of ice-cold 95% ethanol were added to the DNA containing solution, followed by storing the samples at -20^0C for 30 minutes after which time, the samples were centrifuged at 14000 g, the supernatants removed and the pellet air dried. The DNA samples were then resuspended in 100 µl sterile H_2O.

Polymerase chain reaction (PCR) analysis was carried out using primers flanking the V8 region of the 16S rRNA gene (Marchesi 2001). Bacterial DNA fragments were amplified in a total volume of 50 µl. The components of the PCR mixture were as follows: 5 µl of 10 X PCR buffer, 200 µM of deoxynucleotide triphosphate, 0.5 µM each of the universal bacterial primers 986F and 1406R (Invitrogen), 2 units of *Vent* DNA polymerase (New England Biolabs, Mississauga, ON). Thermocycling conditions were as follows: initial denaturation at 94^0C for 3 minutes; 25 cycles of 94^0C for 30 s, 50^0C for 45 s, and 90 seconds at 72^0C; followed by a final extension of 5 minutes. PCR products were analyzed by 1% agarose gel electrophoresis separation and ethidium bromide staining comparing the bands to a 1-kb ladder molecular weight standard.

2.5.3 Extraction of naturally occurring naphthenic acids

Free-water from the NW and WH sites were collected in late August 2001 and the NA's were extracted by a modification of the method of Holowenko (2001). Briefly, 10 L of water from each site (containing the sodium salts of the NA's) were combined and acidified with H_2SO_4 to pH 2-3 to precipitate acids out of solution and was allowed to settle for a week. After settling of the suspended solids, the water was siphoned off until approximately 500 ml remained. The pH of the solution was then brought up to 9 and the undissolved solids were filtered though a funnel, which had been plugged with glass wool. This solution was again acidified to pH 2-3 with H_2SO_4 and transferred to a separatory funnel were it was extracted 6 times with equal volumes of dichloromethane. The organic phase was concentrated *in vacuo* at 37 ^{0}C to a volume of approximately 2 ml and transferred to pre-weighed glass vials. The NA's were then dried under a stream of N_2, weighed and stored at –20 ^{0}C until use.

2.5.4 Microbial degradation of naphthenic acids

Erlenmeyer flasks containing 5 ml of fresh mineral salts medium plus 0.04% (w/v) of either Kodak NA or NA's extracted from NW and WH as the sole carbon source were inoculated in duplicate with 200 µl of the fourth transfer of microorganisms obtained from the enrichment cultures and incubated at 28^{0}C for a period of one, two and four weeks. Single culture studies were performed as described above except that the medium was inoculated with a single colony isolated from the NA-degrading enrichment cultures and incubated for a period of four weeks. Control flasks received an inoculum of heat-killed cells.

After the incubation period, the cultures were transferred to 15 ml polypropylene centrifuge tubes, and centrifuged for 25 minutes at 2500 g. The supernatants were removed and transferred to separatory funnels. The pellets were resuspended in 5 ml of NaH_2PO_4 buffer (pH 10) and centrifuged twice more as described above to remove all traces of NA, and the supernatants were pooled.

A 0.1 ml volume of caprylic acid (C8 straight chain fatty acid, 100ng/µl, Sigma) was added to the separatory funnels containing the supernatants. The samples were acidified to pH <2 using 50 µl of concentrated HCl and then extracted four times with 15 ml dichloromethane (OmniSolv grade, BDH) and the solvent was dried over anhydrous Na_2SO_4 and flash evaporated at 35^0C. The extracts were then transferred to 2 ml vials and the solvent was evaporated under a gentle stream of N_2. Carboxylic acids were derivatized into their *tert.*-butyldimethylsilyl esters by the method of St. John et al. (1998). Briefly, 100 µl of the derivatizing reagent (N-methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA, Aldrich) containing 1% t-BDMS-chloride) was added to the samples, which were then heated at 60^0C for 20 minutes. Following the incubation, the samples were evaporated under a stream of N_2 in order to remove all traces of the derivatizing reagent and stored at -20^0C until used.

The samples were resuspended in 1 ml hexane and 2 µl were injected in splitless mode into a Varian 3800 gas chromatograph fitted with a 30m x 0.25mm i.d. DB-5 capillary column (J & W Scientific, Folsom CA). The temperature program was as follows: 100^0C for three minutes, increasing at 8^0C/min to 300^0C where it as was held for 10 minutes. The injector temperature was 280^0C, and helium was used as the carrier gas.

The eluted samples were detected with a Varian Saturn mass spectrometer in EI mode (approximately 70 eV). The mass spectrometer was controlled using Saturn 2000.40 software. The MS did not collect ions for the initial 6 minutes of the run and thereafter was set to report ions within the mass range of 100 to 555. Upon completion of the run, the data from 10 minutes to the end was averaged to produce a single spectrum of "averaged" ion intensities. No background subtraction was applied and approximately 3000 scans were averaged to obtain this data.

The molecular mass of the $[M-57]^+$ ions and their averaged intensities were grouped into Carbon number and Z family and entered into an Excel spreadsheet as described by Holowenko et al. (2001). The data was normalized as percentages and used to generate three-dimensional plots. Total amounts of NA's were calculated by converting the ion intensities into nanograms by using the regression line from a standard curve generated by analyzing the averaged spectrum generated by 2, 20, 50 and 200 ng of the *tert.*-butyldimethylsilyl esters of caprylic acid as described above (Appendix 5).

2.6 Statistical analysis

The mol % of individual PLFA was entered on a spreadsheet into the JMP program (SAS Institute) and dendrograms were generated according to the Ward method. Means comparisons for microbial biomass determinations and selected PLFA were performed using two way analysis of variance (ANOVA) and Student's t-test analysis respectively also in JMP. In order to simplify the biomass and PLFA data, the samples were combined into the following two categories: Offsite (NA levels < 5 mg/L and comprising H63, FMK, and TR) and Impacted (NA levels > 5mg/L and comprising CL, HS NW, ImCT, SP, WH, P5, MFTS and MFTN). Means comparisons were performed

between the two different categories for each month in the two sampling seasons. For the biomass analysis the sample sizes were as follows: Offsite n= 9, Impacted n= 27 except for the Impacted sites in June of 2001 in which n= 24. For the PLFA analysis data, sample sizes were as follows: Offsite n=3, Impacted n= 9, except for the Impacted sites in August of 2000 and June of 2001 in which n= 8.

CHAPTER THREE: RESULTS

3.1. Microbial community and biomass analysis

3.1.1. Viable Biomass Determinations

The amount of viable biomass in each wetland sediment for the samples collected in 2001 was determined by the quantification of organic phosphate released from the polar lipids in the total lipid extract by the method of Findlay et al. (1989, see Materials and Methods). The amount of the lipid-phosphate in the individual sites is shown in Table 3.1. In Figure 3.1, data from the 12 sites were grouped into Two categories: Offsite (NA levels < 5 mg/L and comprising H63, FMK, and TR), Low and high impact (NA levels > 5 mg/L and comprising NW, CL ,1mCT, SP, WH, HS, P5, MFTS and MFTN)

Table 3.1 shows that the amount of viable biomass was highly variable in the twelve sites ranging from 7.75 nmol lipid phosphate/g dry sediment (P5, August) to 278.89 nmol lipid phosphate/g dry sediment (CL, July). Nevertheless, a pattern emerged such that with the exceptions of NW, WH, and TR, there was a decrease in the amount of viable biomass between June and July followed by an increase in August. NW and WH increased in July from 105.38 and 54.18 nmol/g dry sediment to 167.50 and 98.71 nmol/g dry sediment respectively. TR also increased in July from 69.72 to 101.59 nmol of lipid-phosphate/g dry sediment followed by a smaller increase to 116.47 nmol of lipid-phosphate/g dry sediment in August. The lowest amounts of viable biomass were found in P5 (12.95 and 7.75 nmol of lipid-phosphate/g dry sediment in June and July respectively). This is expected as P5 is an active tailings pond and is continuously

receiving process-affected water, containing large amounts of NA's at concentrations, which may be toxic to microorganisms, i.e., inhibit their own degradation pathway. Another interesting finding was that MFTS always appears to possess half the viable biomass as MFTN. This was unexpected since the two sites are similar in size and depth, receive the same input water, and are located within several meters of one another.

Table 3.1. Viable microbial biomass in various wetland sediments for the year 2001. Assessed by measuring the total amounts of lipid-bound phosphate and reported as the nmols of lipid-bound phosphate/gram dry sediment. Numbers in brackets indicate standard deviation (n = 3).

Site	June	July	August
NW	105.4 (14.4)	167.5 (11.6)	87.6 (6.1)
CL	150.1 (49.7)	284.6 (17.7)	278.9 (22.3)
ImCT	49.2 (11.1)	28.5 (10.4)	130.1 (14.2)
SP	38.9 (25.4)	28.8 (5.5)	124.1 (24.4)
WH	54.2 (2.7)	98.7 (8.1)	73.7 (6.2)
H63	50.9 (7.7)	47.7 (7.3)	89.0 (10.1)
HS	96.9 (7.9)	106.2 (15.6)	111.9 (8.3)
FM	84.0 (18.4)	84.1 (13.4)	50.0 (21.1)
TR	69.7 (10.4)	101.6 (29.3)	116.5 (10.2)
P5*	NA	13.0 (2.8)	7.8 (1.2)
MFTS	25.6 (13.1)	27.6 (0.7)	40.1 (3.0)
MFTN	58.8 (17.7)	60.7 (1.7)	76.7 (4.7)

Figure 3.1 shows that the amount of viable biomass is uniform between the two categories in June, July and, August. This suggest that despite the high input of process-

* NA = data not available.

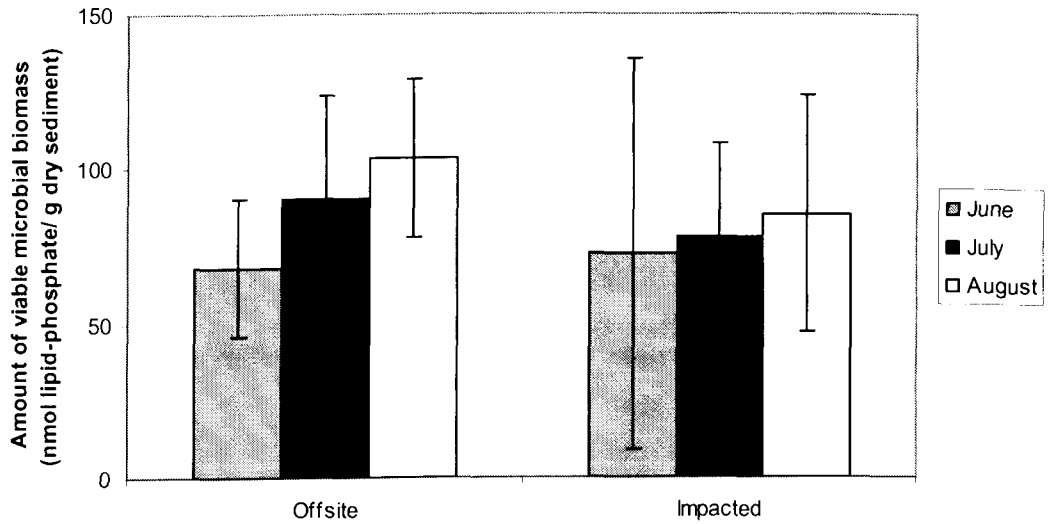


Figure 3.1. The amount of viable microbial biomass in the various wetland sediments in 2001. Viable biomass was estimated by measuring the total amounts of lipid-bound phosphate. Samples are arranged according to their degree of NA contamination: offsite n= 9, impacted (n= 27). The samples were obtained in the months of June, July, and August of 2001. Bars represent the mean of the sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that none of the differences observed between the two categories were statistically significant ($p>0.05$).

affected water and its associated nutrients there may have been toxicity associated with these higher input levels that prevented the microbial community from exploiting the additional carbon present in the impacted sites. It should also be noted that microbial biomass was not affected by particle size. This is supported by the fact that several sites that possess a similar range of particle sizes such as P5, MFTS, and MFTN, (Appendix 2) exhibited had very different amounts of microbial biomass.

3.1.2. Microbial Community Analysis

The differences in the microbial community structures of the various wetland sediments was compared using a technique known as phospholipid fatty acid (PLFA) analysis. In this technique, the fatty acid composition of phospholipids purified from the various sediments was determined and quantified by GC and GC/MS analysis by the method of Frostegard et al. (1997). The mol % of the individual PLFA was determined, for each sediment, and these values were then entered into a spreadsheet in the JMP program and dendograms were generated using Ward's method (Figures 3.2 and 3.3).

Our results for the year 2000 indicate that the differences in microbial community structure assessed by PLFA analysis were unrelated to the amount of NA's in the free water (Figure 3.2). However, the consistent clustering of sites 10, 11, and 12 (P5, MFTS, and MFTN) may indicate an effect of sediment-bound oil on the microbial community. This is possibly due to the hydrocarbons in the oil being a more readily degraded carbon source thereby shifting the community structure towards an oil-degrading community. However, in 2001 there appeared to be no relationship between the sediment's microbial communities and [NA] or sediment-bound oil (Figure 3.3). The dendograms also show that in all three months of 2000 and in June and July 2001, sites 1 and 2 (NW and CL)

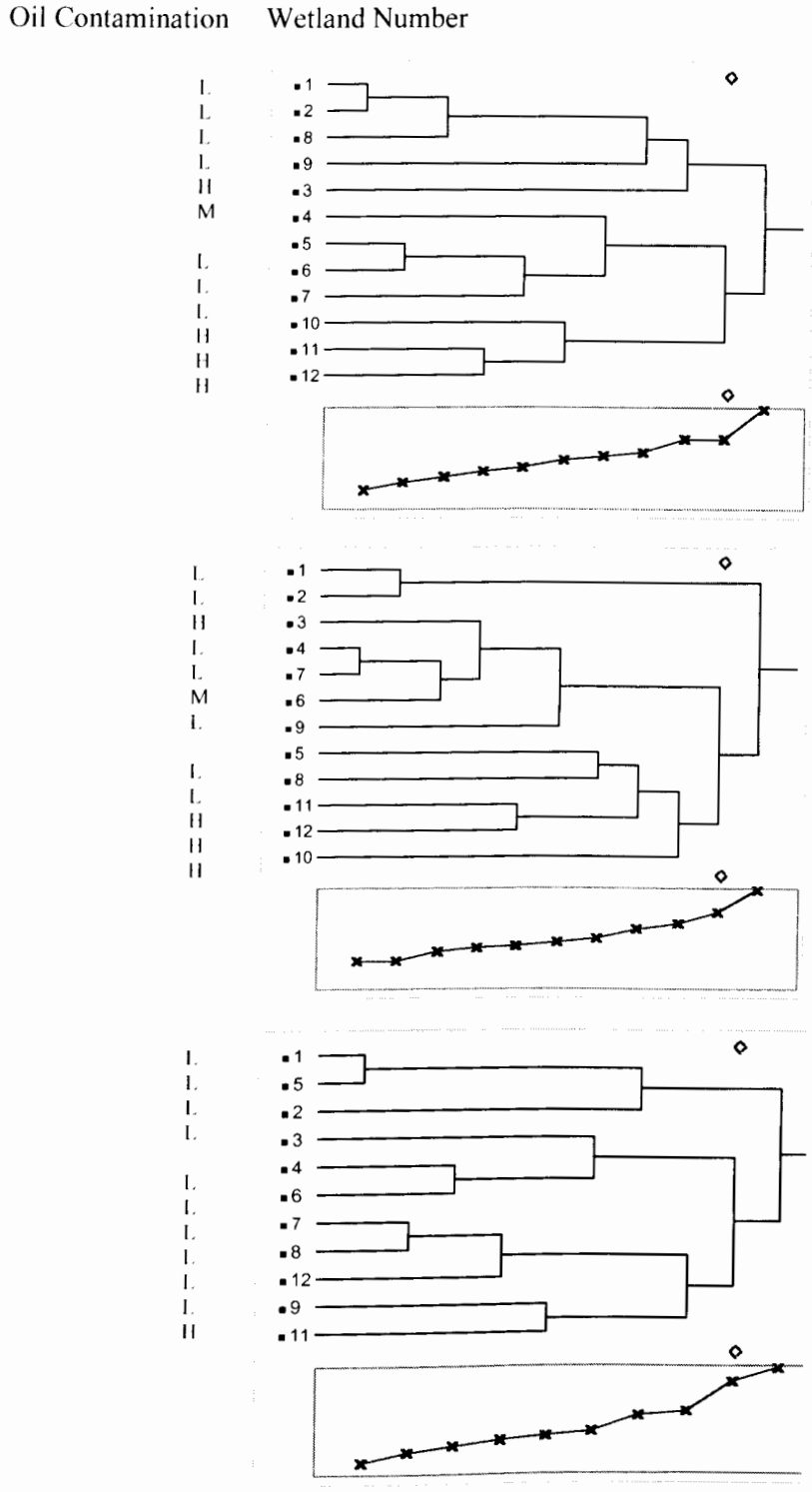


Figure 3.2. Sediment microbial community comparisons based on the PLFA data obtained from sediments sampled in the year 2000. Dendrograms were generated by Ward's method using the total PLFA data in mol %. From top to bottom: June, July and August 2000. L indicates low oil contamination (0-0.49 %), M indicates moderate oil contamination (0.50-0.75 %) and, H indicates high oil contamination (> 0.75%). See Materials and Methods p. 24 for each site's numerical code.

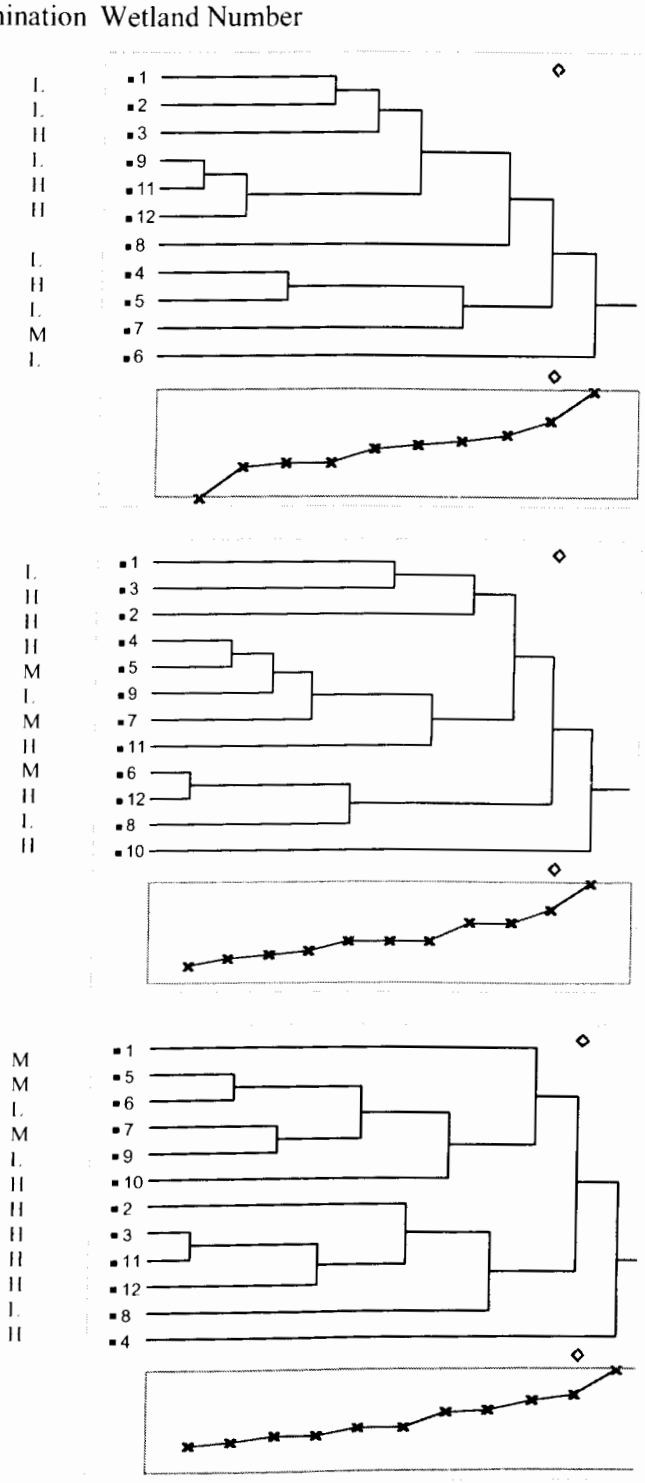


Figure 3.3. Sediment microbial community comparisons based on the PLFA data obtained from sediments sampled in the year 2001. Dendrograms were generated by Ward's method using the total PLFA data in mol %. From top to bottom: June, July and August 2001. L indicates low oil contamination (0-0.49 %), M indicates moderate oil contamination (0.50-0.75 %) and, H indicates high oil contamination (> 0.75%). See Materials and Methods p.24 for each site's numerical code.

cluster together (Figures 3.2 and 3.3) suggesting that their microbial communities are similar. Although the extent of NA input is very different (NW has approximately ten times the amount of NA's as CL), NW and CL are both very shallow (approximately one meter), produce of large amount of anaerobic gas, possess similar amounts of sediment-bound oil (0.2%), and support a wide variety of organisms including birds, frogs, invertebrates and plants. These results indicate that PLFA clustering was influenced by factors other than NA concentration. One similarity between these two wetlands was the presence of large concentrations of macroinvertebrates (mainly chironomid larvae, assessed visually) in the NW and CL wetlands, which may have influenced the PLFA profiles.

The shifts in specific PLFA types as well as individual PLFA's was measured to obtain information on the specific groups of microorganisms, which make up the various microbial communities as well as the communities' physiological status (White et al, 1996, Zellis, 1999). To simplify the interpretation, the 12 sites were grouped into two categories related to their level of NA's: Offsite (NA levels < 5 mg/L and comprising H63, FMK, and TR), Low impact (NA levels between 5 and 10 mg/L and comprising CL and HS) and high impact (NA levels > 5 mg/L and comprising NW, CL, 1mCT, SP, WH, HS, P5, MFTS and MFTN). The PLFA data from these groups is summarized on Table 3.2.

The proportion of Gram-negative anaerobes versus Gram-positive aerobes was measured as the ratios of the *iso*15:0/*anteiso*15:0 and PLFA and *iso*17:0/*anteiso*17:0 PLFA with a ratio of approximately 0.2 being indicative of Gram-positive aerobes and a ratio greater than 5 indicating Gram-negative anaerobes (White et al. 1996). These data

Table 3.2. Mole percentage distribution of PLFA's in sediment samples. Normal Saturates are straight chain saturated PLFA's, Branched saturates are PLFA's possessing a methyl branch in the chain, MUFA are monounsaturated PLFA's, cy, are PLFA possessing a cyclopropyl chain, Lin is linolenic acid, and Hydroxy are PLFA containing a hydroxy group on carbons two or three. Results shown are the mean ± S.D. (in parentheses, offsite n= 3, Low Impact n= 2, and High Impact n= 7).

