EXAMINING OIL SANDS DISSOLVED CARBON AND MICROBIAL DEGRADATION USING STABLE ISOTOPE ANALYSIS

By

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Abstract

Examining Oil Sands Dissolved Carbon and Microbial Degradation Using Stable Isotope Analysis

Oil sands mining operations in northeastern Alberta are rapidly expanding. Upgrading and extracting the bitumen from the sand requires large volumes of water generating large quantities of oil sands process water/materials (OSPM) which is high in organic content. Some of the major organic components found in OSPM include unrecovered bitumen, polycyclic aromatic compounds (PACs), naphthenic acids (NAs) and humic acids. Concerns of acute and chronic toxicity resulting from OSPM have led to provincial legislation preventing the discharge of OSPM into local water and mandating the reclamation of areas affected by oil sands mining. To date, OSPM is stored on lease in settling basins while the mining companies evaluate reclamation strategies.

One of the reclamation strategies involves the use of wetlands constructed with differing amounts of OSPM and organic amendments such as peat. Currently, numerous wetlands, both natural and constructed, are present on oil sands leases. To determine the sustainability of these wetlands for reclamation, the assimilation and flow of carbon and nitrogen within the systems need to be defined. Stable isotope analysis can enhance this understanding. To effectively use stable isotopes in the field, there is the need to determine the changes in stable isotope values occurring from the microbial degradation of organic components such as NAs which contribute a significant portion to the dissolved organic carbon (DOC) in reclamation sites.

This study examined the microbial degradation of commercial and oil sands derived NAs by oil sands derived microbial cultures. Changes in stable isotopes values in the biomass ($\delta^{13}C$, $\delta^{15}N$), DOC and dissolved inorganic carbon (DIC) ($\delta^{13}C$) arising from degradation of the DOC were tracked in both static and semi-continuous tests. Utilization of commercial and oil sands derived NAs resulted in minimal change of the DOC stable isotope values. The biomass was ^{13}C enriched for both the commercial (0.3 to 2.9 per mil (‰)) and oil sands derived NAs (3.7 to 8.5 ‰) relative to the DOC stable isotope values. DIC stable isotope values showed higher variability (-5 to +5.5 ‰). The semi-continuous tests showed biomass that was ^{15}N enriched (3.8 to 8.4 ‰) with the assimilation of ammonium. Isotope trends established in the laboratory study provide further understanding into assimilation of carbon and nitrogen compounds in the field.

DOC and DIC concentration and carbon stable isotope values were determined for water sampled from 13 oil sands aquatic reclamation sites varying in age, construction and organic material. Both DOC and DIC concentrations were elevated in OSPM affected sites, by an average of 40 mg/L for DOC and 83 mg/L for DIC concentrations. DOC concentrations were also elevated by approximately 10 mg/L at high organic sites. $\delta^{13}C$ DOC values were slightly ^{13}C enriched in young sites: 0.6 ‰ compared to $\delta^{13}C$ DOC values at the mature sites. Also, from June to July ^{13}C enrichment (0.3 to 1.9 ‰) of the DOC for all sites was seen. Corresponding with the enrichment seen in the DOC, ^{13}C depletion (-8.8 to -0.3 ‰) of the DIC was seen for most sites from June to July. The trends seen from June to July may be a result of the release of readily degradable organics from the spring thaw stimulating the microbial community. The baseline values determined for DOC and DIC may assist future field food web studies.

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“However long the night, the dawn will break”

African Proverb

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1 Chapter 1. General Introduction

1.1. Oil Sands Overview

In northeastern Alberta, near Fort McMurray, Canada in the Athabasca River basin, lies one of the largest hydrocarbon reserves in the world. The deposit covers an area greater than 42 000 km^2 and contains approximately 1.7 trillion barrels of bitumen (Madill et al. 2001, Holowenko et al. 2002). Bitumen, a natural carbon source deposit, consists of a complex mixture of saturated petroleum hydrocarbons that are either acyclic or cyclic and contain long chain alkyl groups. In addition there are polycyclic aromatic compounds (PACs) (alkyl, azo, and thio PACs) and carboxylic acids such as naphthenic acids (NAs) (Madill et al. 2001). At present, oil sands are removed using surface mining technologies and the bitumen is extracted and refined to produce over 120 million barrels of synthetic sweet crude oil annually. This amount is to be significantly increased to approximately 270 million barrels by 2015, and then eventually to 400 million barrels per year as new companies set-up operations and existing ones expand operations (Holowenko et al. 2002, Alberta Energy and Utilities Board 2006).

Prior to upgrading into sweet crude oil, the bitumen is extracted from the sand via the Clark caustic hot water extraction (flotation) process, for the majority of producers (Mackinnon and Boerger 1986). In this process, the oil sand ore is digested with an alkaline water mixture causing the bitumen to separate as froth from the sand (Holowenko et al. 2002). During extraction, large volumes of wastewater are generated. To process 1 m^3 of oil sands ore, 3 m^3 of water are required, thereby producing 4 m^3 of tailings (solids/fluid) (Holowenko et al. 2002).