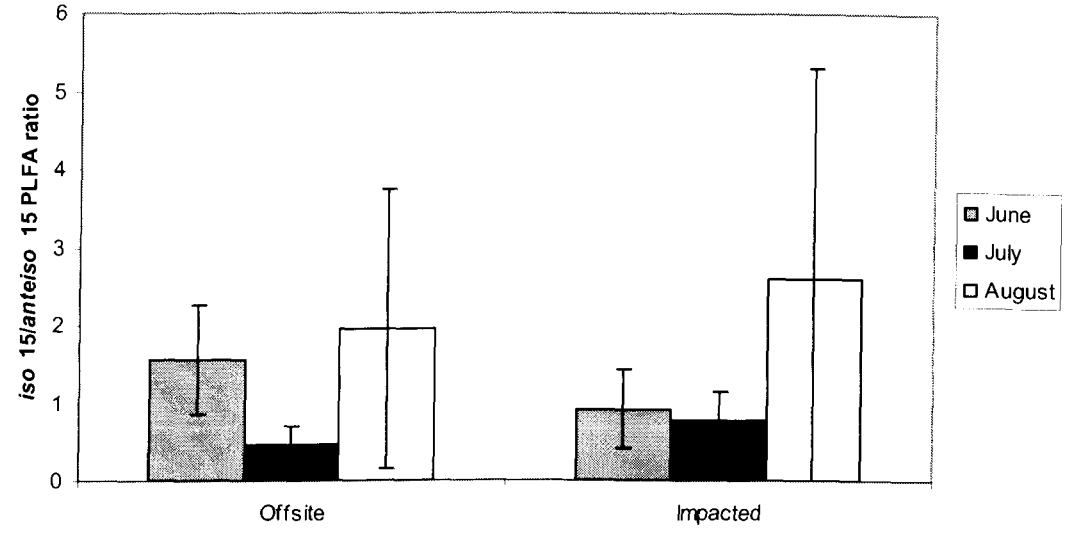
		June			July			August		
		Offsite	Low Impact	High Impact	Offsite	Low Impact	High Impact	Offsite	Low Impact	High Impact
PLFA type	Year									
Normal saturates	2000	38.04	48.32	39.19	68.99	63.45	61.67	36.44	31.92	30.20
		(10.94)	(15.54)	(14.98)	(14.80)	(26.52)	(13.36)	(4.40)	(5.42)	(8.30)
	2001	18.83	39.22	31.35	29.13	33.40	35.09	32.19	28.76	34.19
		(5.89)	(8.87)	(7.09)	(3.98)	95.12)	(9.44)	(4.45)	(5.03)	(5.08)
Branched saturates	2000	15.32	9.83	14.25	6.98	11.28	8.87	23.29	26.68	28.10
		(1.18)	(8.34)	(4.53)	(4.77)	(9.41)	(4.23)	(5.39)	(14.09)	(3.63)
	2001	12.89	11.55	9.32	7.23	16.84	11.14	8.62	11.77	12.26
		(8.80)	(8.25)	(3.59)	(2.63)	(0.14)	(4.65)	(4.23)	(3.80)	(5.52)
MUFA	2000	31.58	30.63	32.27	18.51	19.31	21.13	25.52	27.33	25.27
		(7.57)	(1.63)	(7.77)	(8.04)	(13.59)	(7.69)	(8.16)	(10.33)	(8.25)
	2001	49.87	38.81	46.33	49.35	38.27	41.44	45.82	48.04	37.42
		(13.73)	(0.88)	(8.02)	(5.14)	(0.40)	(9.80)	(6.24)	(13.88)	(10.13)
cy	2000	1.68	1.78	2.38	0.89	1.15	1.91	7.02	5.69	6.65
		(0.14)	(0.19)	(1.21)	(0.49)	(1.23)	(1.54)	(3.29)	(0.21)	(3.71)
	2001	2.86	2.93	2.61	4.07	4.54	4.07	4.24	4.68	3.66
		(2.68)	(0.28)	(0.99)	(1.09)	(2.91)	(1.90)	(2.96)	(2.83)	(1.99)
Lin	2000	0.95	3.03	3.03	2.43	2.04	2.67	2.89	1.22	0.76
		(0.48)	(1.79)	(1.37)	(0.88)	(0.11)	(1.68)	(2.07)	(1.73)	(1.07)
	2001	7.64	6.42	8.22	10.19	6.14	7.95	6.81	5.28	6.63
		(3.74)	(1.54)	(1.56)	(2.32)	(1.75)	(3.47)	(5.00)	(1.20)	(4.85)
Hydroxy	2000	12.43	6.43	8.88	2.29	2.78	3.76	4.85	7.17	9.01
		(2.81)	(7.18)	(3.70)	(2.02)	(2.40)	(2.46)	(2.41)	(3.17)	(6.87)
	2001	7.91	1.09	2.18	0.68	0.81	0.54	2.32	1.48	5.73
		(11.54)	(1.00)	(1.57)	(0.71)	(0.72)	(0.78)	(2.50)	(1.02)	(7.13)

for 2001 and 2001 are shown in Figures 3.4 and 3.5, respectively. In the year 2000, the *iso*15:0/*anteiso*15:0 ratios indicate that the proportion of Gram-negative anaerobes was relatively stable between the two categories in June and July. However a large increase in the variability is observed in August as indicated by the size of the error bars (Figure 3.4). This rather large increase in variability can be partially explained for the impacted sites by an unusually large increase in the proportion of Gram-negative anaerobes in the CL site and is not statistically significant. This is supported by the observation that CL produces a large amount of anaerobic gas. These results indicate a temporal effect in the proportion of Gram-negative anaerobes versus Gram-positive aerobes for the year 2000. In 2001, the *iso*15:0/*anteiso*15:0 was significantly lower and more uniform across the three months within each category, (Figure 3.4). These results suggest that the increase in the proportion of Gram-negative anaerobes versus Gram-positive aerobes observed in August 2000 did not occur in 2001.

In contrast, the large increase in the proportion of Gram-negative anaerobes in the CL site was not observed when the *iso*17:0/*anteiso*17:0 PLFA ratios were calculated (Figure 3.5). The results for 2000 and 2001 show that the proportion of Gram-negative anaerobes was uniform in both categories for the three sampling months. However in June 2001, the off-site wetlands appear to have a higher proportion of Gram-negative anaerobes due to a higher than usual ratio for the month of June in the H63 site (2.85 mol%). However these results are not statistically significant.

The genus *Pseudomonas* is a highly heterogeneous taxon composed of 70 or more species of Gram-negative, strictly aerobic, motile bacilli. They are able to utilize a wide variety of organic molecules as sources of carbon, and are therefore very important in the

2000



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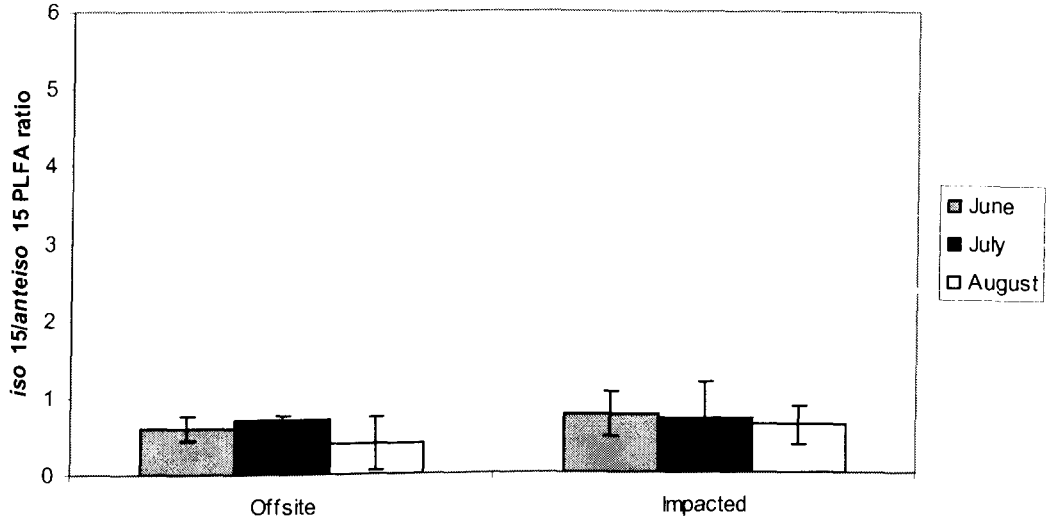

Figure 3.4. The proportion of Gram-positive aerobes vs. Gram-negative anaerobes in wetland sediments, assessed by measuring the amounts of *iso*15:0 and *anteiso*15:0 PLFA and calculating the *iso/anteiso* ratio. A ratio greater than 5 indicates a predominance of Gram-positive anaerobes whereas a ratio of approximately 0.2 indicates a predominance of Gram-positive aerobes. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9). The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the two categories were not statistically significant ($p>0.05$).

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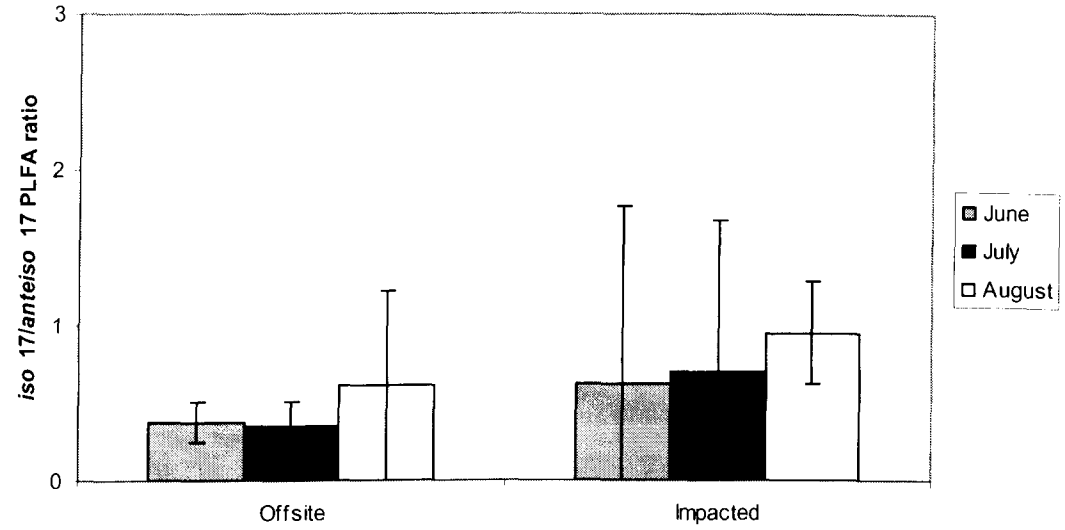

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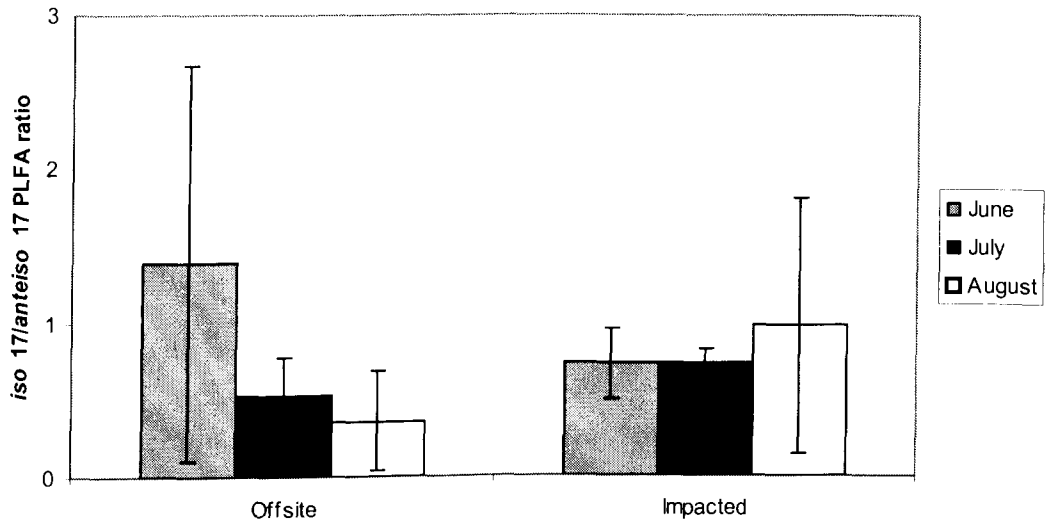
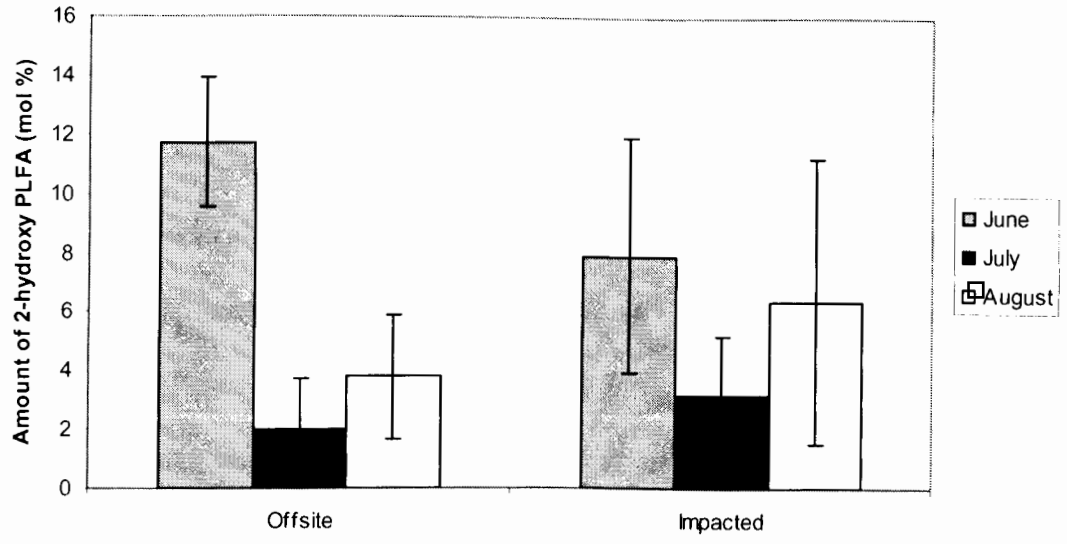


Figure 3.5. The proportion of Gram-positive aerobes vs. Gram-negative anaerobes in wetland sediments. Assessed by measuring the amounts of *iso*17:0 and *anteiso*17:0 PLFA and calculating the *iso/anteiso* ratio. A ratio greater than 5 indicates a predominance of Gram-positive anaerobes whereas a ratio of approximately 0.2 indicates a pedominance of Gram-positive aerobes. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9).. The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the two categories were not statistically significant ($p>0.05$).

mineralization process both in nature and in sewage treatment (Prescott et al, 1996). The proportion of pseudomonads in the sediment samples was measured as the sum of the 2-hydroxy14:0 and 2-hydroxy16:0 PLFA's (Zellis et al. 1999, Figure 3.6.). In 2000, the number of pseudomonads were highest in June (12.42 and 8.86 mol % for the offsite and impacted sites, respectively), decreased in July (1.63 and 3.36 for the offsite and impacted sites, respectively) and increased slightly in August (3.69 and 7.21 mol % for the offsite and impacted sites respectively). There was no relationship between the proportion of pseudomonads and the degree of NA contamination. Rather, it was dominated by the monthly and yearly seasonal changes. In 2001, the proportion of pseudomonads decreased significantly in all sites, nevertheless, as observed in 2000, values in June were the highest (Figure 3.6). It is possible that the differences observed in the proportions of Gram-negative anaerobes and pseudomonads (Gram-negative aerobes) were partly due to large differences in rainfall in 2000 vs. 2001 (possibly due to the increase in nutrients such as carbon sources from decaying plants from run-off water that is associated with higher precipitation).

Another group of soil microorganisms that have a wide metabolic capability are the actinomycetes. These are Gram-positive bacteria that possess filamentous hyphae that do not undergo fragmentation and produce asexual spores. Furthermore, they are widely distributed and can degrade a wide variety of organic compounds. They are also responsible for the production of the majority of the medically useful antibiotics (Prescott, 1996). The proportion of actinomycetes in the sediment samples was measured as the sum of the 10Me16:0, 10Me17:0 and the 10Me18:0 PLFA's (White et al, 1996). The results in Figure 3.7 indicate that in 2000, the number of actinomycetes was uniform

2000



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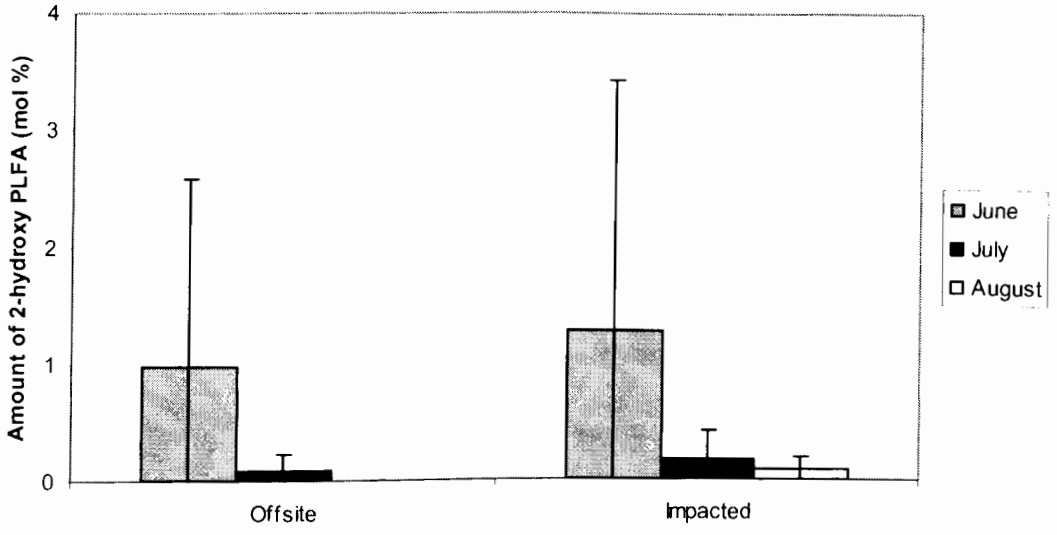


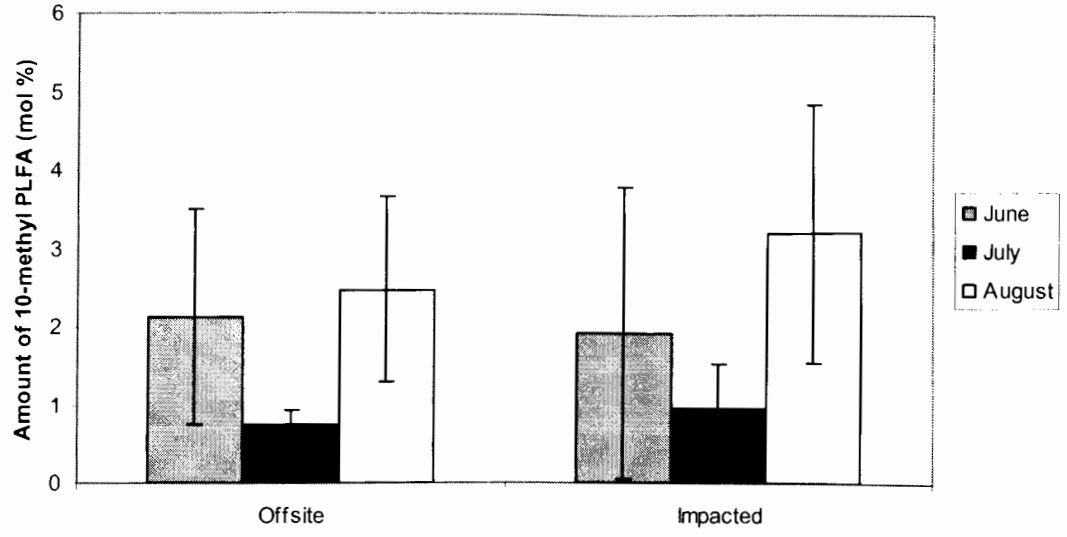
Figure 3.6. The proportion of pseudomonads in the total microbial community of sediment samples, Assessed by measuring the amounts of 2-hydroxy PLFA's. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9). The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the two categories were not statistically significant ($p>0.05$).

across the two categories in June and August, but decreased significantly in July in both categories (from approximately 2.00 mol% to 0.80 and 1.00 mol% for the offsite and impacted sites, respectively). There was no relationship between the number of actinomycetes and the degree of NA contamination. In 2001, the number of actinomycetes was similar between the two categories except for August when the proportion of actinomycetes more than doubled to about 2.00 mol% in the impacted sites (Figure 3.7). These results also show that in August of 2000, the wetland conditions caused a simultaneous increase in both the proportion of actinomycetes, the proportion of Gram-negative anaerobes (Figures 3.4). However, only the results observed in June 2001 were statistically significant.

Microeukarytes such as fungi and protozoans, can be predatory to the bacteria in the wetland sediments thereby maintaining a fine balance between the microrganisms in the sediments and possibly affecting the bioremediation potential of the different sediments (Zelles, 1999). The proportion of microeukaryotes in the sediment samples was measured as the amount of linolenic acid (18:2ω3, White et al, 1996). Figure 3.8 shows that in the 2000 samples, the proportion of microeukaryotes was lower in the offsite wetlands in June, uniform in July and lower in the impacted sites in August. The proportion of microeukaryotes in 2001 was almost four-fold higher than in 2000 although there was no difference between the two categories in the three months. However, only the results observed in June 2000 were statistically significant.

Finally, another factor affecting the degradation of organic compounds by microorganisms in wetland sediments is the presence or absence of nutrients. This is reflected by the physiological state of the microorganisms with those in nutrient-poor

2000



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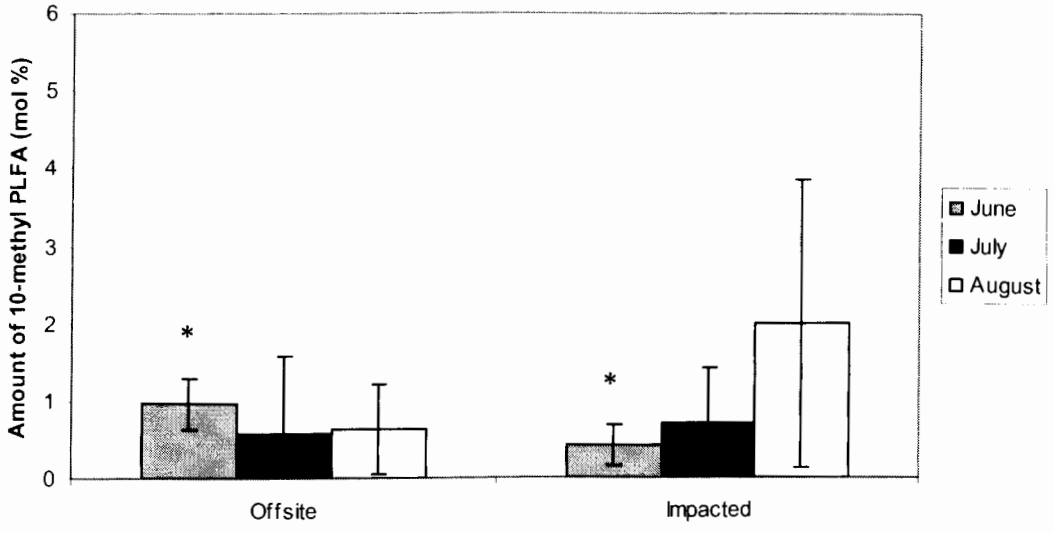
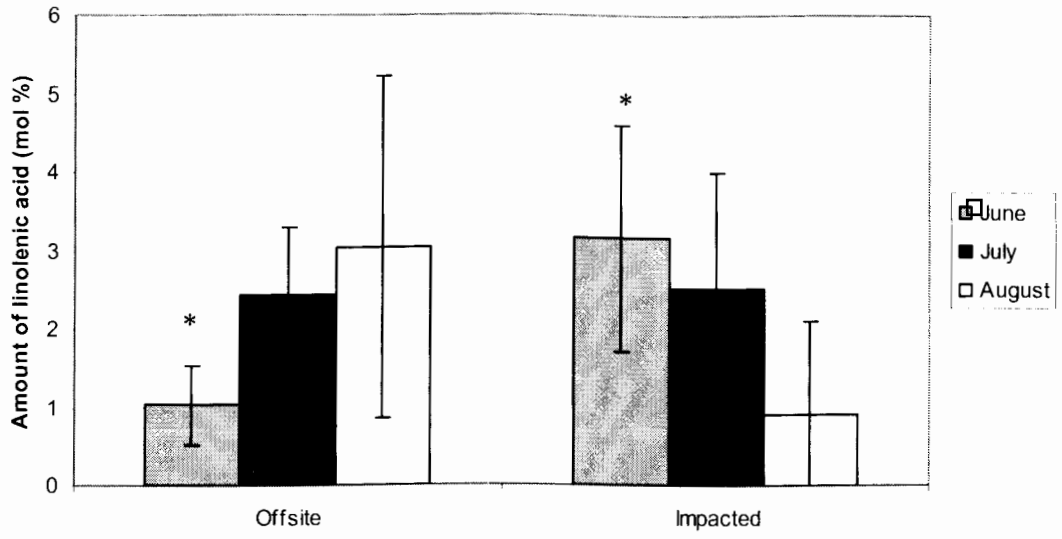


Figure 3.7. The proportion of actinomycetes in the total microbial community of sediment samples, assessed by measuring the amounts of 10-methyl PLFA's. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9). The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the offsite and impacted sites in June of 2001 were statistically significant ($p<0.05$).

2000



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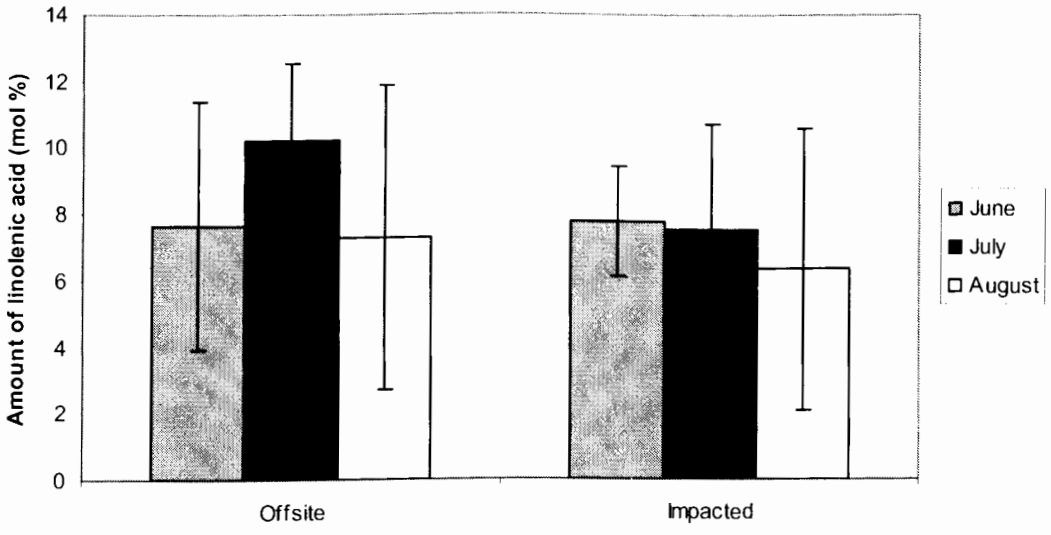

Figure 3.8. The proportion of microeukaryotes such as fungi and algae in the total microbial community. Assessed by measuring the amounts of linolenic acid. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9). The samples were obtained in the months of June (a), July (b), and August (c) of 2000 and 2001. Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the offsite and impacted sites in June of 2000 were statistically significant ($p<0.05$).

sites being mostly in the stationary phase of microbial growth whereas those in nutrient-rich sites are mostly in the logarithmic phase of microbial growth (White et al. 1996). The proportion of microorganisms in stationary phase was measured as the sum of the cy17:0 and cy19:0 PLFA (White et al. 1996, Zellis, 1999, Figure 3.9). In 2000, the proportion of microorganisms in stationary phase was higher in August for both categories when compared to June and July (Figure 3.9). In 2001, the proportion of microorganisms in stationary phase was uniform across the two categories and in the three months. These results indicate that in 2000, nutrients were readily available in June and July of 2000, and became limiting in August. Whereas in 2001, similar amounts of nutrients were probably available in all three months (Figure 3.9). However data obtained from the chemical analysis of the free waters shows that nutrients such as phosphorus and nitrogen were usually below detection level (possibly due to the long period between sample collection and analysis) and that other nutrients such as Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} were present in similar amounts (for example in June 2000, the concentration of Mg^{2+} ranged from 14.8 to 77.1 mg/L in the low and high impact wetlands and from 6.3 to 39 mg/L in the offsite wetlands).

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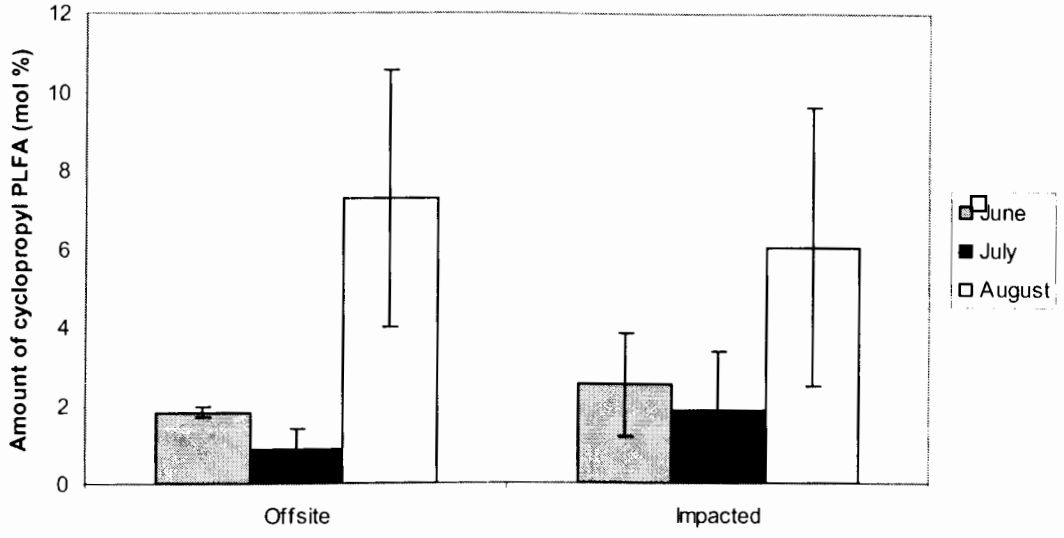

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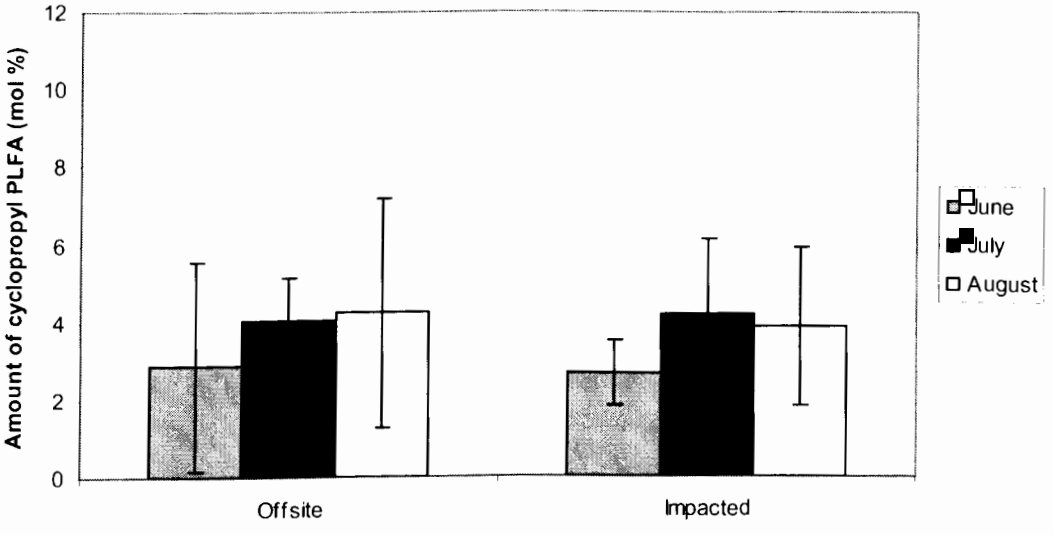


Figure 3.9. The proportion of microorganisms in stationary phase in the total microbial communities of wetland sediments. Stationary phase was assessed by measuring the total amounts of the cyclopropyl PLFA's in each sediment sample. The samples are arranged according to their degree of NA contamination: offsite (n= 3), and impacted (n= 9). The samples were obtained in the months of June (a), July (b), and August (c) of 2000 (top) and 2001 (bottom). Bars represent the mean of composite sediments for each of the three groups ± standard deviation. Student's t-test analysis shows that the differences observed between the two categories were not statistically significant ($p>0.05$).

3.2. Microbial degradation of surrogate naphthenic acids in microcosms

3.2.1. ^{14}C-cyclohexane carboxylic acid (CCA)

The ability of the microbial communities in various wetland sediments to degrade the monocyclic (Z = -2) NA surrogate cyclohexane carboxylic acid (CCA) was assessed by incubating the sediments with their natural waters and ^{14}C-CCA. The release of $^{14}CO_2$ from the microcosms was monitored over a two-week period by trapping the CO_2 in scintillation fluid containing ethanolamine (SCMMEA) by the method of Lai et al. (1996) (described in Materials and Methods pp. 25-27). The degradation rates were then calculated from the linear portion of the ^{14}C-CCA degradation profile (days 0-4, Figure 3.10). The rates or maximum amounts of CCA degraded over 14 days were arranged in order of increasing NA concentration. To simplify the interpretation, and make the results comparable to those obtained from the PLFA analysis, the individual CCA degradation rates were combined into three categories: Offsite (comprising H63, FMK, and TR), Low impact (comprising CL and HS) and High impact (comprising NW, 1mCT, SP, WH, P5, MFTS and MFTN) as described above.

In 2000, the total amount of ^{14}C-CCA mineralized was relatively uniform across all sites with 20 to 30% of the radioactive label being released as $^{14}CO_2$ (Figure 3.11). A noticeable exception occurred in two off-site samples, the FM site in which the total amount of $^{14}CO_2$ released was approximately 14 and 8%, in June and July respectively, and in the H63 site in July in which the amount of $^{14}CO_2$ released was approximately 15%. In August, there was an observable decrease in the total amount of ^{14}C-CCA degraded across the 12 sites with only 8.4 ± 3.2% to 17.9 ± 17.4% of the radioactive label

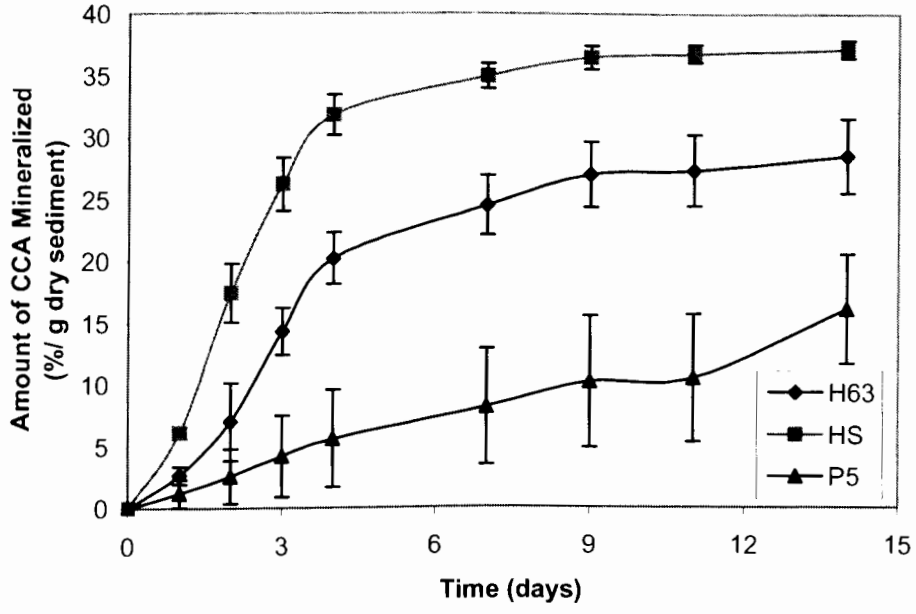
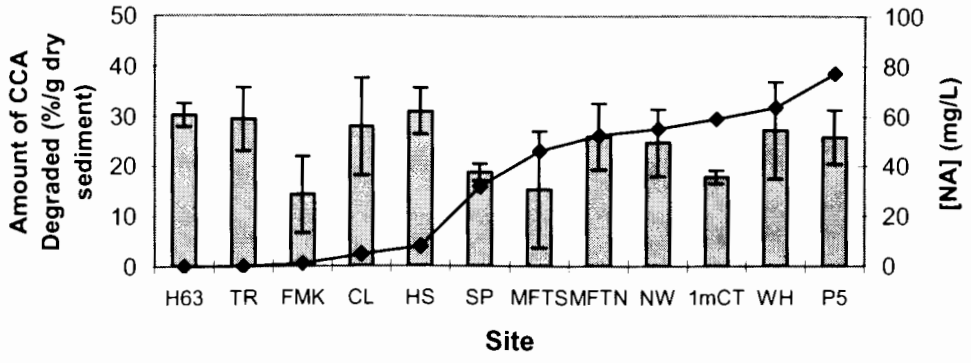
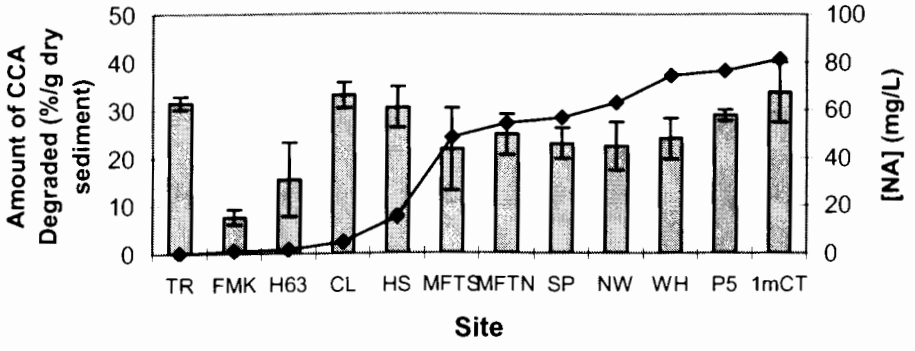


Figure 3.10. A typical CCA mineralization profile by three sediment microbial communities. The production of $^{14}CO_2$ from ^{14}C-labelled CCA was measured over 14 days in a microcosm containing sediment plus water from an off-site wetland (H63), a large wetland in a reclaimed area containing very low levels of NA contamination (HS), and an active tailings pond (P5). Samples were collected and analyzed in July 2001 as stated in Materials and Methods.

June



July



August

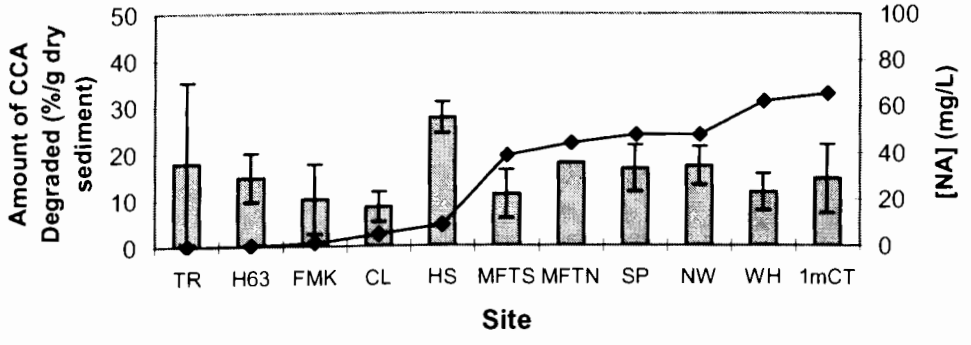


Figure 3.11. ^{14}C-CCA mineralization by sediment samples collected in 2000. The production of $^{14}CO_2$ from ^{14}C-labelled CCA was measured for a period of 14 days in a microcosm containing sediment plus their own water. The samples were obtained in June, July, and August 2000. Sites are arranged in order of increasing NA concentration. Bars represent the mean ± standard deviation for three independent sediment samples A, B, and C. The line represents the concentration of NA's in the free water

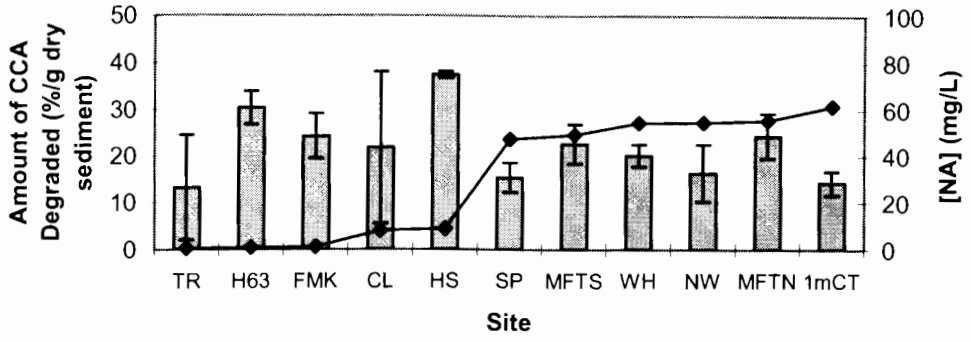
being released. An exception to this finding was observed in the HS site in which no noticeable difference was observed (Figure 3.11).

In 2001 the total amount of ^{14}C-CCA degraded also appeared to be uniform than in 2000 with approximately 20 to 30% of the radioactive label being releases as $^{14}CO_2$. Again, there was a noticeable decrease in the total amount of ^{14}C-CCA degraded across the 12 sites with 12 to 25% of the radioactive label being released (Figure 3.12).

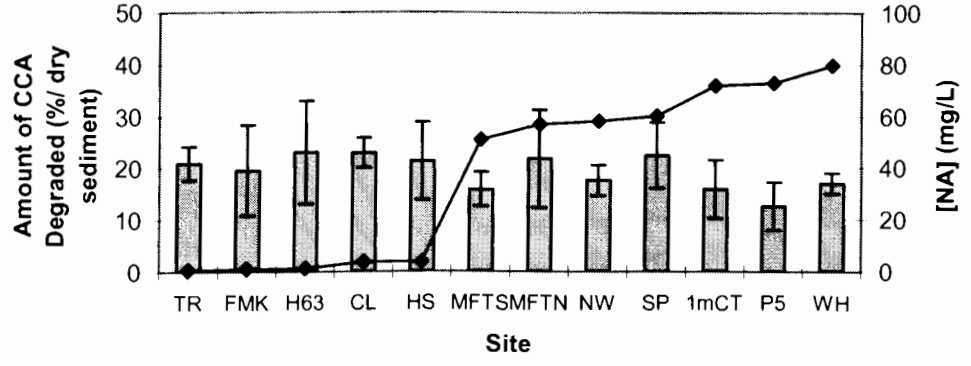
The rate of ^{14}C-CCA mineralization was uniform across the three categories in 2000 ranging between 2.0 and 6.5 %/day/g dry sediment. However it should be noted that the high impact sites exhibited slightly lower mineralization rates and that there was an observable decrease in the overall mineralization in August. In 2001, the ^{14}C-CCA mineralization rates were also relatively uniform ranging between 2 and 6 %/day/g dry sediment with the exception of HS in June, which exhibited an abnormally higher CCA mineralization rate at 8.58 %/day/g dry sediment. As in 2000, the mineralization rates were somewhat lower in the sites with higher amounts of NA contamination and in August although these results were not statistically significant (Figure 3.13).

These results indicate that all sites possess microorganisms capable of degrading ^{14}C-CCA and the there is a temporal effect on the mineralization rates such that the August rates were lower compared to June and July. Figure 3.9 showed that the proportion of microorganisms in the stationary phase of microbial growth increased in August 2000 suggesting that the CCA mineralization rates decreased as the proportion of microorganisms in stationary phase increased.