The tailings contain a mixture of mineral solids, such as sand, silt and clays, as well as dissolved materials including both inorganic and organic compounds such as NAs, phenols, cresols, mercaptans and thiophenols; these are predominantly present as sodium salts. Additional organic components also found include PACs, and unrecovered bitumen (Mackinnon 1989, Holowenko et al. 2002, Quagraine et al. 2005). Since these tailings are acutely toxic to aquatic organisms (Mackinnon and Boerger 1986) there is no permitted release of process material from the oil sands leases to the receiving waters of the Athabasca River drainage. To store the generated process material, settling basins (tailings ponds) have been constructed (Mackinnon 1989). In the settling basins, tailings thicken or become densified and are referred to as "mature fine tailings" (MFT). With gravity, MFT settles to the bottom of the basin with clarifying water on top (Leung et al. 2001, Salloum et al. 2002).

Also, as part of the lease agreements, the oil sands companies must reclaim all processed material by creating landscapes that are equivalent to those that existed prior to initiation of the mining operations. To aid in the reclamation process, wet and dry landscape options have been put forth as two possibilities of sustainable environments. The goal of the wet landscape option is to transfer MFT into the mined-out pits and cap them with process water and/or freshwater to produce new aquatic environments including lakes, ponds and wetlands (Pollet and Bendell-Young 2000, Madill et al. 2001, Leung et al. 2001). In the dry landscape approach, MFT is dewatered by the addition of gypsum (calcium sulfate) creating a more solid mixture known as consolidated tailings (CT) (up to 80% solids) that can be applied to the ground to allow a base for reforestation (Leung et al. 2001, Salloum et al. 2002).

To evaluate the environmental sustainability of the wet landscape option, Syncrude Canada Ltd. created various types of reclaimed aquatic systems such as the 1989 series of 0.16 ha experimental test pits (mesocosms). Each of the test pits was constructed to have similar temperature, water flow, inputs and basin morphology, but they differ in the quality and quantity of process water or MFT used in the construction (Farwell et al. submitted). Differences in the water and sediment quality between the test pits have provided researchers the opportunity to study the impacts of oil sands process affected material (OSPM) on aquatic organisms (Ganshorn 2002, Siwik et al. 2000, Hayes 2005, Nero et al. 2006) and the incorporation of oil sands constituents into aquatic food webs (Leung et al. 2001, Elshayeb 2006, Farwell et al. submitted). In addition, various wetlands with different amendments (e.g. differing levels of peat substrate) have also been created to further evaluate the wet landscape option and its future viability (Smits et al. 2000, Pollet and Bendell-Young 2000, Bendell-Young et al. 2000).

1.2. Naphthenic Acids

NAs encompass a complex group of diverse alkyl-substituted acyclic (highly branched), monocyclic, and polycyclic carboxylic acids with surfactant-like properties (Rogers et al. 2002, Holowenko et al. 2001, Headley and McMartin 2004). The chemical formula is $C_nH_{2n+Z}O_2$, where n denotes the carbon number and Z specifies the hydrogen deficiency that has been produced from ring formation (Figure 1.1). For example a NA with Z = 0 family is acyclic, when Z = -2 family, the acid contains 1 ring, Z= -4 family, 2 rings and so on (Clemente and Fedorak 2005) (Figure 1.1). The ring structures on NAs of oil sands origin are predominantly either cyclopentane or cyclohexane (Herman et al. 1994, Holowenko et al. 2001). NAs commonly found within oil sands process affected

waters can be in the range of n = 7-30 and Z = 0 to -12, but typically are found with a Z = -4 to -6 (Del Rio et al. 2006).