June



July



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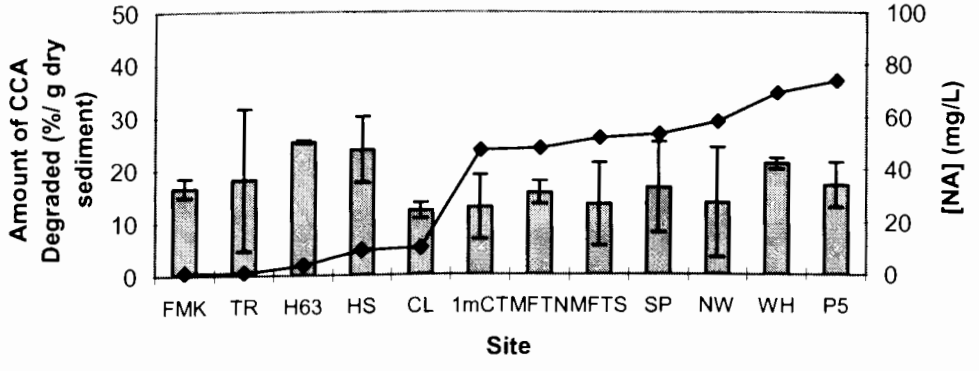
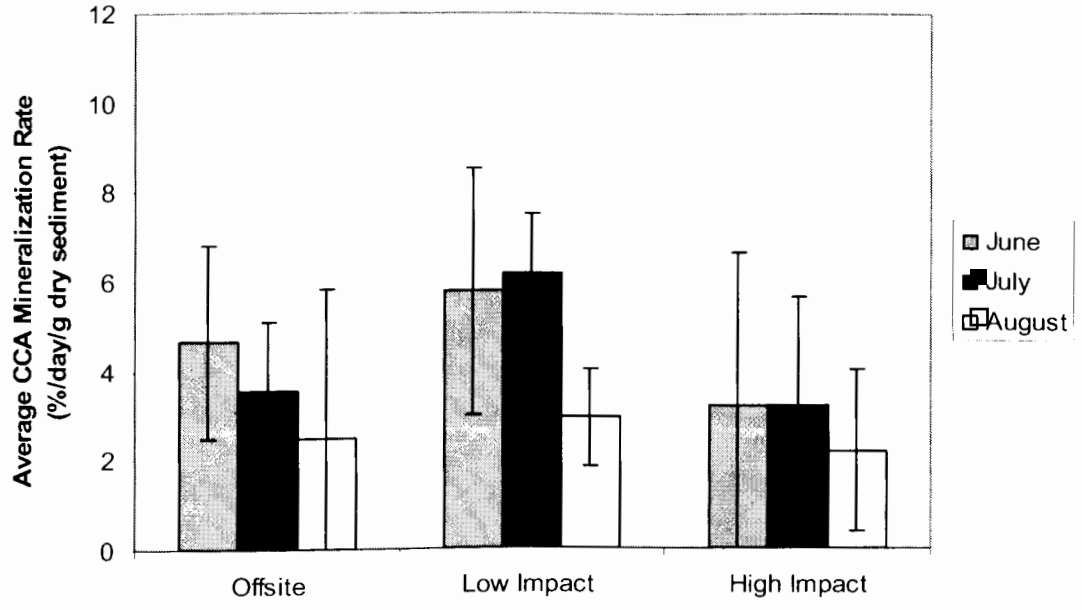


Figure 3.12. ^{14}C-CCA mineralization by sediment samples collected in 2001. The production of $^{14}CO_2$ from ^{14}C-labelled CCA was measured for a period of 14 days in a microcosm containing sediment plus water. The samples were obtained in June, July, and August, 2001. Sites are arranged in order of increasing NA concentration. Bars represent the mean ± standard deviation for three independent sediment samples A, B, and C. The line represents the concentration of NA's in the free water.

2000



2001

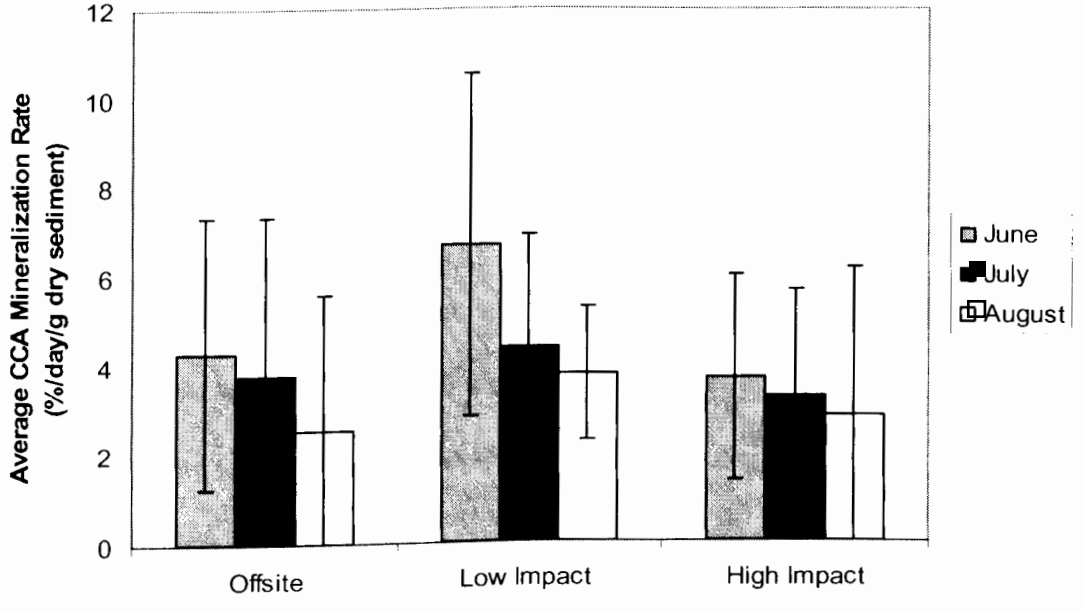


Figure 3.13. CCA mineralization rates of sediment microorganisms assessed by measuring the total production of $^{14}CO_2$ from ^{14}C-labelled CCA in a microcosm containing sediment plus water. Samples are arranged according to their degree of NA contamination: offsite (n= 9), low impact (n= 6), and high impact (n= 21). The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean CCA mineralization rate mean of the sediments for each of the three groups ± standard deviation.

3.2.2. ^{14}C-decahydro-2-naphthoic acid (DHNA)

Due to the fact that the majority of the NA's in samples obtained from oil sands process-affected waters belong to the Z= -4 family and that the rate of biodegradation is inversely proportional to the degree of cyclization (Morales et al. 1993), the ability of the microbial communities in various wetland sediments to degrade the bicyclic (Z = -4) NA surrogate (DHNA) was measured. This was performed by the same methods used to study the degradation of ^{14}C-CCA. Degradation rates and the total amount degraded in 14 days were calculated from the linear portion of the ^{14}C-DHNA degradation profile (Figure 3.14). The rates and maximum amounts of DHNA degraded were arranged in order of naphthenic acid concentration and these data are presented in Figures 3.15 to 3.17. To simplify the interpretation, the 12 wetland sediments were grouped into three categories: Offsite wetlands (H63, FMK, and TR), Low impact (CL and HS) and High impact (NW, 1mCT, SP, WH, P5, MFTS and MFTN) as described above.

The degradation pattern observed with ^{14}C- DHNA, was very different than that observed with ^{14}C-CCA. Whereas the amount and degradation rates of ^{14}C-CCA were relatively constant and high across all wetlands (up to 30% degraded within 14 days), Figure 3.15 shows that in 2000, the levels of ^{14}C- DHNA degraded were much higher for the samples that had been exposed to process-affected water than for the off-site wetlands (from 22.4 ±1.1% in 1mCT compared to 11.2 ± 3.1% in TR, Figure 3.15). It should also be boted tha in August of 2000, the amount of DHNA degraded decreased significantly compared with that observed in June and July (from 2.4 ±2.1% in TR to 11.4 ±3.2% in

In 2001, the total amount of DHNA degraded was more uniform in June and July again with the levels of ^{14}C- DHNA degraded being negligible in the offsite wetlands and

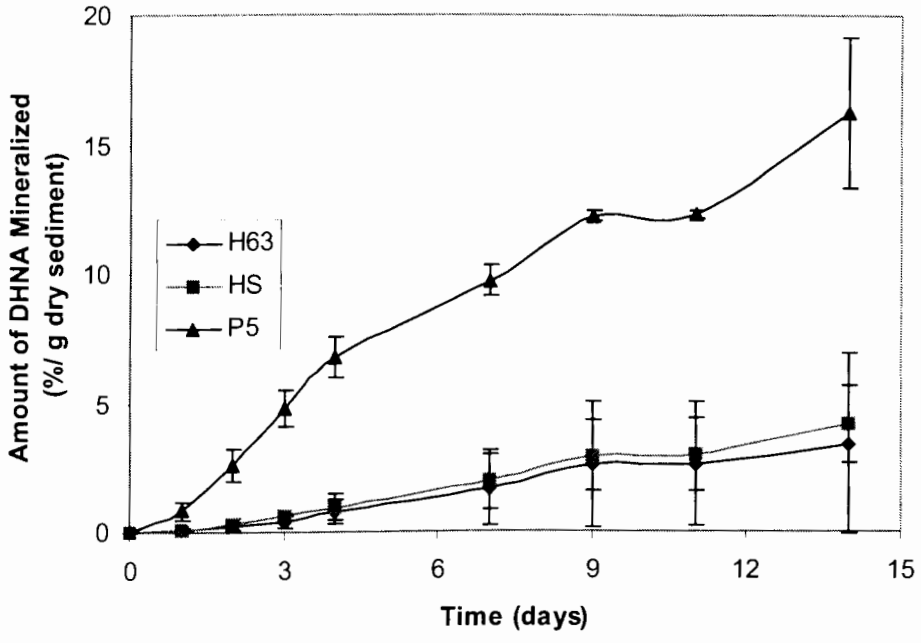
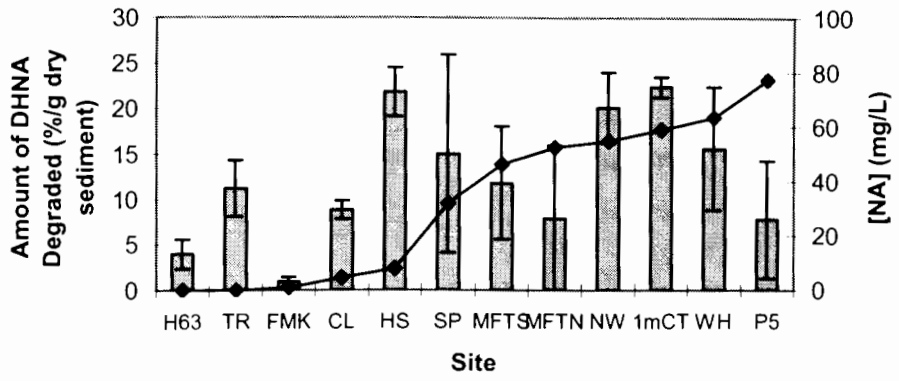
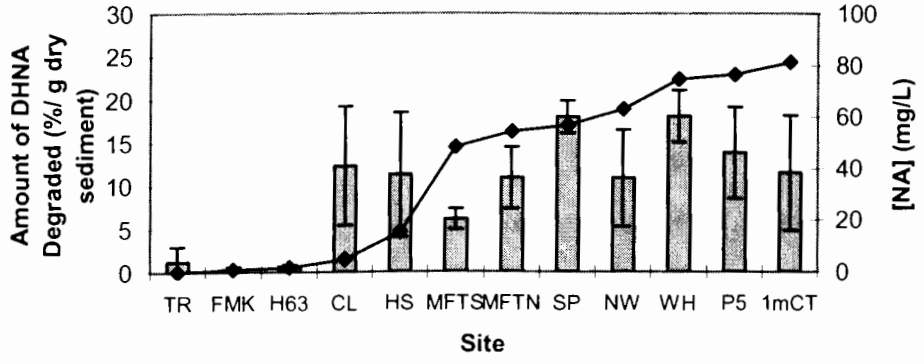


Figure 3.14. A typical ^{14}C-DHNA mineralization profile of sediment samples collected in 2001. The production of $^{14}CO_2$ from ^{14}C-labelled DHNA was measured over 14 days in a microcosm containing sediment plus water from an off-site wetland (H63), a large wetland in a reclaimed area containing very low levels of NA contamination (HS) and an active tailings pond (P5). Samples were collected and analyzed in July 2001. WH).

June



July



August

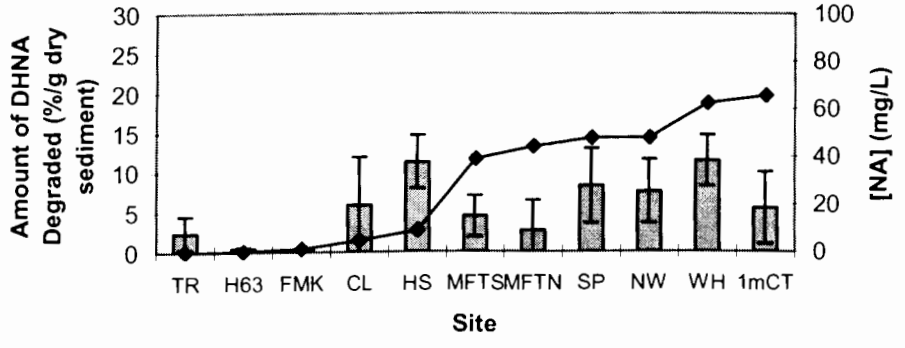


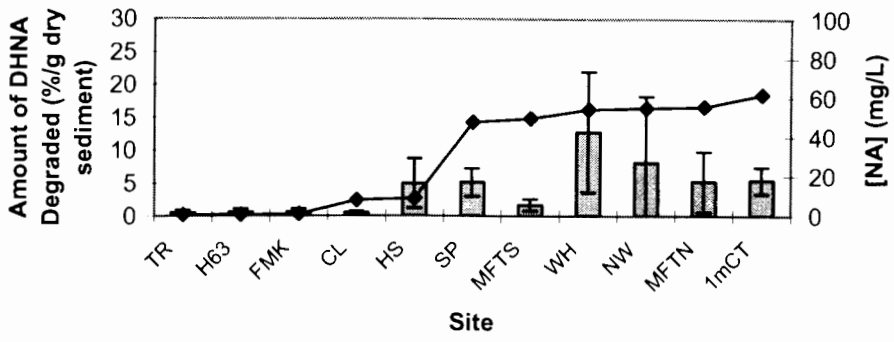
Figure 3.15. Total ^{14}C-DHNA mineralization by wetland sediments collected in 2000. The production of $^{14}CO_2$ from ^{14}C-labelled DHNA was measured for14 days in a microcosm containing sediment plus water. The samples were obtained in June, July, and August, 2000. Sites are arranged in order of increasing NA concentration. Bars represent the mean ± standard deviation for three independent sediment samples A, B, and C. The line represents the concentration of NA's in the free water.

much higher for the samples that had been exposed to process-affected water (up to 15%, Figure 3.16). However, it should be noted that in contrast to the results obtained in August of 2000, the maximum amount of DHNA degraded increased in August of 2001 (Figures 3.15 and 3.16).

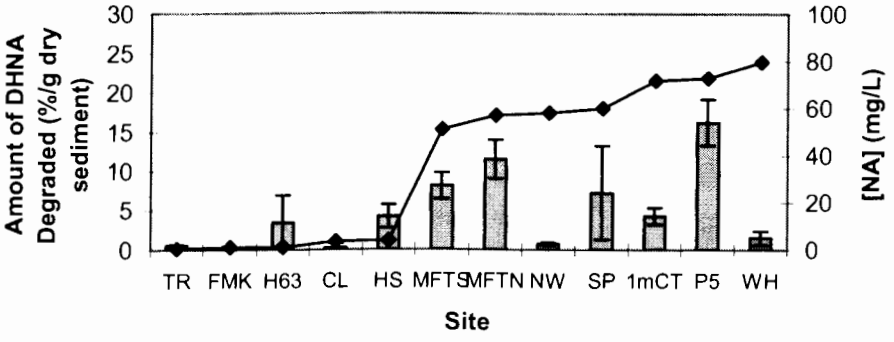
The ^{14}C-DHNA mineralization rates supported these observations. Together, these data indicate that the mineralization rates of ^{14}C-DHNA were low to negligible in off-site wetlands compared to sites that received even low amounts of process-affected water. This pattern is consistent in all three months in 2000 and 2001(Figures 3.15 to 3.17). It should be noted that the DHNA mineralization rates were six to fifty times lower than the CCA mineralization rates (from 0.65 to 1.5 %/day/g dry sediment for DHNA versus 2.47 to 6.20 %/day/g dry sediment for CCA in 2000 and from 0.04 to 1.13 %/day/g dry sediment for DHNA compared versus 2.54 to 6.70 %/day/g dry sediment for CCA in 2001).

These results show that the proportion of microorganisms responsible for the mineralization of ^{14}C-DHNA decreased drastically in 2001. This correlates with the results shown in Figure 3.6, which show that the proportion of pseudomonads decreased significantly in 2001 thereby suggesting that pseudmonads may be at least partially responsible for the mineralization of DHNA and therefore of NA's belonging to the Z= -4 family and higher.

June



July



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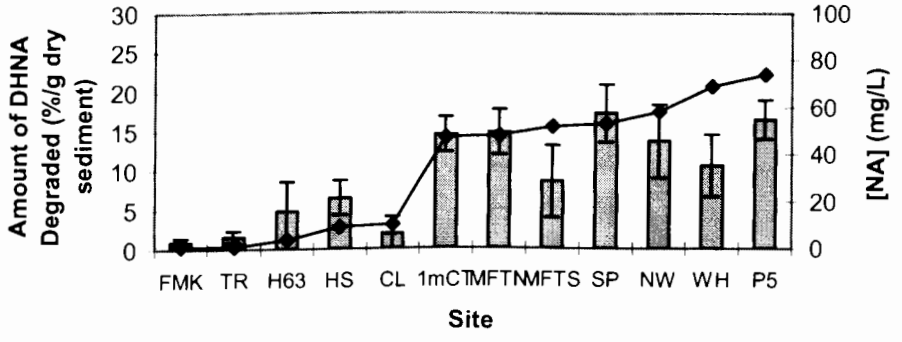
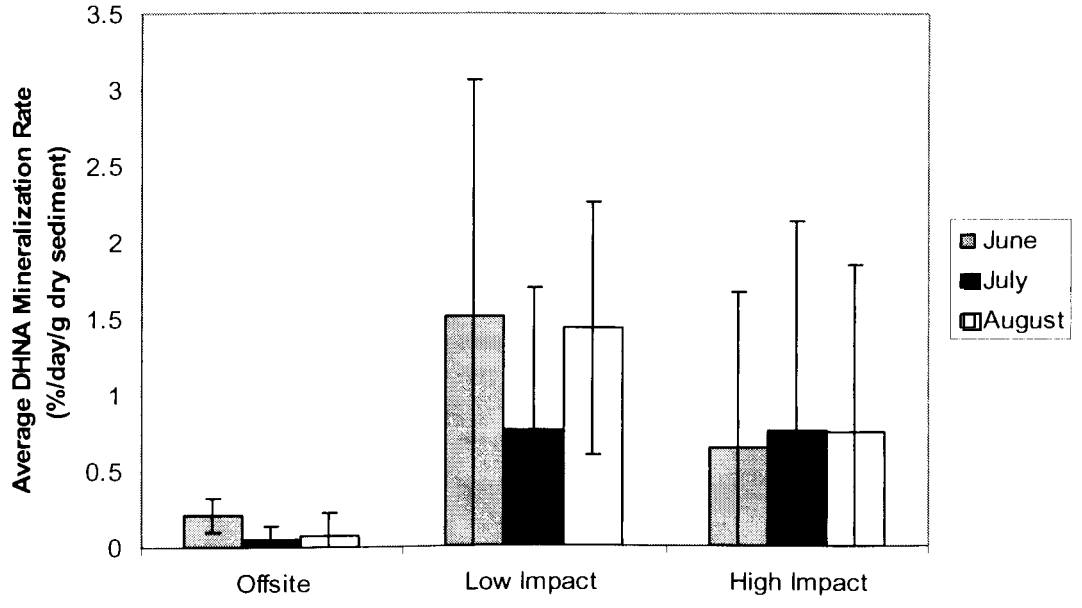


Figure 3.16. ^{14}C-DHNA mineralization by wetland sediments collected in 2001. The production of $^{14}CO_2$ from ^{14}C-labelled DHNA was measured for 14 days in a microcosm containing sediment plus water. The samples were obtained in the months of June, July, and August, 2001. Sites are arranged in order of increasing NA concentration. Bars represent the mean ± standard deviation for three independent sediment samples A, B, and C. The line represents the concentration of NA's in the free water.

2000



2001

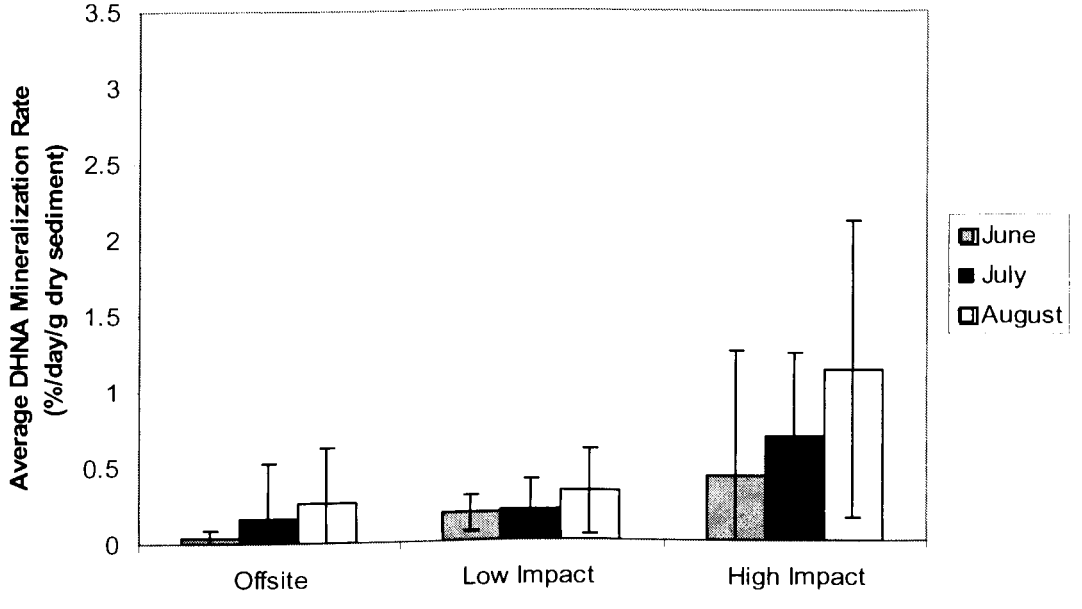


Figure 3.17. DHNA mineralization rates of wetland sediment samples assessed by measuring $^{14}CO_2$ production from ^{14}C-labelled DHNA in a microcosm containing sediment plus water. Samples are arranged according to their degree of NA contamination: offsite (n= 9), low impact (n= 6), and high impact (n= 21). The samples were obtained in the months of June, July, and August of 2000 (top) and 2001 (bottom). Bars represent the mean DHNA mineralization rate mean of the sediments for each of the three groups ± standard deviation.