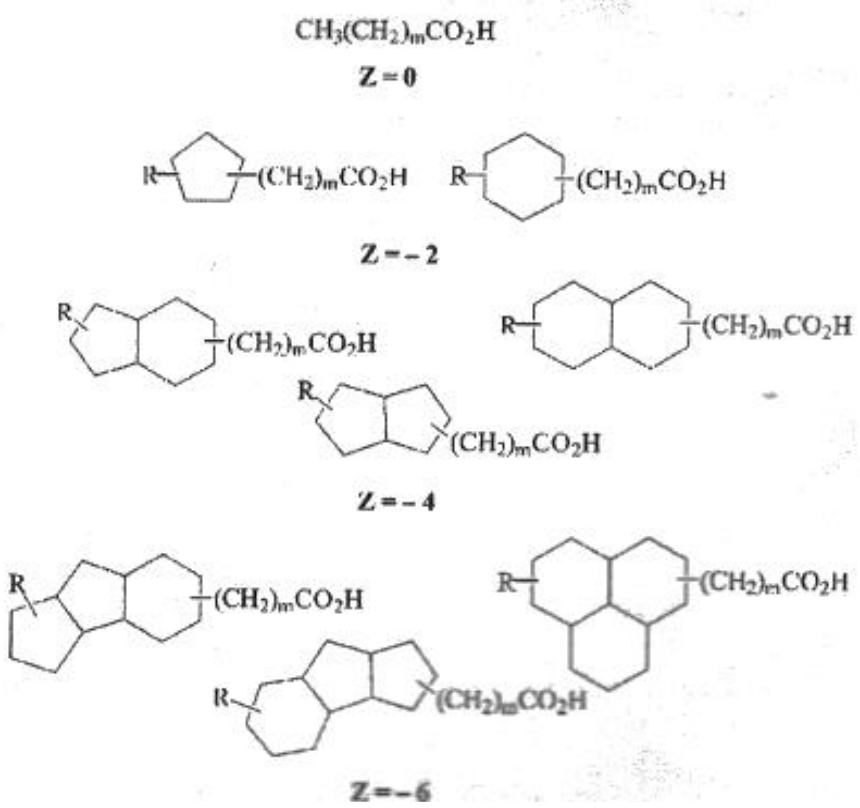

Figure 1.1. Examples of typical naphthenic acid structures where R is an alkyl group and m ≥ 0 (from Clemente et al. 2004).

Physical and chemical properties of the NAs are variable due to the high degree of structural complexity. Typically, NAs have a boiling point between 250ºC and 350°C and mixtures are a viscous liquid with a musty hydrocarbon odour. The colour can vary from pale yellow through black depending on the concentrations present. Molecular weights range between 140-450 amu and the density is between 0.97 to 0.99 g/cm^3. Solubility in water tends to be less than 50 mg/L at pH 7. The octanol water partition coefficient is approximately 2.4 at a pH of 7 and 2 at a pH of 10 (Headley and McMartin 2004).

The alkaline environment created during extraction of the oil sand ore solubilizes the NAs and concentrates them as a mixture of sodium salts (Clemente and Fedorak 2005). On the oil sands leases, NA concentrations range between <2 to 120 mg/L for settling basins and reclaimed sites, significantly elevated compared to the 0.1 to 0.9 mg/L of NAs found in the Athabasca River (Holowenko et al. 2002, Clemente and Fedorak 2005, Del Rio et al. 2006). Increased solubility of the NAs makes them more bioavailable to organisms (Mackinnon and Boerger 1986, Rogers et al. 2002, Clemente and Fedorak 2005). Extracted oil sands ore contains approximately 9-12% bitumen and 200 mg/kg of NAs; therefore, for 500 000 tonnes of ore processed there is the potential for 100 tonnes of NAs to be released (Clemente and Fedorak 2005).

1.3. Quantification of Naphthenic Acids

Due to the high degree of complexity of the NAs, quantifying and characterizing them is an ongoing challenge. Fourier transformed-infrared spectroscopy (FT-IR) developed by at Syncrude Canada in 1995 has been employed by Holowenko et al. (2001) and Rogers et al. (2002) to determine total NA content. Other technologies used

to determine total NA concentrations in mixtures include high performance liquid chromatography (HPLC). HPLC analysis is advantageous due to the time efficiency in sample preparation compared to FT-IR; however, the large variability that occurs between "identical" samples hinders this method. The results obtained also seem to be influenced by the particular commercial NA mixture selected to construct a calibration curve. Reasons for the variation have not been determined (Clemente et al. 2003).

1.4. Characterization of Naphthenic Acids

To characterize the NAs by the number of carbons, rings and their proportions within a mixture, gas-chromatography mass spectrometry (GC-MS) has been employed (Holowenko et al. 2002, Clemente et al. 2003, Clemente et al. 2004). Until recently, this method provided the most detail of a given NA mixture by showing the distributions and proportions of the C numbers, and Z families through a 3-D graph ("fingerprint") (Figure 1.2). Recently, HPLC/Quadrupole Time-Of-Flight (QTOF)-MS has been developed and has shown differences in the "fingerprints" compared to the GC-MS method (Bataineh et al. 2006). Further work is needed to gain a more thorough understanding of the differences.