3.3. Isolation of *Pseudomonas* spp from Naphthenic acid-degrading enrichment cultures

Although it is well known that NA's are removed from process-affected waters by microbial degradation, the precise metabolic pathways remain unknown (Nix et al. 1993, Lai et al. 1996, Schley et al. 1998). Holowenko et al. (2002) have shown that in samples obtained from aged process-affected waters, the majority of NA's had 22 or more carbon atoms (termed the "C22+ cluster"). This increase in the C22+ cluster was also found to correlate with a decrease in toxicity when compared with fresh process-affected waters (Holowenko et al. 2002). The purpose of this study was to isolate NA-degrading microrganisms in an effort to increase our understanding of the NA degradation process.

Using commercially-available NA's from Kodak as the sole carbon source and sediments obtained from an active tailings pond, we isolated microbes of two distinct colony types. One isolate grew much faster than the other on R2A media, and grew in well-defined colonies. The other isolate appeared slimy with somewhat translucent colonies. Both colony types secreted a diffusible yellow pigment and were Gram-negative, oxidase-positive rods. PCR was used to amplify the V8 region of the 16S rRNA gene (Marchesi et al, 2001). DNA sequencing and analysis of the amplicon using the BLAST database identified these two organisms as members of the *Pseudomonas* genus. Identification to species level was performed using the API 20 NE identification strips, which are specific for Gram-negative microorganisms isolated from environmental samples. The fast-growing bacterial isolate was identified as *Pseudomonas putida* and was designated as *P. putida* LD1 and the slow-growing isolate was identified as *Pseudomonas fluorescens* and was designated P. fluorescens LD2.

3.4. Microbial degradation of naphthenic acids

Biodegradation studies were carried out using a mixed culture of the two *Pseudomonas* isolates, and commercially available NA's (Kodak) as well as a combination of NA's extracted from the NW and WH sites (2.0 mg in a 5ml culture). After 48 hours, the cultures became turbid when compared to the autoclaved controls indicating that the mixed culture was able to grow on NA's as a sole carbon source.

To quantify the ability of the enrichment cultures to degrade the commercially available NA mixture, culture supernatants were acidified, the remaining NA's were extracted from the growth medium, derivatized into their *tert.*-butyldimethylsilyl esters and analyzed by GC/MS. Upon electron impact, these derivatives give rise to very few fragments of which the base ion is the $[M\text{-}C_4H_9]^+$ ion (loss of the t-butyl group or M-57). Thus by derivatizing the NA's mixture, it is possible to obtain molecular mass and structural information for its components by entering the ion intensities corresponding to the M+57 peaks into a matrix such as the one shown in Appendices 6 and 7 (Holowenko et al., 2001). The major limitation of this method is that it cannot distinguish between the various isomers, such as *cis* and *trans*, R and S, and positional isomers of the R-group. GC/MS analysis of the *tert.*-butyldimethylsilyl esters of the pure Kodak NA's showed a series of unresolved peaks that created a "hump" in the chromatographic profile that eluted just after the internal standard caprylic acid (Figure 3.18 A). When the NA mixture was inoculated with the NA-degrading enrichment culture a reduction in the size of the hump was observed (Figure 3.18 C). Within one week, the NA "hump" was much smaller, compared to the control flasks that contained autoclaved microorganisms (Figure 3.18 B). Samples taken two and four weeks after inoculation showed further decreases in

the NA"hump" size (Figure 3.18 D and E). Partial resolution and quantification of individual NA's was achieved by a modification of the method by Holowenko et al. (2001, see Materials and Methods pp.36-37). Figure 3.19 shows that the mixed culture of two pseudomonads was able to mineralize over 95 % of the NA's present in the culture flasks. In contrast, GC/MS analysis of the NA mixture inoculated for four weeks with either *P. putida*LD1 or *P. fluorescens*LD2 alone did not show a noticeable reduction in the size of the NA hump. Quantification of the remaining NA's showed that after four weeks, the single-organisms were each able to remove approximately 15% of the NA's whereas the mixed culture removed over 95% of the NA's present (Figure 3.20). Partial resolution of the NA's by GC/MS showed that the pure cultures primarily degraded the NA's belonging to the Z = 0 and –2 families compared to the autoclaved controls (Figure 3.21, controls not shown). It should also be noted that incubation of the mixed culture of strains LD1 and LD2 with "aged" NA purified from sites number 1 and 5 (Natural Wetland and West Hummock, respectively) resulted in undetectable changes to the NA profile. Partial resolution of the aged NA showed that their composition (measured as their percent abundance) was almost identical to that of the kodak NA after four weeks of degradation by the mixed culture (Figure 3.22). This indicates that aged NA from the Suncor Inc. site are recalcitrant to degradation by these *Pseudomonas* species.

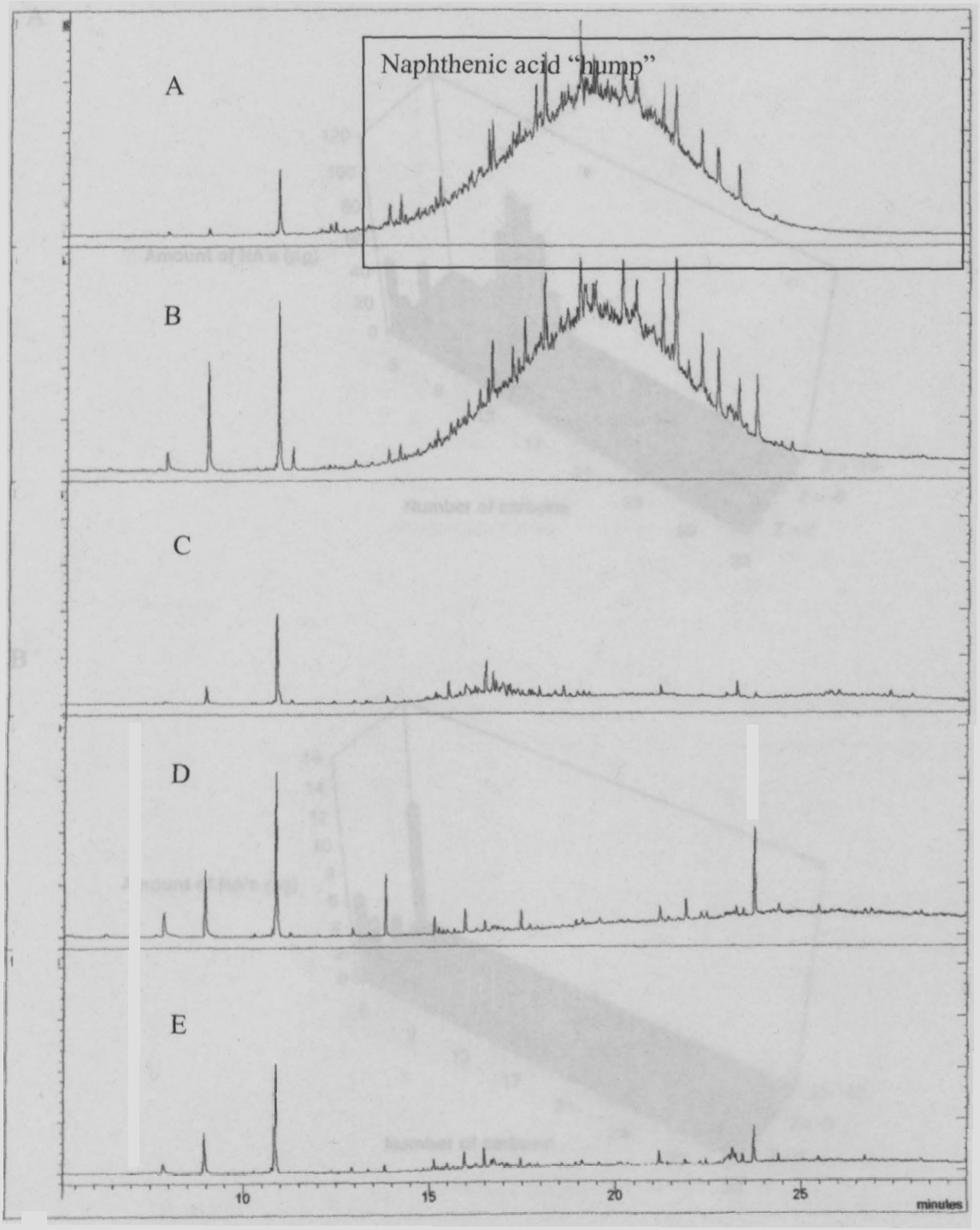
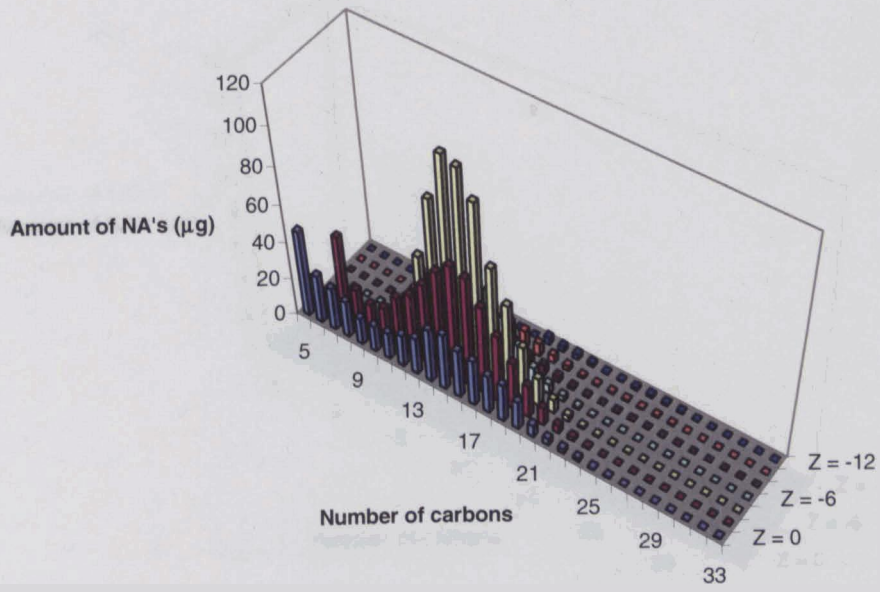


Figure 3.18. GC/MS (RIC) profiles showing the biodegradation of commercially available NA's. by a mixed culture composed of *Pseudomonas putida* and *Pseudomonas fluorescens*. Stock solution (A), killed control (B), after one week (C), after 2 weeks (D) and after 4 weeks (E). Peak at 10.75 minutes corresponds to the internal standard (caprylic acid) at a concentration of 10ng/μl.

A



B

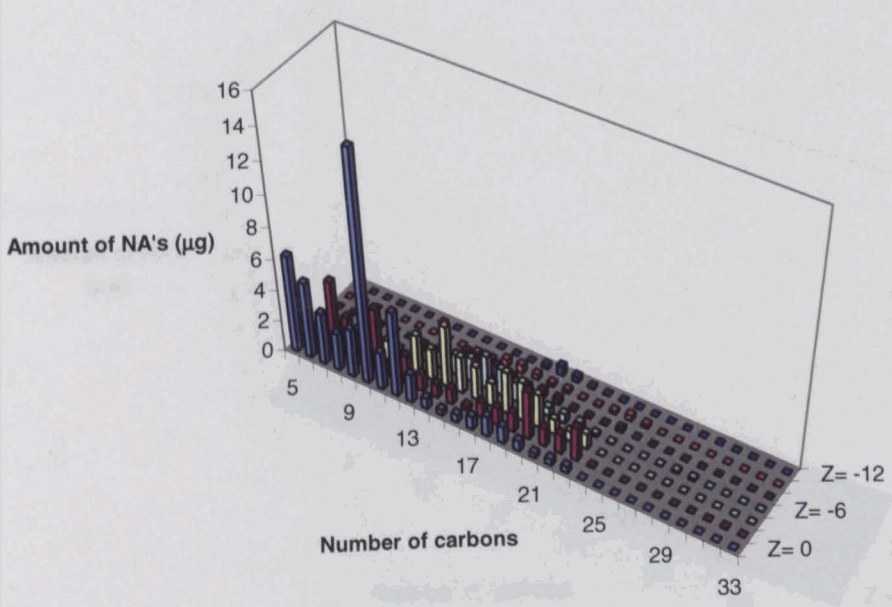
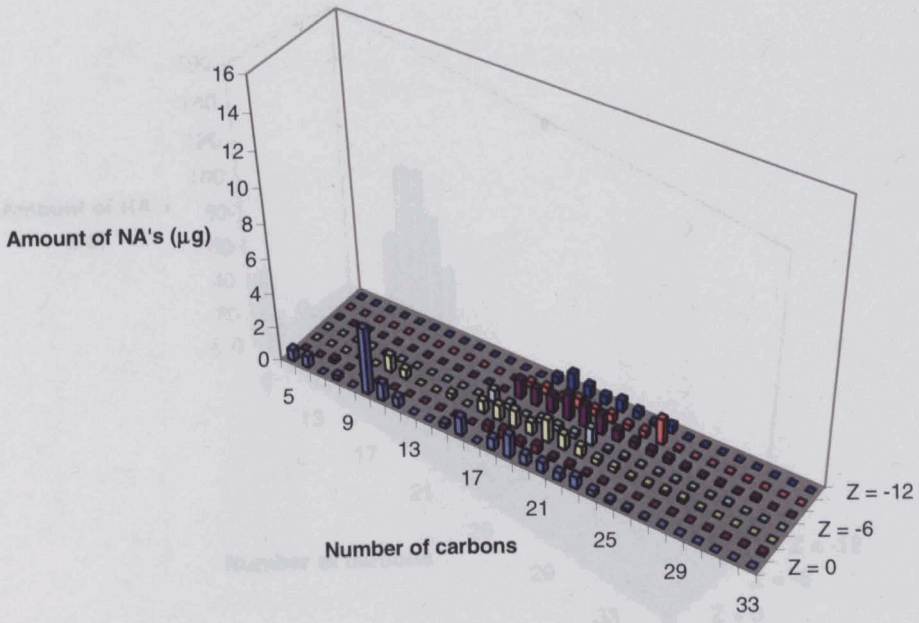


Figure 3.19 continued next page

C



D

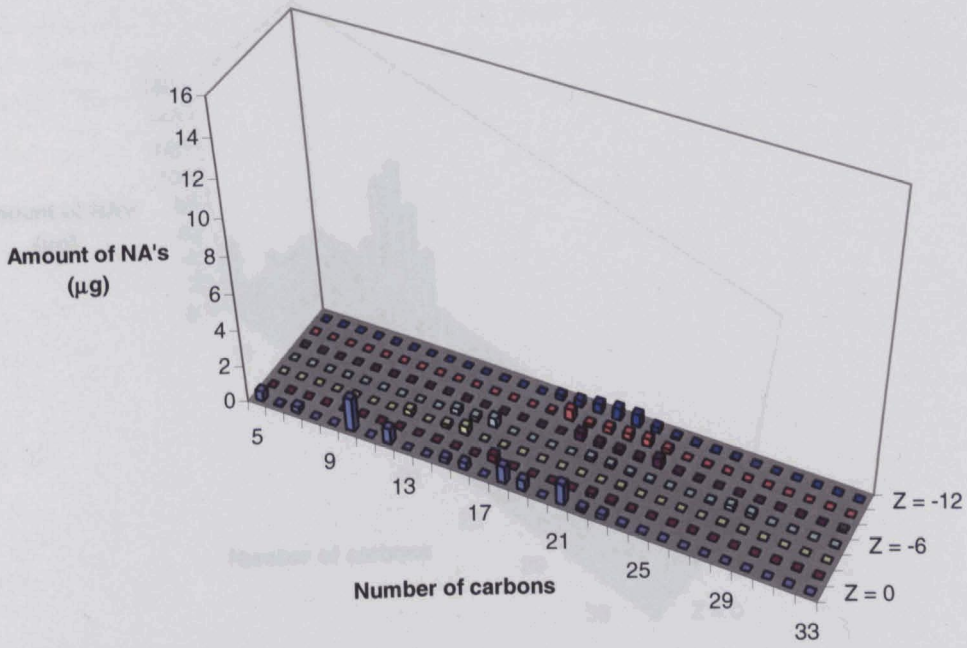
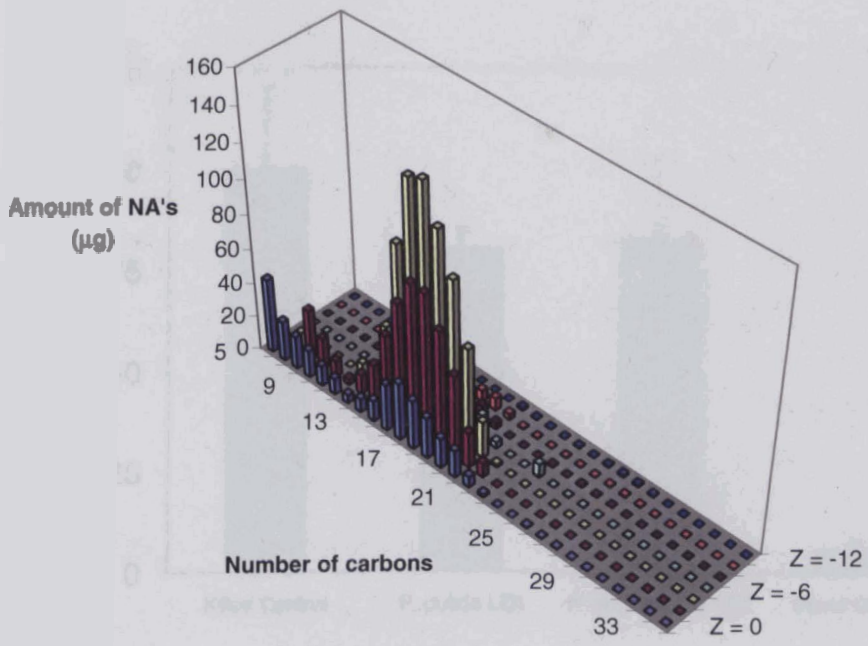


Figure 3.19. NA degradation profile after incubation with by a mixed culture of *P. fluorescens* LD1 and *P. putida* LD2. Mixed cultures consisting of *Pseudomonas fluorescens* LD1 and *Pseudomonas putida* LD2 were inoculated into minimal medium containing 400 µg/ml Kodak NA's for up to four weeks. Autoclaved control (A), sample after one week incubation (B) sample after two weeks incubation, (C), and sample after four weeks incubation (D). Note differences in the scale of the y-axis.

A



B

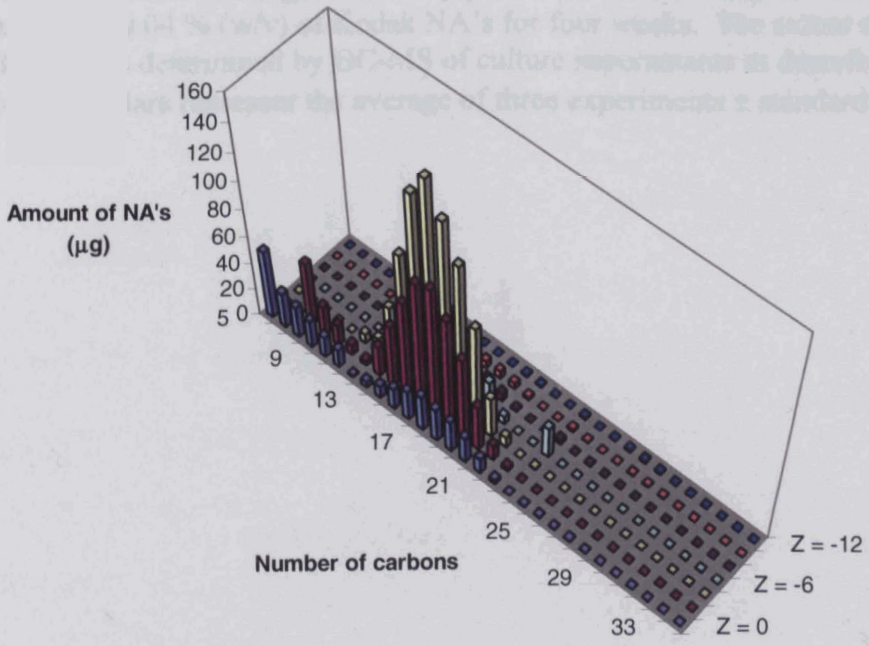


Figure 3.20. NA degradation profile by pure cultures. Minimal salts medium plus 400 µg/ml Kodak NA were inoculated with *Pseudomonas fluorescens* LD1 (A) or, *Pseudomonas putida* LD2 (B) as well as with autoclaved controls of each isolate (not shown). After four weeks at 28^{0}C, the NA profiles of culture supernatants were determined by GC/MS.

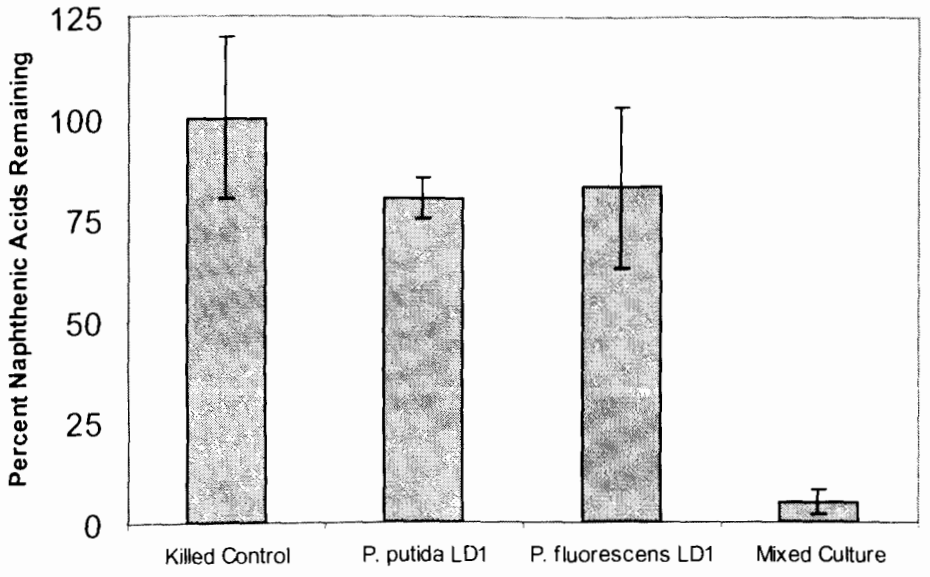
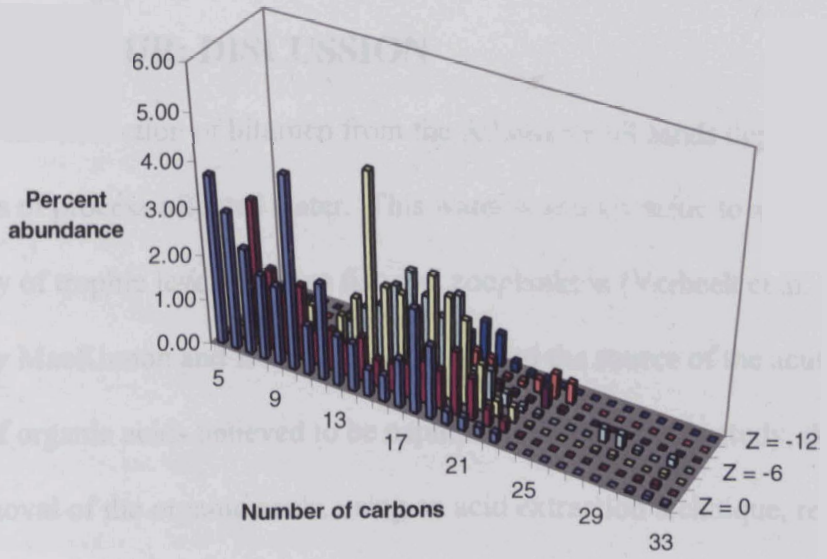


Figure 3.21. Kodak NA degradation by pure and mixed cultures. Cultures were incubated with 0.04 % (w/v) of Kodak NA's for four weeks. The extent of NA degradation was determined by GC-MS of culture supernatants as described in Material and Methods. Bars represent the average of three experiments ± standard deviation.

A



B

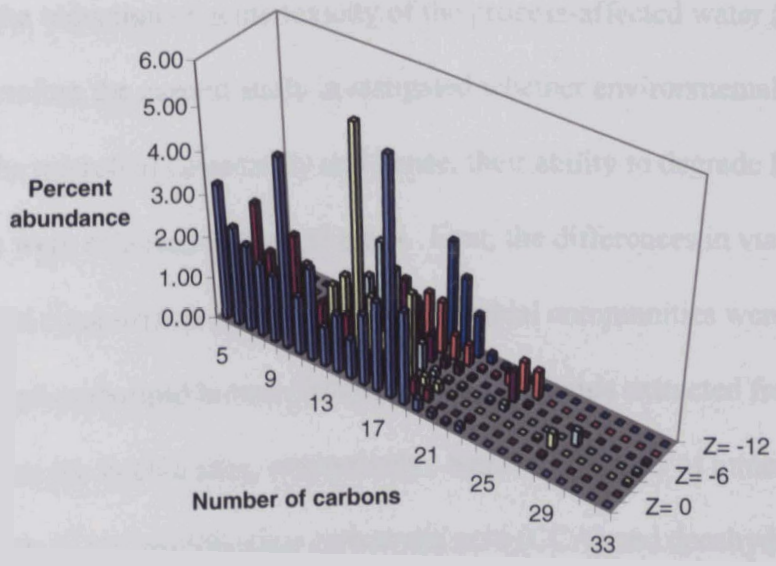


Figure 3.22. Comparison of "aged" Suncor NA's Vs. Kodak Na's after 4 weeks of incubation with the mixed culture. NA extracted from sites NW and WH were derivatized, and analyzed by GC/MS (A) and their percent abundance profile was compared with that obtained after four weeks of incubation of Kodak NA with the mixed culture (B).

CHAPER FOUR: DISCUSSION

The extraction of bitumen from the Athabasca oil sands deposit generates large volumes of process-affected water. This water is acutely toxic to aquatic organisms from a variety of trophic levels such as fish and zooplankton (Verbeek et al. 1993). A previous study by MacKinnon and Boerger (1986) showed the source of the acute toxicity to be a group of organic acids believed to be naphthenic acids. In this study, they demonstrated that removal of the organic acids, using an acid extraction technique, rendered the waters non-toxic as shown by the Microtox assay as well as fish and zooplankton assays.

Microbial degradation of oil sands NA's has been shown to be the predominant factor in the reduction of acute toxicity of the process-affected water (Nix and Bishay, 1997), therefore the current study investigated whether environmental parameters affected the microbial community and hence, their ability to degrade NA's. The objectives were achieved in several ways. First, the differences in viable microbial biomass and composition of the sediment microbial communities were measured by analysis of phospholipid-bound phosphate and fatty acids extracted from phospholipids purified from the twelve sites, respectively. Second, the rates of mineralization of the two surrogate NA's, cyclohexane carboxylic acid (CCA) and decahydro naphthoic acid (DHNA), belonging to the Z = -2 and –4 families, respectively, were measured in twelve sediments obtained from wetlands that contained varying degrees of NA contamination. Third, degradation of a commercially available mixture of NA's by an enrichment culture

composed of microorganisms isolated from a wetland whose waters contained high levels of NA's.

The viable microbial biomass was assessed as the amount of phospholipid-bound phosphate by the method developed by Findlay et al. (1989). This method was developed specifically for the analysis of lipid-bound phosphates in sediments and has become the method of choice for the determination of microbial biomass in environmental samples (White et al. 1996a). Lipid phosphate analysis is an attractive alternative to other methods used in the determination of microbial biomass such as ATP content, acridine orange direct counts and chloroform fumigation. This is because it is fast and inexpensive, does not require specialized equipment and has been shown to correlate with the results obtained with the chloroform fumigation method (White et al. 1996a).

Lipid-phosphate analysis provides a quantitative measure of the viable biomass because all intact cells contain phospholipids and organisms without intact cellular membranes are not viable. In cell death, phospholipases rapidly transform the phospholipids in cell membranes to diglycerides by removing the phosphate-containing head groups (White and Tucker, 1969). The major limitation of this technique is the difficulty in converting the amount of lipid-phosphate into cell numbers. This is due to the fact that bacterial volumes can vary over three orders of magnitude (Guckert et al. 1985 and Norland, 1993) and therefore different organisms possess different amounts of phospholipids. For example, Brinch-Iverson and King (1990) have shown that in bacterial enrichments and isolates from the sea, the lipid-phosphate content varies between 34 and 380 μmol per gram of carbon for aerobes, compared to 118 and 250 μmol per gram of carbon for anaerobes.

Figure 3.1 shows that the viable microbial biomass did not vary significantly within each wetland category, indicating that the sites are at equilibrium. This is in agreement with the PLFA data, which showed that in 2001 (the year for which the biomass determinations were made), the proportion of organisms in stationary phase was constant in the three months and across the three categories. An interesting observation is the fact that the offsite and high impact sites possessed similar amounts of viable biomass. However, the sites in the low impact category (namely CL and HS) had twice as much viable biomass as the other sites. This is in agreement with the visual observations, which showed that the low impact sites were able to sustain a variety of higher organisms such as frogs and birds. It is possible that in these low-impact sites, the low levels of NA contamination are sufficient to stimulate microbial activity, which in turn would be able to support higher trophic levels. Conversely, in the high impact sites, the high amount of NA's inhibited the survival of microbes (except for those adapted to NA degradation as indicated by the high DHNA degradation rates in the high impact sites) as well as higher trophic levels. A study by Bendell-Young et al. (2000), supports this hypothesis. They compared off-site wetlands to a low-impact wetland created by seepage of process-affected water from the tailings ponds (NW in this study) and found that NW supported the growth of large numbers of microinvertebrates such as chironomid larvae and plants such as cattail; however, fish did not survive in these wetlands. Pollet and Bendell-Young, (2000) exposed tadpoles of the northern Canadian toad (*Bufo boreas*) and the wood frog (*Rana sylvatica*) to process-affected waters and the effects on parameters such as survival, growth, development rate and physical deformities were measured. Their data showed that wetlands created from process-

affected water seepage were unable to support amphibian populations thereby demonstrating that process-affected water is toxic to higher trophic levels.

PLFA analysis has been used as a measure of microbial community in several studies involving a variety of soil as sediments such as Antarctic continental shelf sediments (Bowman et al. 2003), hydrothermal vents (Summit et al. 2000) and manure hot spots (Frostegard et al. 1997). PLFA analysis is a useful measure because phospholipids are essential components of all biological membranes and are rapidly degraded upon cell death. Therefore, unlike techniques based on nucleic acid analysis, PLFA analysis is specific for the viable components of the microbial community.

The dendrograms obtained in 2000 as well as June and July of 2001 showed that the NW and CL sites cluster together (Figures 3.2 and 3.3) indicating that their microbial composition is very similar. This is also suggested by their physical features: both wetlands are shallow, produce a high amount of anaerobic gas, and support a wide variety of organisms including birds, frogs, invertebrates and plants. The clustering of these two wetlands was unrelated to process-affected water exposure since NW has approximately ten times the amount of NA's in the free water compared to CL (Appendix 8). However, they do possess similar amounts of sediment-bound oil (approximately 0.2 %). Similarly, in 2000, the sites P5, MFT-s and, MFT-n clustered together strongly suggests that the differences in community structure may be more closely related to the amount of oil in the sediments than on the amount of NA's in the free water. This is supported by the fact that other sites with comparable amounts of NA's such as SP and WH as well as the off-site wetlands also did not cluster with the above-mentioned sites. Straight chain hydrocarbons in the oil are more amenable to biodegradation due to their ease of

oxidation into fatty acids via aerobic and anaerobic pathways (Cozzarelli et al. 1995). Therefore, the changes in community structure in 2000 may reflect the amount of oil in the sediments. This relationship was not as clear in 2001, indicating that other factors may have influenced the changes in community structure.

Although individual components of the microbial community cannot be identified from the PLFA profile, certain groups of microorganisms possess distinct PLFA's, and the presence or absence of these biomarkers is an indicator of the presence or absence of these groups (Summit et al. 2000). For example, White et al. (1996) has shown that monounsaturated PLFA are indicative of Gram-negative bacteria, while the branched saturated PLFA are found in Gram-positive organisms. Cyclopropyl PLFA are found in Gram-negative bacteria and their abundance increases as the organisms enter stationary phase (Kieft et al. 1994). However, the absence of biomarkers does not mean that the specific groups of microorganisms are not present in the communities under study since other factors such as environmental parameters and phase of growth may also influence the types of PLFA present (Summit et al, 2000).

The results of the PLFA analysis show that although it is possible to observe differences in community structure, the results are highly variable and change from month to month thus indicating that seasonal changes have a great impact on community structure. The data also indicates that the PLFA profiles of the twelve sites are highly dynamic. However, due to the fact that composite sediments (of three samples A, B, C) were used to generate the PLFA data, it was not possible to evaluate the community dynamics within a given wetland.

It is also possible that the differences in community structure were caused by other physico-chemical parameters such as an abnormally high amount of rainfall observed in 2001, which may have introduced other nutrients or microbes due to runoff from the adjacent soil. This may explain the observed relationship between the amount of sediment-bound oil and community structure in 2000 but not in 2001. The PLFA-based community structure analysis differed from that obtained from a study by A. Hadwin on the same sediments (personal communication). Using DGGE profiles, Hadwin (personal communication) found that the microbial community structures in June and July of 2001 were affected by NA concentration. This is possibly due to the fact that PLFA analysis focuses on the major biomarkers present in the functional (i.e. active) members of the microbial community whereas DGGE analysis is a PCR-based technique and therefore may detect even the inactive members of the community. It is also possible that DGGE analysis provides a greater degree of community resolution due to the fact that several PLFA biomarkers exhibit a significant overlap between different microorganisms.

The biodegradation studies using ^{14}C-CCA as the Z = -2 surrogate NA showed that for both sampling seasons the ^{14}C-CCA mineralization rates were relatively uniform across all sites and that they tended to decrease in August. This indicates that all sediments, including those obtained from off-site wetlands possess microorganisms capable of degrading monocyclic NA's (i.e., those belonging to the Z = -2 family). These results agree with those of Herman et al. (1993), which showed that microbes indigenous to the oil sands tailings readily degraded carboxylated cycloalkanes, and that recalcitrance was due partially to alkyl substitutions in the ring structures.

The fact that the CCA mineralization rates decreased in August suggested that the microorganisms responsible for CCA mineralization entered the stationary phase of growth. This was supported by the PLFA data especially in 2000, which showed that the amount of cyclopropyl PLFA, which are indicative of stationary phase growth (Kieft et al. 1994) increased in August (Figure 3.9). An exception to this was the HS site, which showed a high rate of CCA degradation across the three months in both sampling seasons regardless of the amount of cyclopropyl PLFA. This implies that although the majority of the microbial community entered stationary phase, the CCA-degrading organisms did not.

The results also showed that the sites with the highest degree of NA contamination, particularly P5, which receives the greatest amount of process-affected water, exhibited lower CCA mineralization rates. It is unlikely that the lower rate was due to nutrient limitation since the nutrient levels in P5 were comparable if not higher than those found in other sites. For example, in June of 2000, the concentration of Mg^{2+} in P5 was 15.8 mg/L compared with 11.7 mg/L in TR. The amounts of the limiting nutrients such as nitrogen and phosphorus were found to be below detection limit for the majority of the sites studied except for Pond 5 for which the concentration of nitrogen available as ammonia was high (for example 9.3 mg/L in June of 2000). The decrease in the CCA mineralization rates may therefore have been caused by the presence of inhibitory compounds in the contaminated sites. Finally, the fact that no lag period was observed when ^{14}C-CCA was added to the sediments *in vitro* indicates that an active microbial community was already present.

A different pattern was observed when ^{14}C- DHNA, (Z = -4) was used as a NA surrogate. Although the amount and degradation rates of ^{14}C-CCA were relatively uniform in all wetlands regardless of their level of NA contamination, the levels of ^{14}C-DHNA degradation by the off-site sediments were significantly lower compared to the sites that received input of process-affected water (Figures 3.13 and 3.17). This suggests that off-site sediments are not exposed to significant levels of compounds related to DHNA, despite the high natural levels of petroleum in the surface sediments throughout the Fort McMurray area. However, once sediments are exposed to even low levels of process-affected water (e.g., the low impact sites, CL and HS), the microbial communities adapted to degrade the Z = -4 compounds. This finding is supported by a study by Morales et al. (1993), which found that the majority of NA's in natural effluents belonged to the Z= -4 family. Unlike the CCA degradation, P5 did not exhibit lower rates of DHNA degradation, suggesting that either compounds that interfere with DHNA degradation were not abundant or that the microorganisms in this site adapted to metabolize DHNA.

In both 2000 and 2001, the DHNA mineralization rates were significantly lower than those observed for CCA. For example in June of 2000, the CCA mineralization rates in the low impact sites were six times higher than those observed for DHNA whereas in June of 2001, the CCA mineralization rate in the off-site wetlands were 150 times higher than those observed for DHNA. This suggests that the bicyclic surrogates are less amenable to biodegradation than the Z = -2 surrogates. This supports the observation that the rate of NA biodegradation is inversely proportional to the degree of cyclization (Morales et al. 1993). In August of 2000, there was a significant decrease in

the amounts of DHNA degraded indicating that the sediment microorganisms had entered the stationary phase of growth (Figure 3.16). However, in August of 2001, there was a major increase in the amount of DHNA degraded. Indicating that the sediment microorganisms were still actively metabolizing DHNA. This was supported by the PLFA data, which showed that the amount of cyclopropyl PLFA increased in August 2000 whereas it remained uniform throughout 2001.

Another interesting observation was the fact that based on their ^{14}C-DHNA mineralization profiles, (Figures 3.15 and 3.16), the low-impact sites (CL and HS) behaved more like the high impact sites (i.e. higher DHNA mineralization) in 2000 whereas in 2001 they behaved more like off-site wetlands (i.e. DHNA mineralization decreased over 10 fold). This phenomenon may be explained by the decrease in the proportion of pseudomonads observed in the sediment samples collected in 2001 (Figure 3.6.), which may also partially explain the overall decrease in DHNA mineralization observed in 2001.

It is well known that biodegradation studies involving complex mixtures of industrial pollutants can present several difficulties (Herman et al. 1994). Naphthenic acids extracted from oil sands are an extremely complex mixture from which individual compounds have not yet been isolated, and representative compounds are not commercially available. At present, it is impossible to separate the individual NA's in a mixture by classical chromatographic methods; the method developed by St. John et al. (1998) and modified by Holowenko et al. (2001) is capable of only identifying NA groups based on their carbon number and Z family distribution. Previous studies have only been able to determine the relative percentage of each Z number and carbon group.

Therefore, in this study an internal standard was validated so that quantitative changes in NA composition could be monitored.

A mixed culture composed of *Pseudomonas putida* LD1 and *Pseudomonas fluorescens* LD2 was isolated from enrichment on minimal medium containing commercially available NA's as their sole carbon source. GC analysis of the culture supernatants showed a reduction in the size of the unresolved NA's hump indicating that the NA's were metabolized and almost completely degraded after approximately four weeks at 28 ^{0}C (Figure 3.20). Quantification of NA's was achieved by GC/MS analysis using a modification of the method by Holowenko et al. (2001), in which caprylic acid was used as the internal standard. The results showed that over 95 % of the NA's present were removed by the mixed culture. Moreover, these results showed that all NA's in the mixture were degraded regardless of carbon number or Z family. However due to the fact that neither CO_2 evolution nor dry weight of the NA-degrading cultures were performed, it is impossible to determine what proportion of NA's were mineralized versus those that were degraded to a terminal metabolite. Nevertheless, our studies with radiolabelled surrogates, (CCA and DHNA) shows that microbial mineralization of both Z= -2 and Z= -4 NA's can occur.

When either *P. fluorescens* LD1 or *P. putida* LD2 were tested individually, only 15 % of the NA's were removed. Moreover, the majority of these belonged to the Z = 0 and Z = -2 families (Figures 3.20 and 3.21). The synergistic effect of the two organisms in mixed culture indicates that unique metabolic pathways in both organisms are necessary to completely degrade the NA mixture. These results agree with those of Herman et al. (1994) in which they were able to isolate a mixed culture composed of

Pseudomonas stutzeri and *Alcaligenes denitrificans*, which was capable of degrading NA's (assessed as a visible reduction of the NA hump) but no single NA's-degrading microorganism was found. The synergistic effect observed in the mixed culture is possibly due to a co-metabolism process in which one organism partially metabolizes the NA's and the other one completes the mineralization process.

Unfortunately, we were unable to isolate any NA metabolites in the culture supernatants. However, since the ion peaks analyzed corresponded to aliphatic and saturated carboxylic acids it is possible that the metabolites were missed during the analysis. It is also possible that biodegradation occurred via β-oxidation thereby converting the NA's into a series of homologous organic acids which would be hidden within the components of the NA's hump. Evidence for the β-oxidation hypothesis of microbial metabolism of NA's is provided by two studies performed by Blakley (1978) and Blakley and Papish (1982). In these studies, a strain of *Pseudomonas putida* and an *Alcaligenes* spp were grown on minimal medium containing the monocyclic surrogate NA CCA as their sole carbon source. Following extraction and identification of the major metabolites, they postulated that CCA is degraded via β–oxidation. Therefore it is quite possible that degradation of at least monocyclic NA occurs via the β–oxidation pathway. Although the degradation pathway for bicyclic NA's has not yet been elucidated, several studies have identified DHNA as a terminal metabolite of naphthalene degradation under anaerobic conditions (Annweiler et al. 2001 and 2002).

The fact that DHNA is observed as a terminal metabolite of naphthalene under anaerobic conditions implies that NA's belonging to the Z= -4 family are not degraded via β–oxidation. This is due to the fact that β–oxidation is a widely available catabolic

pathway for a variety of organic compounds under aerobic and anaerobic anaerobic conditions. For example Rontani et al, (1999) found that two different bacterial communities isolated from marine sediments under aerobic and denitrifying conditions were able to completely mineralize (E)-phytol (3,7,11,15-tetramethylhexadec-2(E)-en-1-ol) and that in both cases the mineralization ocurred by alternating β–decarboxymethylation and β–oxidation. A possible mechanism for the aerobic biodegradation of bicyclic NA's is the β–oxidation of the carboxylated sidechain followed by a decarboxylation reaction to give the bicyclic hydrocarbon, which in turn can be hydroxylated followed by an oxidation reaction to give the corresponding cyclic ketone. The resulting ketone can then undergo a biological Baeyer-villiger oxidation reaction mediated by a specialized type of cytochrome P450 to give the corresponding ketone, which is followed by ring cleavage via hydrolysis and finally oxidized via β–oxidation. This hypothesis is supported by a study by Schwab et al, (1994) which found that the organophosphorus insecticide Heptenophos (7-chlorobicyclo(3,2,0)hepta-2,6-dien-6-yl dimethyl phosphate, which is somewhat similar in structure to DHNA) was metabolized by a dephosphorylation reaction followed by mineralization by the above mechanism.

Conclusion

This study was part of a larger study, which attempted to determine whether there are any physico-chemical features of wetlands that receive process-affected water from the oil sands extraction process, that are associated with high rates of microbial degradation of NA's. It is hoped that an understanding of these parameters will lead to

improvements in the design of artificial wetlands so as to optimize the extent of NA degradation thereby providing an economically viable water treatment method.

This study has determined that significant microbial degradation of the Z= -2 NA surrogate CCA was observed across the twelve sites under study (between and 2.47 and 6.2 %/day/g dry sediment) thus indicating that all wetland sediments possess microbial communities capable of mineralizing Z= -2 NA's regardless of prior exposure to process-affected waters. In contrast, microbial degradation of the Z= -4 NA surrogate DHNA only occurred in wetlands containing intermediate to high levels of NA exposure. This implies that only those sediments that have been previously exposed to process-affected waters will contain microbial communities capable of degrading at least some Z= -4 NA's. We have also determined that for although there are significant differences in microbial community structures as determined by PLFA analysis, in the year 2000, the major differences do not appear to be due to the NA concentration in the free water but rather to the levels of sediment-bound oil although this relationship was not observed in 2001. It is possible that the results obtained in 2001 were affected by an abnormally large amount of precipitation compared to previous years and which carried additional nutrients into the wetlands. Finally, this study has also resulted in the isolation of a mixed culture composed of two microorganisms, which together, are capable of degrading over 95% of the NA's present in a commercially available mixture of NA's. However incubation of the mixed culture with aged NA's isolated from a shallow wetland receiving some input of process-affected water exhibited negligible degradation thus suggesting that some of these compounds are recalcitrant. However more research is needed to understand the mechanisms responsible for the microbial metabolism of NA's

although circumstantial evidence points to a biological Baeyer-Villiger type oxidation reaction. These results suggest that it may be possible to manipulate the existing environmental conditions to select for a microbial community exhibiting higher rates of NA degradation.