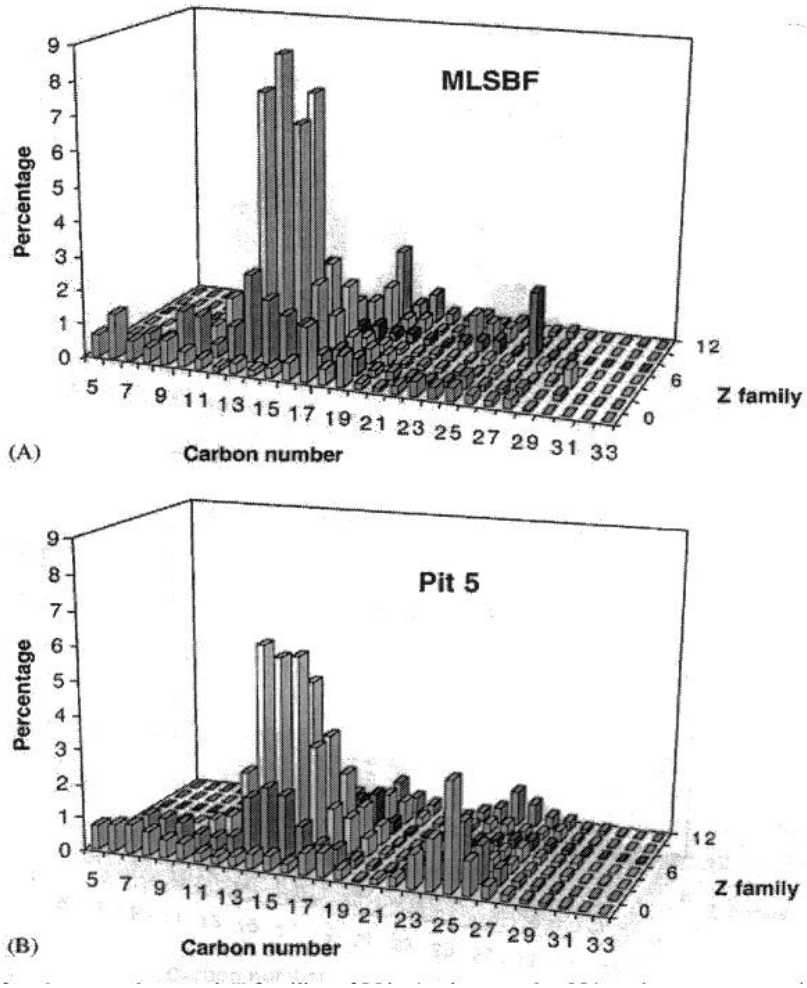

Figure 1.2. The distribution of carbon numbers and Z families (GC-MS) of fresh NAs from Mildred lake settling basin (MLSBF) and aged NAs (Pit 5) (from Holowenko et al. 2002).

1.5. Microbial Communities Present in the Oil Sands

Enumeration and identification of microbial communities present in oil sands affected areas have been conducted by various researchers over the years (Wyndham and Costerton 1981, Foght et al. 1990, Herman et al. 1993, Herman et al. 1994, Del Rio et al. 2006, Hadwin et al. 2006). Early on, it was found that natural microbial communities present in oil sands affected areas could degrade hydrocarbons (Wyndham and Costerton 1981). Further work characterized individual microbial species and determined which hydrocarbons they could degrade. Microbial isolates included: filamentous Actinomycetes, *Arthrobacter*, *Acinetobacter*, *Pseudomonas* and *Alicaligenes*. However, the importance of having a diverse microbial community was demonstrated in studies that showed that no single strain could degrade a mixture of hydrocarbons (Foght et al. 1990).

To better understand what species were involved in the degradation of hydrocarbons, challenges were experienced in obtaining microbial isolates from oil sands process affected (Herman et al. 1993 and 1994). Initially, *Acinetobacter* was the only microbial isolate that was identified as degrader of surrogate NAs. Mixed cultures were able to degrade methylated mixtures of carboxylic acids, however, no individual degrader isolates could be determined (Herman et al. 1993). The need to have a mixed culture for degrading NAs was further emphasized when studies identified that together *Pseudomonas stutzeri* and *Alcaligenes denitrificans* could degrade commercial NAs and that *Pseudomonas fluorescens*, *Acinetobacter calcoaceticus* and *Kurthia* sp. could degrade oil sands derived NAs (Herman et al. 1994). Using degradation as an endpoint, community differences between microbial cultures isolated from process and non-process affected sites were noted by Del Rio et al. (2006). Further research identified changes in

microbial community structures depending on the level of process material and the season. The number of *Actinomycetes* increased which corresponded with decreases in NA degradation. Examinations of community structure showed that microbial communities from no oil sands process material (OSPM) sites were less homogeneous compared to OSPM sites (Hadwin et al. 2006).

1.6. Microbial Degradation of Naphthenic Acids

Due to the high degree of complexity of the natural NA mixtures and a lack of sources of individual NA compounds, surrogate NAs were used in early microbial degradation studies (Herman et al. 1993, Herman et al. 1994, Lai et al. 1996). Examples of surrogate NAs used include hexadecane, palmitic acid, cyclopentane and cyclohexane and their methylated derivatives. Within a 1-week period, with sufficient amounts of nitrogen and phosphorus, and using bacteria originating from OSPM, mineralization of each of the surrogate NAs had occurred, however, degradation of the methylated ones occurred to a lesser extent (Herman et al. 1993). Acyclic surrogate NAs were found to degrade more rapidly; 10-15 % in 4 weeks compared to 2-4 % in 8 weeks for the cyclic surrogate NAs (Lai et al. 1996). Also, results from Del Rio et al. (2006) showed significantly slower degradation (1-20 % in 14 days) with a complex surrogate NA (decahydronaphthoic acid (DHNA); bicyclic) compared to the surrogate NA (cyclohexane carboxylic acid; monocyclic) with less cyclization (15-30 % degradation in 14 days). Decreased dissolved oxygen levels (<1 mg/L) and temperature (from 15 °C to 5 °C) were also found to decrease degradation, conversely, additions of phosphorus increased microbial activity (Lai et al. 1996).