Future Directions

Previous exposure to NA's predicts the ability of a microbial community's ability to degrade NA's. At present, it is unknown how long it takes for an optimal NA-degrading community to develop upon exposure of pristine sediments to process-affected waters. Therefore, a future study in which pristine microcosms are incubated with process-affected waters for a set time period after which the mineralization rates radiolabelled NA surrogates can be determine would help determine the time after exposure required for enhanced NA degradation to occur. In this study the differences in community structure could be analyzed over time using a combination of the PLFA, DGGE and BIOLOG techniques. Concurrently GC-MS analysis could be used to determine the types of NA's (carbon and Z number) degraded at different times throughout the study.

Another future study would be the determination of the DHNA degradation pathway. A variety of DHNA-degrading microcosms could be incubated with C^{14}-labelled DHNA for a predetermined time followed by separation of the sediments by centrifugation and acidification and extraction of the DHNA metabolites. HPLC or TLC could then be used separate these metabolites and determine their R_f values and elution times. This could be followed by a repeat of the experiment using unlabelled DHNA, followed by tentative identification via GC, GC/MS, IR and NMR. Finally, the enzyme(s) responsible for DHNA degradation could be purified and tested against a variety of NA surrogates.

APPENDICES

Appendix 1: Calibration curve for glycerol-3-phospate

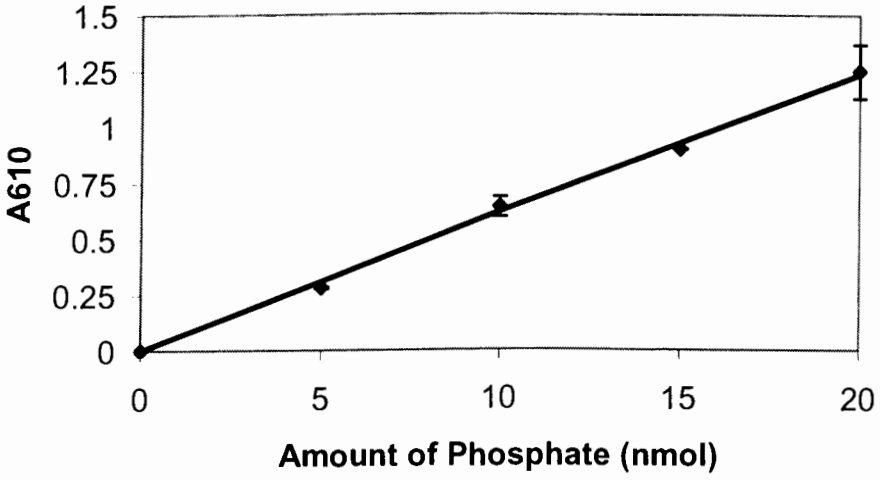


Figure A1: Calibration curve for biomass determination. Each point represents the mean of triplicate analyses ± standard deviation.

Appendix 2: Particle size distribution for the twelve wetland sediments (% less than)
Determined by Dr. Mike MacKinnon at the Syncrude Research Centre, Edmonton, Alta.

June 2000

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	96.0	79.6	50.9	37.8	25.7	15.8	9.0	3.6
CI	95.0	79.2	52.4	38.7	27.2	17.5	10.3	4.0
1mCT	87.0	68.2	45.9	35.4	26.9	19.5	12.7	4.6
SP	87.0	67.8	45.0	36.9	29.0	20.8	13.0	4.6
WH	96.0	85.4	53.8	38.4	25.6	15.7	9.0	3.7
H63	98.0	89.0	52.8	40.2	29.7	20.0	12.0	4.4
HS	84.0	65.6	41.1	30.2	21.4	14.1	8.4	2.9
FM	77.0	62.1	47.6	38.4	29.5	20.3	12.6	4.6
TR	93.0	81.4	67.6	58.9	49.6	38.0	25.2	8.5
P5	78.0	34.2	20.2	16.4	12.7	9.3	6.4	2.8
MFTS	87.0	67.0	45.5	36.6	28.5	20.2	12.7	4.7
MFTN	95.0	83.9	66.3	57.5	48.0	35.0	21.7	7.3

July 2000

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	75.0	42.4	24.3	18.4	13.5	9.1	5.7	2.4
CI	59.0	19.6	9.4	7.8	6.3	4.5	2.9	1.1
1mCT	83.0	59.3	38.7	30.3	23.3	16.9	11.0	4.1
SP	91.0	69.8	44.2	36.5	29.3	21.5	13.6	4.7
WH	80.0	33.9	15.6	11.3	8.2	5.8	3.9	1.7
H63	88.0	68.7	45.4	36.1	27.9	19.7	12.5	4.8
HS	93.0	80.5	53.2	38.3	26.6	17.3	10.3	3.7
FM	82.0	72.1	58.1	47.4	36.2	24.6	15.0	5.7
TR	89.0	73.5	60.2	52.8	45.0	35.3	24.1	8.4
P5	95.0	53.5	29.5	24.2	19.3	14.0	9.1	3.5
MFTS	95.0	83.5	66.7	57.0	46.8	34.3	21.1	6.5
MFTN	96.0	86.6	70.3	61.5	52.1	38.5	23.6	7.3

August 2000

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	94.4	70.6	40.0	30.0	21.4	13.7	8.1	3.2
CI	42.7	18.5	11.1	9.5	7.9	6.0	4.1	1.6
1mCT	73.6	47.8	28.1	20.0	14.4	10.2	6.8	2.9
SP	97.7	81.0	55.2	46.9	38.4	28.3	17.6	5.6
WH	54.1	21.3	11.0	8.2	6.1	4.4	2.9	1.3
H63	68.5	42.5	24.2	19.1	15.2	11.3	7.6	3.0
HS	51.7	38.7	29.4	23.5	17.6	12.2	7.6	2.8
FM	22.3	14.0	11.5	9.8	8.0	5.9	4.1	1.6
TR	98.0	82.7	66.9	58.7	50.6	40.2	28.1	10.1
P5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MFTS	81.7	61.5	42.2	32.4	24.2	17.2	11.1	4.2
MFTN	79.3	38.8	20.5	16.7	13.4	9.9	6.6	2.7

June 2001

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CI	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1mCT	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
WH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H63	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
HS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
FM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TR	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MFTS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MFTN	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

July 2001

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	95.0	71.0	40.7	30.2	21.1	13.4	8.0	3.3
CI	96.0	74.0	45.6	33.7	23.3	14.6	8.6	3.6
1mCT	92.0	73.0	51.6	40.4	30.7	22.2	14.8	5.8
SP	96.0	82.0	56.1	45.7	35.8	25.5	16.2	6.0
WH	84.0	65.0	43.0	31.6	22.3	15.0	9.5	3.8
H63	80.0	60.0	37.4	29.6	23.6	17.8	12.3	5.1
HS	62.0	45.0	30.1	22.9	16.9	11.7	7.5	3.1
FM	15.0	7.0	4.8	4.0	3.2	2.4	1.7	0.8
TR	100.0	94.0	80.8	70.9	60.9	48.4	33.8	12.3
P5	84.0	59.0	39.0	32.5	26.4	19.4	12.5	4.6
MFTS	100.0	98.0	88.0	80.4	70.3	54.2	36.0	13.0
MFTN	95.0	82.0	69.4	61.6	52.7	39.8	25.6	8.6

August 2001

Site	250 μm	125 μm	44 μm	22 μm	11 μm	5.5 μm	2.8 μm	1.0 μm
NW	90.7	71.7	45.1	32.2	21.7	13.5	8.1	3.4
CI	93.5	77.1	52.1	38.8	26.9	17.0	10.0	4.1
1mCT	99.9	81.4	57.1	43.9	32.9	24.1	16.7	7.1
SP	100.0	82.9	53.1	43.2	34.0	24.9	16.8	7.0
WH	99.2	78.5	55.2	42.1	30.9	21.6	14.0	5.6
H63	99.4	75.5	46.9	36.7	29.3	22.4	15.9	6.7
HS	98.2	80.4	52.8	38.7	27.5	18.5	11.4	4.5
FM	63.7	45.8	34.0	27.5	21.3	15.1	10.1	4.6
TR	100.0	100.0	88.3	79.0	69.4	56.2	40.1	14.9
P5	100.0	83.1	58.4	50.1	41.6	30.9	19.8	7.3
MFTS	100.0	100.0	90.6	82.4	72.3	56.1	37.2	13.4
MFTN	99.9	77.7	54.9	46.0	37.8	28.5	19.0	7.1

Appendix 3: Relative retention times (rrt) for bacterial fatty acid methyl ester (BAME) standards.

BAME standard	RRT
11:0	0.348
2-OH 10:0	0.354
12:0	0.402
13:0	0.465
2-OH 12:0	0.475
3-OH 12:0	0.496
14:0	0.539
i15:0	0.590
a15:0	0.597
2-OH 14:0	0.622
3-OH 14:0	0.637
i16:0	0.663
16:1ω7c	0.678
16:0	0.694
i17:0	0.712
cy17:0	0.772
17:0	0.794
2-OH 16:0	0.807
18:2ω6,9	0.826
18:1ω9	0.873
18:1 ω7	0.879
18:0	0.885
cy19:0	0.903
19:0	0.984
20:0	1.000

Appendix 4: Mol% distribution of individual fatty acids in wetland sediments used in PLFA analysis.

June 2000	Wetland											
Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	0.40	bdl[3]	1.84	1.47	0.56	0.33	1.28	0.84	0.36	bdl	0.94	0.35
i15:0	0.37	0.34	1.19	2.06	0.66	1.36	2.66	1.95	2.24	1.57	1.74	1.20
a15:0	0.59	0.70	1.20	1.10	1.08	0.61	1.95	2.36	1.38	bdl	1.69	3.21
15:0	1.78	0.53	3.00	4.08	1.63	4.48	5.53	4.06	4.81	bdl	2.95	3.85
2-OH14:0	1.51	bdl	7.99	10.74	3.39	11.69	9.50	8.77	9.18	3.04	6.65	8.99
i16:1	bdl	bdl	2.37	7.21	1.31	6.02	4.95	4.15	4.76	1.31	4.74	5.45
3-OH14:0	bdl	bdl	2.45	bdl	bdl	1.94	bdl	bdl	1.79	bdl	bdl	bdl
i16:0	1.55	0.53	8.71	6.18	3.12	7.34	5.47	4.68	5.86	3.53	4.73	4.47
a16:0	2.13	bdl	3.77	5.77	2.41	6.38	4.44	bdl	bdl	4.61	bdl	bdl
16:1ω9	4.26	5.60	10.06	14.99	8.97	14.82	11.38	12.74	10.79	12.01	16.58	12.40
16:1ω7c	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
16:1ω7t	1.69	0.37	6.65	10.26	4.52	13.00	8.29	bdl	9.15	8.11	12.54	8.76
16:1ω5	0.82	0.84	3.35	4.77	2.33	6.07	3.88	4.43	4.30	4.52	10.12	5.02
16:0	31.07	22.16	20.74	15.90	24.85	10.75	14.17	16.44	11.32	15.38	1.83	12.79
10Me16:0	bdl	bdl	bdl	bdl	1.70	bdl	1.31	1.24	1.09	bdl	2.06	2.63
i17:0	0.48	0.44	bdl	bdl	1.71	1.75	1.28	0.96	1.40	1.89	4.41	2.83
a17:0	2.21	1.22	2.38	4.02	4.02	3.94	3.41	4.15	2.90	8.97	1.21	7.36
cy17:0	0.47	1.20	1.12	1.10	1.79	1.41	1.48	0.81	1.38	2.69	2.83	2.27
17:0	0.81	2.30	1.80	1.00	1.97	4.19	1.79	1.53	27.93	7.49	0.00	5.39
2-OH16:0	1.31	1.35	1.55	1.41	1.57	2.55	1.27	1.27	1.77	5.09	2.63	3.34
10Me17:0	bdl	bdl	bdl	bdl	0.55	0.43	0.26	0.30	0.25	bdl	bdl	bdl
18:2ω6,9	2.82	4.33	2.25	1.64	3.20	1.14	1.85	1.48	0.48	2.51	6.26	3.58
18:ω9	10.89	13.73	6.65	3.96	7.61	1.50	3.19	3.89	0.88	7.47	10.70	2.08
18:1ω7	1.90	9.19	1.79	1.79	2.74	1.16	1.04	2.54	0.88	2.71	bdl	bdl

[3] bdl = below detection limit.

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
18:0	31.54	32.85	15.35	5.39	16.30	5.36	11.71	14.77	1.56	11.54	8.50	8.11
10Me18:0	0.92	0.72	0.75	0.41	0.94	0.66	0.49	2.15	0.28	bdl	1.47	3.01
cy19:0	0.54	0.46	0.59	0.31	0.84	0.41	0.52	1.15	0.32	2.06	1.64	0.90
20:0	1.34	1.44	0.86	0.32	0.77	0.22	0.56	0.65	0.12	0.65	0.79	0.53
21:0	bdl	bdl	bdl	bdl	0.22	0.20	0.13	0.26	bdl	bdl	bdl	0.17
22:1	bdl	bdl	bdl	bdl	1.97	bdl	bdl	bdl	bdl	bdl	bdl	0.37
22:0	0.41	0.46	bdl	bdl	0.37	bdl	0.24	0.55	bdl	bdl	bdl	bdl
23:0	0.77	0.57	0.57	2.40	0.89	1.37	3.31	9.23	2.10	bdl	bdl	0.26
24:0	bdl	bdl	bdl	bdl	0.41	bdl	0.26	0.67	0.65	bdl	0.55	0.41

July 2000	Wetland											
Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	6.85	1.99	5.16	1.10	0.93	0.60	3.16	0.28	1.17	bdl	1.76	1.47
i15:0	0.58	0.40	0.39	0.91	2.04	1.42	2.79	0.08	0.79	0.54	0.78	0.87
a15:0	0.90	0.58	0.55	1.48	3.00	3.28	6.47	0.29	1.09	0.32	0.84	1.26
15:0	1.14	0.89	1.27	2.25	2.46	1.46	3.03	0.12	1.68	0.89	2.17	1.22
2-OH14:0	0.72	0.93	0.58	4.44	3.82	2.12	3.96	bdl	2.36	2.42	4.49	2.20
i16:1	0.30	0.50	0.23	1.70	2.25	0.97	1.62	bdl	0.53	bdl	1.64	0.29
3-OH14:0	0.00	bdl	bdl	1.76	0.51	bdl	bdl	bdl	0.80	0.49	bdl	bdl
i16:0	0.61	0.72	0.61	3.30	2.78	2.37	3.05	0.19	1.65	1.55	2.51	1.21
a16:0	0.69	0.62	0.51	3.70	2.87	1.58	2.05	bdl	bdl	1.07	2.58	0.96
16:1ω9	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	4.85	bdl	3.53	bdl
16:1ω7c	1.08	1.49	0.71	7.92	10.78	8.17	10.71	1.50	bdl	6.60	2.15	4.52
16:1ω7t	bdl	bdl	bdl	bdl	bdl	bdl	bdl	0.18	bdl	2.87	3.47	2.66
16:1ω5	0.73	0.88	bdl	5.15	5.90	5.35	5.30	0.46	3.08	bdl	1.85	bdl
16:0	35.84	30.93	49.93	18.87	14.88	14.02	20.91	15.70	21.62	17.90	20.86	27.32
10Me16:0	0.03	0.16	0.14	bdl	0.46	0.54	0.57	0.12	0.32	bdl	0.71	0.42
i17:0	0.24	0.39	0.62	0.91	0.91	0.91	0.78	0.32	0.35	0.58	1.13	0.59
a17:0	1.04	1.32	0.19	2.27	1.36	1.75	1.70	1.05	1.58	1.67	3.07	1.91
cy17:0	0.03	0.20	0.22	1.19	1.59	1.20	2.00	0.29	0.45	2.87	0.86	1.14
17:0	1.42	1.85	2.82	0.92	0.94	1.13	1.02	1.50	0.98	1.20	1.38	1.78
2-OH16:0	0.04	0.15	0.08	0.99	0.58	0.74	0.45	0.05	0.81	1.07	1.26	0.82
10Me17:0	0.26	0.39	1.38	0.34	0.09	0.10	bdl	0.53	bdl	bdl	0.40	bdl
18:2ω6,9	1.77	2.12	6.28	1.62	2.30	3.23	1.93	2.57	1.49	3.11	1.68	1.92
18:ω9	5.04	5.50	13.34	4.39	5.18	6.44	4.71	6.37	4.99	8.63	5.33	4.55
18:1ω7	1.17	1.33	5.31	2.68	8.33	6.67	6.20	3.80	1.79	9.15	3.36	4.73
18:0	37.79	44.70	1.93	26.58	23.86	32.06	12.30	60.57	39.04	26.96	26.37	33.47
10Me18:0	bdl	0.03	0.12	bdl	0.27	0.19	0.27	0.14	0.29	1.08	0.96	0.53
cy19:0	0.09	0.08	0.16	3.01	0.14	0.14	1.37	0.08	0.50	0.75	0.63	0.34
20:0	0.88	1.00	3.52	0.73	0.87	1.44	0.72	2.04	1.73	1.16	1.03	1.36
21:0	0.14	0.13	0.91	bdl	bdl	0.14	2.23	0.10	bdl	0.33	0.21	bdl

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
22:1	bdl	bdl	0.08	bdl	bdl	bdl	bdl	bdl	0.41	bdl	bdl	bdl
22:0	0.56	0.57	2.43	0.48	0.62	1.37	0.72	1.37	5.64	4.60	0.96	1.00
23:0	bdl	0.07	0.28	1.21	0.15	0.09	bdl	bdl	bdl	2.21	1.55	0.89
24:0	0.07	0.06	0.26	0.11	0.15	0.53	bdl	0.32	bdl	bdl	0.46	0.58

August 2000	Wetland											
Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	2.22	1.58	3.22	1.22	1.46	1.36	2.48	4.50	0.94	N/A	0.91	2.49
i15:0	4.27	10.45	6.52	1.92	2.94	2.45	2.30	3.10	4.17	N/A	6.92	3.77
a15:0	2.81	1.35	5.01	1.69	1.83	2.15	4.36	4.48	1.04	N/A	1.16	3.76
15:0	7.18	2.34	4.84	1.12	6.81	1.52	1.31	1.53	1.18	N/A	1.79	3.50
2-OH14:0	13.29	5.66	bdl	1.96	11.33	3.71	2.69	2.01	0.90	N/A	1.84	2.16
i16:1	3.65	1.38	bdl	bdl	3.53	0.74	bdl	0.47	bdl	N/A	bdl	1.42
3-OH14:0	3.91	2.99	1.25	0.48	4.22	1.39	1.98	1.05	1.16	N/A	1.92	1.66
i16:0	5.06	4.40	0.70	bdl	6.58	2.78	2.36	1.73	1.57	N/A	1.94	3.25
a16:0	6.27	7.68	0.73	2.31	5.82	4.49	1.38	bdl	1.69	N/A	bdl	4.25
16:1ω9	10.33	bdl	0.91	bdl	12.30	5.18	2.22	bdl	bdl	N/A	bdl	bdl
16:1ω7c	bdl	6.44	bdl	4.40	bdl	bdl	7.91	9.57	5.42	N/A	5.43	10.25
16:1ω7t	8.97	4.62	2.51	3.93	8.58	4.10	bdl	bdl	bdl	N/A	bdl	2.69
16:1ω5	4.40	5.38	bdl	2.81	4.84	2.43	4.72	5.40	5.20	N/A	6.82	3.30
16:0	10.21	17.11	18.45	14.20	10.00	13.36	19.49	20.84	15.16	N/A	16.21	16.50
10Me16:0	2.30	2.58	5.95	4.79	2.31	3.69	1.42	1.35	2.41	N/A	3.77	1.38
i17:0	3.50	4.13	9.45	8.33	3.80	7.37	2.59	1.64	5.12	N/A	3.20	1.54
a17:0	3.58	4.90	5.88	7.63	3.93	6.11	2.74	2.71	bdl	N/A	4.47	3.36
cy17:0	2.35	3.83	7.15	7.48	3.29	5.20	4.02	2.08	3.84	N/A	4.73	2.43
17:0	bdl	3.41	4.33	5.58	bdl	4.05	1.74	bdl	3.30	N/A	1.07	1.81
2-OH16:0	1.30	0.75	2.50	3.36	1.94	2.53	0.39	0.55	1.66	N/A	1.36	0.61
10Me17:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	N/A	bdl	bdl
18:2ω6,9	bdl	bdl	bdl	bdl	0.32	0.66	2.50	4.94	3.53	N/A	2.20	2.35
18:ω9	1.24	1.07	1.51	6.56	0.82	2.75	7.88	8.31	5.85	N/A	4.30	4.76
18:1ω7	bdl	0.98	2.30	3.56	0.90	2.39	11.38	11.67	6.34	N/A	9.41	9.62
18:0	1.48	2.54	1.82	6.09	0.44	5.19	5.37	6.82	14.48	N/A	7.29	4.94
10Me18:0	bdl	1.14	bdl	bdl	bdl	bdl	bdl	bdl	bdl	N/A	bdl	bdl
cy19:0	0.28	2.01	3.80	3.47	0.75	4.84	1.67	1.58	4.27	N/A	3.63	0.98
20:0	bdl	0.08	bdl	bdl	bdl	bdl	0.79	0.44	2.16	N/A	0.72	0.61
21:0	bdl	0.40	2.60	1.27	bdl	2.59	0.46	0.38	2.14	N/A	2.34	0.40

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
22:1	0.63	0.16	3.10	2.05	0.44	2.10	1.42	0.87	1.04	N/A	1.91	1.96
22:0	bdl	bdl	bdl	bdl	bdl	bdl	0.70	0.39	1.56	N/A	0.45	0.27
23:0	0.79	0.29	4.17	2.58	0.81	3.23	0.91	0.76	2.57	N/A	2.25	3.65
24:0	bdl	0.35	1.31	1.22	bdl	1.63	0.84	0.84	1.31	N/A	1.96	0.31