Degradation experiments were also conducted using commercial and oil sands derived NAs (Herman et al. 1994, Clemente et al. 2004, Scott et al. 2005, Del Rio et al. 2006). Commercial NAs degraded more readily and to a greater degree, than oil sands derived NAs. This was true when NAs were incubated directly with OSPM, and also with NA-degrading enrichment cultured developed from OSPM. In a 24-30 day period, Herman et al. (1994) showed greater microbial growth on a commercial NA extract (Kodak NAs) (60 mg/L), with 50 % of the NAs converted to CO_2 compared to 20 % converted to CO_2 on oil sands derived NA extract (Mildred Lake Settling Basin). Clemente et al. (2004) compared the degradation of two commercial NAs (Kodak and Merichem Refined) that had significantly different NA composition based on C number and Z family. In a 10-day period, Merichem Refined NA concentrations decreased from 100 mg/L to approximately 10 mg/L. The Kodak NA concentrations decreased from 82 mg/L to less than 3 mg/L during a 36-day period. Both showed approximately 60% of the NAs being detected as CO_2. Analysis of the mixtures with GC-MS showed that the lower molecular weight NAs had been degraded (Clemente et al. 2004). Scott et al. (2005) and Del Rio et al. (2006) confirmed earlier results found by Herman et al. (1994) that NAs originating from the oil sands tailings water were not as easily degradable as the commercials NAs. This is likely because lower molecular weight NAs are dominant in commercial mixtures, and these are more readily degradable. On average commercial NAs were over 80% biodegraded within a 10-day period compared to minimal degradation (17 % NA decrease) in a 40-day period when Syncrude OSPM acted as the sole NA source. In addition, Del Rio et al. (2006) demonstrated that *Pseudomonas* isolates were only able to degrade 15 % of the Kodak NAs (400 mg/L) in 4 weeks as

compared to 95 % by a mixed culture, demonstrating the effectiveness of co-metabolism and the need for biodiversity in environments (Del Rio et al. 2006).

1.7. Stable Isotopes

Carbon 13 ($\delta^{13}C$) and nitrogen 15 ($\delta^{15}N$) stable isotope ratios are used to trace energy flow (food sources) and determine trophic structure in food webs (DeNiro and Epstein 1978 and 1981, Fry and Sherr 1984, Minagawa and Wada 1984, Vander Zanden and Rasmussen 2001). Stable isotope ratios of carbon and nitrogen can be defined as $\delta X = [(R_{sample}) - (R_{standard})/(R_{standard})] \times 1000$, where X is equal to ^{13}C or ^{15}N and R_{sample} is equal to $^{13}C/^{12}C_{sample}$ or $^{15}N/^{14}N_{sample}$ and $R_{standard}$ is equal to $^{13}C/^{12}C_{standard}$ or $^{15}N/^{14}N_{standard}$ (Fry and Sherr 1984). $\delta^{13}C$ isotopes are typically used to identify dietary sources of a consumer because $\delta^{13}C$ values are minimally altered (<1 per mil (‰) from one trophic level to another (DeNiro and Epstein 1978, Peterson and Fry 1987, Hobson and Welch 1992). Using the concept of minimal alteration, $\delta^{13}C$ isotopes have also been used as to trace origins of organic material (Peterson and Fry 1987, Kirkluk et al. 1995, Mazeas et al. 2002, Hall et al. 1999, Meckenstock et al. 1999). In contrast, $\delta^{15}N$ values have shown a greater degree of change, approximately 3-4 ‰ per increasing trophic levels (Minagawa and Wada 1984, Peterson and Fry 1987, Hobson and Welch 1992).

Various factors can cause the ratios of $^{13}C/^{12}C$ ($\delta^{13}C$) or $^{15}N/^{14}N$ ($\delta^{15}N$) to change (Peterson and Fry 1987). Changes in the isotope values occur when organisms preferentially select or excrete the lighter or heavier isotope, thereby increasing or decreasing its original proportion and thus altering the isotope signature. For example, if the lighter isotope (^{12}C) of a substrate is preferentially incorporated into the biomass of the substrate-consuming organism then the $\delta^{13}C$ of the biomass of an organism will

become ^{13}C depleted (more negative). An organism preferentially excreting the lighter isotope (^{14}N) will become more enriched in the heavier isotope (^{15}N) and the $\delta^{15}N$ will be more positive (DeNiro and Epstein 1978 and 1981, Minagawa and Wada 1984, Meckenstock et al. 1999, Barth et al. 2000). Isotope changes at the microbial level will be evident in other parts of the food web (DeNiro and Epstein 1978 and 1981, Fry and Sherr 1984, Peterson and Fry 1987, Persson et al. 2001, McCutchan Jr. et al. 2003).

1.7.1 Oil Sands Related Stable Isotope Studies

Recent studies in the oil sands have used stable isotopes to gain a better understanding of carbon and nitrogen cycling in food webs present in areas containing OSPM (Elshayeb 2006, Farwell et al. submitted, Daly 2007). Stable isotope analysis (SIA) of carbon demonstrated that food webs in ponds with and without OSPM greater than 10 years old did not differ in food chain length. The $\delta^{13}C$ values of variables such as DOC, DIC, sediments and particulate organic carbon also showed no differences. Variability in carbon isotope values of the primary producers was observed, but attributed to other possible sources of carbon such as atmospheric CO_2. Unlike the results found by Elshayeb (2006), trends of ^{13}C depletion were detected in predatory invertebrates in ponds with greater OSPM material, indicating uptake of OSPM (Farwell et al. submitted).

Studies by Elshayeb (2006) and Farwell et al. (submitted) also examined uptake of nitrogen at different levels in the food web in the reclaimed sites. Both found a trend towards ^{15}N enrichment in the biota and sediment from OSPM sites. Contributions of nitrogen in these systems may include inputs of sewage at the time of construction of the reclaimed sites, the presence of amines from oil sands upgrading, and the possible

leaching of nitrates and ammonia from the additions of peat amendments. These inputs of nitrogen are continuously recycled through the food webs in the reclaimed systems (Elshayeb 2006, Farwell et al. submitted).

1.7.2 Stable Isotopes and Degradation

Degradation of carbon and nitrogen compounds at the microbial level to determine correlations with changes in stable isotope values in various by-products has been examined (Macko and Estep 1984, Macko et al. 1987, Hoch et al. 1992, Wattiaux and Reed 1995, Hall et al. 1999, Meckenstock et al. 1999 and 2002, Barth et al. 2000, Morasch et al. 2001, 2002, 2004, Lehmann et al. 2002, Mazeas et al. 2002, Zyakun et al. 2003). By-products of degradation or endpoints examined included $\delta^{13}C$ measurements of the substrate, microbial biomass, CO_2 and DIC (Macko and Estep 1984, Macko et al. 1987, Meckenstock et al. 1999, Hall et al. 1999, Barth et al. 2000, Morasch et al. 2001, Lehmann et al. 2002). Nitrogen isotope studies have mainly been used to examine the assimilation of different nitrogen compounds by microbial cultures. Endpoints measured using $\delta^{15}N$ isotopes included substrate and microbial biomass (Macko and Estep 1984, Macko et al. 1987, Hoch et al. 1992, Wattiaux and Reed 1995, Lehmann et al. 2002).

Carbon isotope changes in degradation experiments are highly variable between studies and changes may be dependent on the microbial species used (Hunkeler and Aravena 2000; Meckenstock et al. 2002; Wick et al. 2003), the substrates of degradation interest, measured endpoints, and the environment; aerobic or anaerobic (Hunkeler and Aravena 2000, Morasch et al. 2004, Meckenstock et al. 2004). Mazeas et al. (2002) found no differences in the $\delta^{13}C$ of the remaining substrate mixture consisting of saturated hydrocarbons and alkylated and non-alkylated PACs after degradation by a

mixed culture. Another study examined the individual degradation of n-hexadecane ($\delta^{13}C$ = -44.6 ‰) and naphthalene ($\delta^{13}C$ = -21 ‰) by *Burkholderia* sp. and *Pseudomonas putida* (Zyakun et al. 2003). Greater changes in isotope values were noted with *Burkholderia* sp. than with *P.putida*. $\delta^{13}C$ for the substrate, CO_2, biomass and exometabolites (DOC and DIC combined) were measured at the end of each experiment. With approximately 50 % degradation of n-hexadecane, isotope values for both the CO_2 and biomass were found to be ^{13}C depleted (–5.6 ‰ and -2 ‰) and the exometabolite was ^{13}C enriched (3.1 ‰) relative to the initial n-hexadecane isotope value. Degradation of naphthalene resulted with CO_2 that was ^{13}C depleted (–3.1 ‰) relative to the initial naphthalene isotope value as was the exometabolite (1.9 ‰), similar to degradation of n-hexadecane, but oppositely to what was previously observed, the biomass was ^{13}C enriched (1.8 ‰) (Zyakun et al. 2003). Hall et al. (1999) monitored the carbon isotope values of the CO_2 and biomass after complete degradation of phenol ($\delta^{13}C$ = -28.5 ‰) by *P.putida* and found that the CO_2 was ^{13}C depleted (–2.5 ‰) relative to the substrate (phenol) and the biomass was ^{13}C enriched (2.5 ‰) relative to the substrate, similar results occurred for a different species, except, the biomass had increases of 3 ‰. Degradation of benzoate by the same species showed similar trends but decreases and increases were different. $\delta^{13}C$ for the CO_2 had decreased by –5 ‰ and the biomass had increased 0.7 ‰ relative to the substrate (Hall et al. 1999).

Nitrogen isotope studies have focused on uptake and assimilation of different nitrogen sources. Macko and Estep (1984) and Macko et al. (1987), examined $\delta^{15}N$ values of the biomass relative to the substrate which were amino acids or amino sugars. Results showed ^{15}N enrichment of up to 22.3 ‰ with amino acids such as glutamic acid

and aspartic acid and ^{15}N depletion down to -12.9 ‰ with amino acids such as alanine of the biomass relative to the initial substrate isotope values. Amino acids such as alanine directly enter the cell and are deaminated by deaminating enzymes to produce ammonia which enters the TCA cycle. The ammonia is fixed into glutamate by glutamate synthetase which is passed onto amino acids used for protein synthesis. The depletion of the ^{15}N was thought to occur either through deamination. ^{15}N depletion was attributed to a loss of ^{15}N of ammonia during glutamate synthesis. Glutamic and aspartic acid enter the metabolic pathway directly and both have different C/N ratios, therefore, if energy is needed, more aspartate will need to be deaminated compared to glutamate because of a higher C/N ratio thereby causing ^{15}N enrichment (Macko and Estep 1984, Macko et al. 1987).