June 2001	Wetland											
Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	1.67	1.67	1.41	0.47	3.26	2.90	3.07	6.35	0.39	N/A	1.60	1.67
i15:0	1.22	1.22	0.92	2.78	1.89	2.33	3.00	3.61	0.77	N/A	1.01	1.70
a15:0	2.37	2.37	1.64	2.41	1.87	3.20	2.58	8.55	1.16	N/A	1.49	2.98
15:0	1.07	1.07	0.85	1.53	2.95	1.88	0	2.24	0.77	N/A	0.66	2.12
2-OH14:0	bdl	bdl	bdl	0.87	2.84	1.96	5.78	0.12	bdl	N/A	bdl	bdl
i16:1	bdl	bdl	bdl	bdl	1.05	0.80	1.98	bdl	bdl	N/A	bdl	bdl
3-OH14:0	0.56	0.56	0.38	1.32	1.22	1.25	14.56	1.67	0.59	N/A	0.45	1.81
i16:0	0.90	0.90	1.04	0.98	3.69	4.02	3.83	2.28	0.88	N/A	0.81	0.99
a16:0	bdl	bdl	bdl	bdl	4.71	bdl	3.59	bdl	bdl	N/A	bdl	bdl
16:1ω9	3.20	2.82	2.75	4.66	4.75	bdl	4.50	2.03	1.68	N/A	2.2	1.92
16:1ω7c	6.32	1.08	1.85	9.33	9.68	13.9	13.11	8.08	14.36	N/A	10.27	11.05
16:1ω7t	0.61	0.98	bdl	bdl	1.20	2.63	1.17	1.16	1.38	N/A	bdl	bdl
16:1ω5	1.00	1.35	3.55	4.01	2.54	5.68	1.85	1.77	2.47	N/A	1.64	1.84
16:0	35.24	35.88	25.69	16.00	18.22	11.13	21.86	20.84	17.39	N/A	16.54	18.74
10Me16:0	0.32	0.34	0.64	0.49	0.78	0.97	0.65	0.59	1.09	N/A	0.25	bdl
i17:0	0.68	0.54	1.28	0.85	0.84	6.35	0.90	0.69	0.93	N/A	0.75	0.8
a17:0	0.96	1.23	1.17	0.88	1.58	2.23	1.39	1.48	1.09	N/A	0.86	1.34
cy17:0	0.70	1.53	2.14	1.00	1.48	0.79	1.98	1.28	1.86	N/A	0.75	1.09
17:0	1.02	1.03	1.13	1.08	0.78	0.45	0.80	1.82	1.47	N/A	0.82	0.77
2-OH16:0	0.04	bdl	bdl	0.30	0.39	0.87	bdl	bdl	bdl	N/A	bdl	bdl
10Me17:0	bdl	bdl	bdl	bdl	bdl	0.22	bdl	bdl	bdl	N/A	bdl	bdl
18:2ω6,9	10.17	7.51	6.33	8.57	9.66	3.81	5.33	11.29	7.81	N/A	7.94	6.54
18:ω9	17.06	18.67	14.63	16.25	15.73	6.27	11.11	18.26	16.50	N/A	17.18	13.28
18:1ω7	10.06	12.99	18.10	8.55	8.49	3.88	7.70	8.71	17.89	N/A	28.52	22.67
18:0	1.88	3.97	8.21	3.04	2.42	1.49	1.69	4.04	3.53	N/A	2.77	2.41
10Me18:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	N/A	bdl	bdl
cy19:0	2.33	1.20	0.47	0.19	0.35	0.15	1.14	0.44	4.07	N/A	2.24	2.85
20:0	0.23	0.23	0.52	0.24	0.29	0.10	bdl	0.56	0.52	N/A	0.35	0.45
21:0	bdl	0.11	0.01	0.08	0.09	0.15	bdl	0.27	bdl	N/A	bdl	bdl

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
22:1	bdl	0.29	bdl	0.13	0.24	0.14	bdl	bdl	bdl	N/A	0.15	bdl
22:0	0.13	0.25	0.34	0.11	0.30	0.11	bdl	0.97	.65	N/A	0.32	0.52
23:0	0.17	1.49	0.53	0.62	1.52	0.30	bdl	bdl	bdl	N/A	bdl	0.36
24:0	0.09	0.29	0.34	0.13	0.36	bdl	bdl	1.57	.76	N/A	0.41	0.71

July 2001 Wetland

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	1.50	3.12	1.88	2.93	1.48	0	2.56	0.36	1.41	3.38	6.70	0.35
i15:0	2.27	4.62	0.67	1.87	2.27	0.64	3.54	0.99	1.32	2.70	5.99	0.65
a15:0	3.47	8.14	2.62	2.72	3.79	0.87	7.88	1.52	1.86	1.40	8.98	1.20
15:0	0.70	1.49	0.95	1.17	0.63	0.25	1.48	0.52	0.91	0.92	1.92	1.01
2-OH14:0	0.05	0.09	bdl	bdl	0.10	bdl	0.22	0.24	bdl	0.78	bdl	bdl
i16:1	0.22	0.54	bdl	0.19	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
3-OH14:0	0.16	0.15	bdl	0.19	0.28	bdl	1.1	0.38	1.42	1.52	0.37	bdl
i16:0	2.43	1.77	1.46	2.06	1.56	0.41	1.68	0.99	1.51	2.04	1.80	0.75
a16:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
16:1ω9	2.29	bdl	2.19	2.76	1.83	0.80	1.93	0	1.99	0	2.27	1.04
16:1ω7c	9.01	bdl	14.46	13.64	10.11	5.08	9.30	6.67	14.12	4.58	10.34	6.65
16:1ω7t	0.82	26.07	bdl	1.12	0.76	0.66	0.76	0.86	bdl	bdl	1.01	bdl
16:1ω5	1.46	2.21	2.19	1.87	1.34	1.15	1.91	1.95	2.01	3.78	2.10	1.30
16:0	32.18	24.67	29.86	18.30	15.90	15.68	17.80	18.57	18.27	14.48	20.83	19.03
10Me16:0	0.33	0.51	0.98	0.51	0.60	bdl	0.67	0.72	bdl	2.41	bdl	bdl
i17:0	0.57	1.21	1.28	0.95	0.98	0.97	1.36	1.51	0.73	3.04	1.01	0.96
a17:0	0.68	1.93	1.78	1.17	1.42	1.35	1.80	2.43	2.84	3.41	1.60	1.58
cy17:0	0.82	2.28	2.36	1.83	2.12	1.21	3.63	1.45	1.77	3.62	1.63	1.93
17:0	0.77	1.65	1.82	1.24	1.00	1.15	1.11	bdl	1.01	4.32	1.30	1.55
2-OH16:0	0	0.06	0.22	0.12	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
10Me17:0	0	0.33	bdl	bdl	bdl	bdl	bdl	1.02	bdl	bdl	bdl	bdl
18:2ω6,9	13.11	4.9	8.18	8.08	9.78	11.47	7.37	11.60	7.51	3.06	3.95	9.47
18:ω9	bdl	bdl	2.27	21.13	18.31	27.41	12.28	18.14	20.50	6.46	10.59	19.40
18:1ω7	24.22	9.65	17.08	12.80	17.17	19.07	11.81	16.18	11.42	5.68	12.29	18.56
18:0	1.38	3.04	4.62	bdl	2.93	5.30	3.57	6.40	3.92	21.25	2.72	7.62
10Me18:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
cy19:0	0.26	0.20	0.42	2.04	3.98	2.73	2.97	1.59	3.44	2.64	1.58	3.26
20:0	0.37	0.58	0.40	0.36	0.50	0.89	1.24	1.19	0.86	2.56	0.29	1.40
21:0	0.07	0.08	0.25	0.10	bdl	0.30	0.36	0.29	bdl	1.81	bdl	bdl

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
22:1	0.08	0.09	0.77	0.30	bdl	bdl	bdl	0.12	bdl	2.08	bdl	bdl
22:0	0.15	0.32	bdl	0.25	0.53	1.22	0.76	1.63	0.57	bdl	0.31	0.93
23:0	0.32	bdl	1.00	bdl	bdl	bdl	bdl	0.54	bdl	bdl	bdl	bdl
24:0	0.31	0.3	0.29	0.29	0.63	1.39	0.89	2.15	0.61	2.1	0.42	1.36

August 2001	Wetland											
Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
14:0	1.50	0.71	0.70	bdl	5.55	5.52	2.53	0.43	1.34	9.73	0.55	1.03
i15:0	1.11	1.10	0.94	3.15	2.48	2.79	2.16	0.61	2.03	3.08	0.96	1.66
a15:0	1.92	3.42	1.53	3.26	4.84	5.32	6.06	bdl	3.17	2.84	1.89	2.56
15:0	0.61	0.63	0.47	3.90	1.07	1.09	1.00	bdl	0.93	0.78	0.47	1.05
2-OH14:0	0.10	0.10	bdl	bdl	0.25	bdl	0.29	bdl	bdl	bdl	bdl	bdl
i16:1	0.68	1.45	1.24	bdl	1.42	2.72	1.57	2.02	2.59	3.18	1.39	0.85
3-OH14:0	0.57	0.66	1.60	19.62	3.20	3.56	1.90	bdl	1.99	11.38	1.88	1.54
i16:0	5.41	1.34	1.27	bdl	1.81	1.40	2.24	0.30	2.07	1.04	0.58	0.65
a16:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
16:1ω9	4.07	bdl	1.29	0.00	2.21	2.32	2.12	bdl	2.25	bdl	bdl	bdl
16:1ω7c	5.33	21.85	9.41	1.73	8.61	11.96	9.20	3.82	11.83	3.75	6.26	5.94
16:1ω7t	0.59	0.77	bdl	0.00	0.87	1.73	0.71	bdl	1.25	bdl	bdl	bdl
16:1ω5	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
16:0	20.02	17.73	22.04	18.13	21.36	23.41	20.25	12.72	19.47	21.64	17.57	21.03
10Me16:0	0.23	0.39	bdl	1.21	bdl	bdl	0.48	bdl	bdl	bdl	bdl	1.20
i17:0	0.35	0.81	0.63	7.43	0.57	0.63	0.92	bdl	0.98	3.29	0.77	2.99
a17:0	0.67	1.73	1.32	2.63	1.43	1.38	1.92	1.63	1.56	2.09	1.41	1.98
cy17:0	0.54	2.04	2.16	0.00	1.63	1.49	2.99	0.99	2.37	2.72	1.83	2.27
17:0	0.85	1.68	1.16	2.89	0.94	0.95	1.12	1.23	1.05	1.64	1.54	1.76
2-OH16:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
10Me17:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
18:2ω6,9	16.18	4.43	7.50	1.67	5.90	4.97	6.13	12.56	4.30	1.76	6.50	6.92
18:ω9	27.42	10.79	17.92	13.73	13.12	12.35	12.36	25.15	13.51	10.20	15.49	16.36
18:1ω7	7.50	22.99	17.82	6.20	10.14	9.65	12.27	21.80	12.52	9.18	22.61	15.42
18:0	1.73	3.28	5.26	4.64	6.61	4.32	3.54	7.17	5.17	7.81	9.49	5.24
10Me18:0	0.17	0.29	1.11	5.05	1.19	bdl	0.67	1.22	0.73	2.04	1.39	2.41
cy19:0	1.66	0.64	2.31	bdl	1.80	0.80	3.69	1.80	5.27	1.84	3.57	3.31
20:0	0.27	0.41	0.59	bdl	0.70	bdl	1.69	1.12	1.01	bdl	0.89	0.74
21:0	0.00	0.04	bdl	bdl	bdl	bdl	0.19	0.35	bdl	bdl	bdl	0.16

Fatty Acid (mol%)	NW	CL	1mCT	SP	WH	H63	HS	FM	TR	P5	MFT-s	MFT-n
22:1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
22:0	0.23	0.31	0.46	bdl	0.66	0.34	0.82	1.62	0.96	bdl	0.70	0.68
23:0	0.13	0.10	0.81	4.76	0.71	0.76	0.35	1.32	0.66	bdl	1.17	1.27
24:0	0.15	0.32	0.49	bdl	0.94	0.54	0.83	2.13	0.99	bdl	1.09	0.97

Appendix 5: t-test analysis of the differences between selected PLFA for the three categories: Offsite, Low Impact and, High Impact. Positive values show pairs of means that are statistically significant.

Year 2000:

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-1.018	-0.211	-0.627	-0.144	-0.789	-0.850
Impacted	-0.211	-0.666	-0.144	-0.411	-0.850	-0.948

i17/a17 PLFA ratios.

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-0.314	-0.126	-1.713	-0.984	-0.865	-0.639
Impacted	-0.126	-0.205	-0.984	-1.121	-0.639	-0.499

2-hydroxy PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-7.505	-2.785	-3.650	-1.352	-9.444	-4.659
Impacted	-2.785	-4.913	-1.352	-2.389	-4.659	-6.678

10-Me PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-1.569	-1.194	-0.991	-0.553	-6.309	-4.746
Impacted	-1.194	-1.027	-0.553	-0.649	-4.746	-4.461

18:2 PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-2.381	0.069	-1.728	-1.960	-2.763	-0.262
Impacted	0.069	-1.558	-1.960	-3.234	-0.262	-1.953

total cyclopropyl PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-1.201	-0.843	-1.635	-1.187	-6.334	-5.752
Impacted	-0.843	-1.834	-1.187	-2.497	-5.752	-7.758

Year 2001:

i15/a15 PLFA ratios.

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-0.477	-0.213	-0.533	-0.546	-0.348	-0.171
Impacted	-0.213	-0.584	-0.546	-0.998	-0.171	-0.228

i17/a17 PLFA ratios.

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-1.266	-0.567	-0.177	-0.022	-0.910	-0.422
Impacted	-0.567	-1.551	-0.022	-0.272	-0.422	-1.391

2-hydroxy PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-4.146	-2.479	-0.530	-0.378	-0.166	-0.091
Impacted	-2.479	-5.078	-0.378	-0.432	-0.091	-0.108

10-Me PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-0.537	0.078	-1.872	-1.533	-2.031	-0.987
Impacted	0.078	-0.380	-1.533	-1.528	-0.987	-3.103

18:2 PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-3.071	-2.546	-5.710	-2.326	-8.361	-7.352
Impacted	-2.546	-5.319	-2.326	-6.993	-7.352	-10.241

total cyclopropyl PLFA

	June		July		August	
	Offsite	Impacted	Offsite	Impacted	Offsite	Impacted
Offsite	-3.587	-2.612	-4.293	-3.445	-5.290	-3.224
Impacted	-2.612	-2.071	-3.445	-3.505	-3.224	-2.827

Appendix 6: Calibration curve for the t-BDMS derivative of caprylic acid

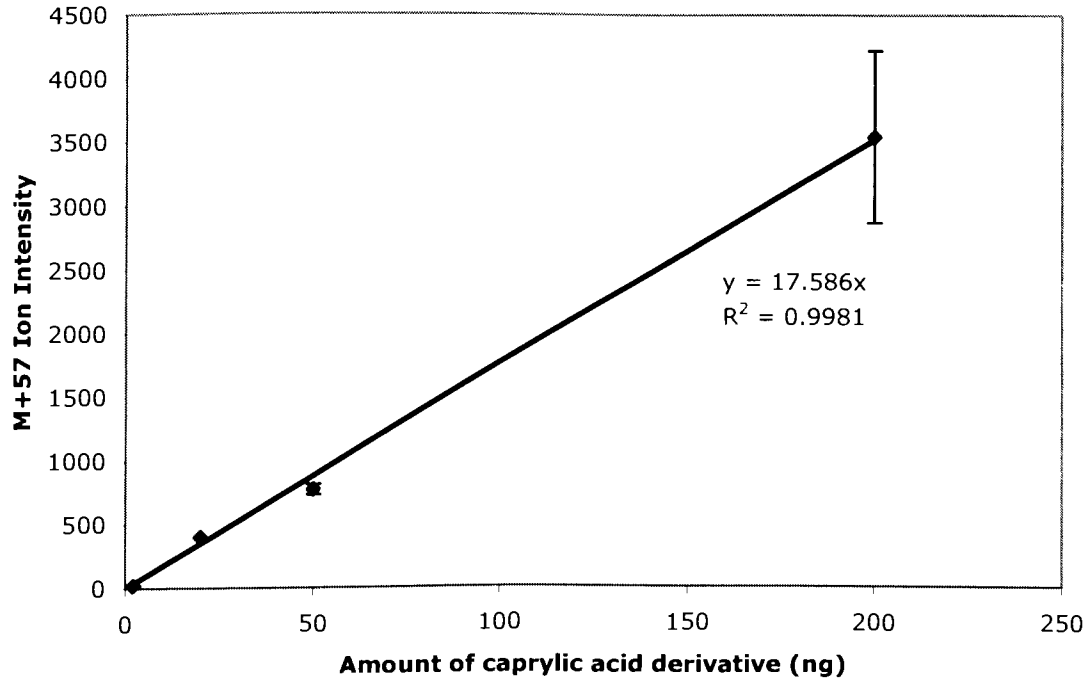


Figure A2: Calibration curve for NA biodegradation. Each point represents the mean of triplicate analyses ± standard deviation.

Appendix 7: Expected M+57 base peak ions for a homologous series of t-BDMS derivatized naphthenic acids. Adapted from St. John et al. (1998) as modified by Holowenko et al. (2002).

Carbon number	Z number						
	0	-2	-4	-6	-8	-10	-12
5	159	NA	NA	NA	NA	NA	NA
6	173	NA	NA	NA	NA	NA	NA
7	187	185	NA	NA	NA	NA	NA
8	201	199	NA	NA	NA	NA	NA
9	215	213	NA	NA	NA	NA	NA
10	229	227	225	NA	NA	NA	NA
11	243	241	239	NA	NA	NA	NA
12	257	255	253	251	NA	NA	NA
13	271	269	267	265	NA	NA	NA
14	285	283	281	279	277	NA	NA
15	299	297	295	293	291	NA	NA
16	313	311	309	307	305	303	NA
17	327	325	323	321	319	317	NA
18	341	339	337	335	333	331	329
19	355	353	351	349	347	345	343
20	369	367	365	363	361	359	357
21	383	381	379	377	375	373	371
22	397	395	393	391	389	387	385
23	411	409	407	405	403	401	399
24	425	423	421	419	417	415	413
25	439	437	435	433	431	429	427
26	453	451	449	447	445	443	441
27	467	465	463	461	459	457	455
28	481	479	477	475	473	471	469
29	495	493	491	489	487	485	483
30	509	507	505	503	501	499	497
31	523	521	519	517	515	513	511
32	537	535	533	531	529	527	525
33	551	549	547	545	543	541	539

Appendix 8: Sample matrix showing the amounts of different NA's (in μg) found in 2 mg of the commercially available NA's mixture.

Carbon number	Z number						
	0	-2	-4	-6	-8	-10	-12
5	46.39	0.00	0.00	0.00	0.00	0.00	0.00
6	25.84	0.00	0.00	0.00	0.00	0.00	0.00
7	22.92	45.41	0.00	0.00	0.00	0.00	0.00
8	19.48	20.19	0.00	0.00	0.00	0.00	0.00
9	14.23	15.82	0.00	0.00	0.00	0.00	0.00
10	14.22	18.11	12.69	0.00	0.00	0.00	0.00
11	14.46	27.98	20.13	0.00	0.00	0.00	0.00
12	17.34	33.68	48.90	7.80	0.00	0.00	0.00
13	20.06	43.57	82.81	16.77	0.00	0.00	0.00
14	29.20	54.76	108.55	28.23	11.67	0.00	0.00
15	31.12	62.15	105.95	35.47	13.08	0.00	0.00
16	26.10	59.81	92.74	40.73	12.72	10.36	0.00
17	25.05	48.36	62.99	29.92	9.88	9.71	0.00
18	20.98	36.56	47.00	18.37	6.37	6.70	4.09
19	20.39	26.03	28.29	10.22	5.13	4.28	3.13
20	15.23	17.56	14.97	5.09	3.00	2.64	2.94
21	6.45	9.68	7.56	2.84	1.45	1.18	2.18
22	3.34	4.17	3.00	1.25	0.67	0.63	1.04
23	2.40	1.89	1.28	0.55	0.36	0.57	0.92
24	1.69	0.94	0.64	0.38	0.57	0.80	1.24
25	0.77	0.41	0.28	0.27	0.41	0.75	1.52
26	0.50	0.20	0.17	0.21	0.28	0.80	1.41
27	0.18	0.07	0.13	0.16	0.21	0.50	0.92
28	0.13	0.04	0.11	0.24	0.14	0.23	0.38
29	0.04	0.06	0.10	0.21	0.16	0.14	0.14
30	0.06	0.03	0.01	0.07	0.11	0.11	0.06
31	0.09	0.06	0.06	0.03	0.10	0.10	0.10
32	0.18	0.11	0.04	0.06	0.04	0.09	0.11
33	0.41	0.34	0.06	0.10	0.21	0.17	0.28

Appendix 9: Concentration of NA's (mg/L) in free water samples obtained from the twelve sites under investigation. Determined by Dr. Mike MacKinnon at the Syncrude Research Centre, Edmonton, Alta.

Site	2000			2001		
	June	July	August	June	July	August
NW	54.9	63.2	47.7	54.7	58.1	58.3
CL	4.6	4.8	5.0	8.0	3.6	10.2
1mCT	58.9	81.2	65.5	61.7	71.8	47.2
SP	31.9	56.8	47.7	47.5	60.1	53.4
WH	63.5	74.5	62.2	54.3	79.7	69.2
H63	0.01	1.7	0.2	0.7	1.1	3.7
HS	7.8	15.3	9.0	9.0	3.8	9.5
FM	1.0	1.2	1.3	1.1	1.1	N/A
TR	0.1	0.0	0.0	0.5	0.5	1.0
P5	77.2	76.4	N/A	N/A	73.0	74.0
MFT-s	46.2	48.4	38.9	49.4	50.9	52.0
MFT-n	52.4	54.3	44.1	55.4	56.9	48.0

Appendix 10: Amount of sediment-bound oil (weight %) obtained from the twelve sites under investigation. Determined by Dr. Mike MacKinnon at the Syncrude Research Centre, Edmonton, Alta.

Site	2000			2001		
	June	July	August	June	July	August
NW	0.21	0.27	0.14	0.24	0.36	0.64
CL	0.23	0.13	0.26	0.14	1.13	0.87
1mCT	0.98	1.24	0.06	0.85	1.17	0.92
SP	0.69	0.48	0.32	0.89	0.87	1.14
WH	0.29	0.13	0.03	0.39	0.57	0.57
H63	0.21	0.54	0.20	0.24	0.55	0.47
HS	0.38	0.41	0.20	0.67	0.60	0.62
FM	0.22	0.26	0.08	0.25	0.17	0.25
TR	0.05	0.03	0.00	0.04	0.08	0.08
P5	1.41	1.26	N/A	N/A	3.75	1.47
MFT-s	0.78	1.31	0.78	0.81	2.34	3.90
MFT-n	1.40	1.40	0.44	1.52	1.38	1.91

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