Hoch et al. (1992) examined the uptake of different low and high concentrations of ammonium by microbial cells and found large variability in isotope change, which were attributed to differences in uptake pathways, which are largely concentration dependent. ^{15}N enrichment of 13 ‰ was seen in microbial cells that were provided with high concentrations of ammonium, compared to ^{15}N depletion of -15 ‰ was seen with cells provided low amounts of ammonium. When concentrations of ammonium are high, uptake occurs through passive diffusion and is assimilated via glutamate dehydrogenase and at lower concentrations of ammonium uptake occurs through active transport and is assimilated via glutamine synthetase (Hoch et al. 1992).

1.8. Overall Objectives

The primary aim of this study was to use aerobic microbial microcosms to correlate the degradation of commercial and oil sands derived NAs with changes in the stable isotope values of the microbial biomass, DOC and DIC. The second objective was

to conduct a brief survey of various aquatic reclamation sites in the oil sands mining area to identify trends in DOC and DIC concentrations and stable isotope values, associated with sites of differing age of construction, construction materials and organic content levels. The stable isotope trends established from both the laboratory and field studies should enhance the interpretation of future field-based data in terms of carbon and nitrogen source utilization.

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2 Chapter 2. Examining the Microbial Degradation of Naphthenic Acids Using Stable Isotope Analysis

Abstract

Naphthenic acids (NAs) are a complex group of naturally occurring oil sands constituents that may constitute a significant portion of the dissolved organic carbon (DOC) pool available for microbial degradation in oil sands aquatic reclamation. One approach to better understand the utilization of oil sands process-derived carbon and nitrogen in reclamation involves the use of stable isotope analyses. In order to improve the usefulness of stable isotope analyses in the field, there is a need to determine the stable isotope values ($\delta^{13}C$, $\delta^{15}N$) associated with microbial degradation of a complex mixture of NAs and nitrogen sources.

In static and semi-continuous laboratory microcosms, utilization of commercial NAs by oil sands derived microbial cultures resulted in microbial biomass that was similar or ^{13}C enriched (0.3 to 2.9 ‰) relative to the DOC source, depending on the length of incubation. Utilization of the oil sands NA extract resulted in greater ^{13}C enrichment of microbial biomass (3.7 to 8.5 ‰) relative to the DOC source. Overall, the $\delta^{13}C$ of the DOC comprised of complex mixtures of NAs showed minimal change (-0.5 to +0.2 ‰) during the incubation period whereas the $\delta^{13}C$ of the DIC was more variable (-5 to +5.5 ‰). In some semi-continuous tests, the final $\delta^{15}N$ biomass values were ^{15}N enriched (3.8 to 8.4 ‰) relative to the initial biomass. The isotope trends established in this study enhance our interpretation of field-based data, particularly in terms of carbon source utilization and ^{15}N enrichment.

2.1 Introduction

Naphthenic acids (NAs) are one of the major groups of naturally occurring oil sands constituents that are of environmental concern in terms of oil sands reclamation, followed by polycyclic aromatic compounds (PACs) and salinity. Concentrations of NAs are elevated in process water generated during the caustic (sodium hydroxide) hot water extraction of oil sands to remove bitumen from the sand (Mackinnon and Boerger 1986). It is estimated that 3 m^3 of water is required to process 1 m^3 of oil sands, thereby producing 4 m^3 of tailings (solids/fluid) (Holowenko et al. 2002) which must be reclaimed based on the provincial lease requirements of zero discharge. Both the quality (particularly NA concentration) and quantity of process water are issues as the oil sands industry continues to expand operations, resulting in oil sands reclamation on a sub-watershed scale.

A complex group of carboxylic acids containing alkyl-substituted acyclic, monocyclic and polycyclic carboxylic acids make up NAs. Their prevalence and acute toxicity in oil sands tailings (Mackinnon and Boerger 1986) has led to research on the toxicity of oil sands NAs to fish (Nero et al., 2006; Farwell et al. submitted) as well as on the toxicity to dietary items such as phytoplankton (Hayes, 2005). Reviews by Headley and McMartin (2004), Quagraine et al. (2005) and Clemente and Fedorak (2005) have highlighted the recent advances in the qualitative and quantitative analyses of NAs following biodegradation. The qualitative analyses of NAs continues to be a challenge for assessing environmental risk associated with NAs. Studies have shown that over time, the acute toxicity of the NAs is reduced because of microbial degradation of the NAs (Herman et al. 1994, Holowenko et al. 2002, Clemente et al. 2004). Although the

acute toxicity may be reduced, there are still questions regarding the chronic toxicity and sustainability associated with different oil sands reclamation strategies. Interest lies in using stable isotope analyses as a tool to characterize the utilization of oil sands derived carbon sources by microbes in oil sands aquatic reclamation. This is one of the approaches used to study carbon dynamics as part of a larger project to assess the sustainability of different oil sands reclamation strategies.

In order to use stable carbon and nitrogen isotopes to determine the contribution of oil sands constituents as a carbon source within a reclamation option, there is a need to better understand the isotope change from source to consumer. In a series of experimental reclaimed ponds of similar age and size, there were trends of ^{13}C depletion and ^{15}N enrichment for some benthic invertebrates along a gradient of increased levels of oil sands processed-material suggesting that oil sands constituents may contribute to the carbon pool utilized by consumers in some reclamation options, depending on the construction materials (Farwell et al. submitted).

While there is information on the rate of degradation of various surrogate NAs (Herman et al. 1993; Lai et al. 1996; Del Rio et al., 2006) as well as commercial and oil sands derived NA mixtures (Herman et al., 1994; Clemente et al., 2004; Scott et al., 2005; Del Rio et al. 2006), there is no information on stable isotope analysis for NA degradation. Numerous studies have used stable carbon isotope analysis as a tracer and have focused on measuring changes in the stable isotope values of the substrate and CO_2 only. Mazeas et al. (2002) used a mixed culture to degrade a mixture of saturated hydrocarbons and PACs and found no changes in the $\delta^{13}C$ values for the substrate over time. In the study by Zyakun et al. (2003), there was variable ^{13}C enrichment (1.9 to 3.1

‰) of the substrate depending on the hydrocarbon examined and the rate of degradation. Hall et al. (1999) found differences in the $\delta^{13}C$ values of CO_2 depending on the chemical degraded (phenol vs. benzoate) and the species used, but found no change in the $\delta^{13}C$ values of microbial biomass.

To effectively use stable isotope analysis as a tool to trace oil sands derived carbon sources in aquatic reclamation, there is a need to further understand the stable isotope values associated with microbial degradation of complex mixtures such as NAs, which may comprise a significant portion of the dissolved organic carbon (DOC) within reclamation sites. Therefore, the objective of this study was to use oil sands derived microbial cultures in aerobic laboratory microcosms to degrade commercial and oil sands derived NAs and determine the relationship between the stable isotope values of the DOC source ($\delta^{13}C$) and nitrogen source ($\delta^{15}N$) and products, specifically microbial biomass ($\delta^{13}C$, $\delta^{15}N$) and respired carbon dioxide in the water as dissolved inorganic carbon (DIC) ($\delta^{13}C$). The results of this laboratory study will assist in the interpretation of field based stable isotope analyses examining carbon flow and dynamics of food webs in aquatic environments with varying reclamation strategies.

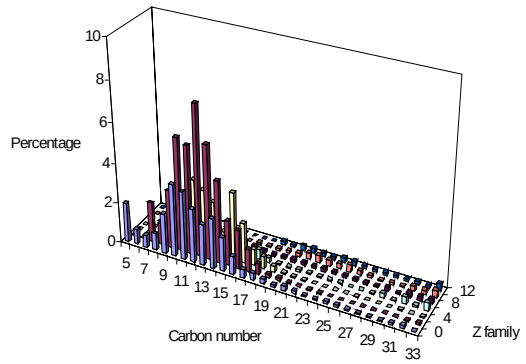
2.2 Materials and Methods

2.2.1 Naphthenic Acids

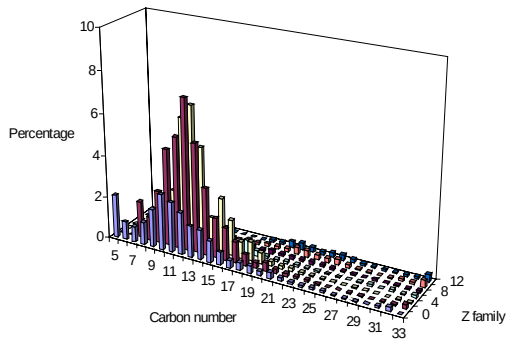
The complex NA mixtures used as the sole carbon substrate in these biodegradation tests included Merichem Crude and Merichem Refined (Merichem Chemicals and Refinery Services LLC, Houston, TX, USA) and an oil sands NA extract derived from oil sands process water. Merichem NA mixtures were prepared for use in

degradation tests in a solution of 0.1 M NaOH (EMD Chemicals, Gibbstown, NJ, USA). The oil sands NA mixtures were extracted from process water collected from Mildred Lake Settling Basin (MLSB) located on the Syncrude Canada Ltd. lease (Alberta, Canada) following the method of Nero et al. (2006). Briefly, process water was acidified to pH 2 and centrifuged at 3000 rpm for 8 minutes to collect the precipitant containing NAs, which was then dissolved in 0.1 N NaOH. Following centrifugation to remove suspended material, the supernatant was acidified, extracted with dichloromethane and then back-extracted with 0.1 N NaOH and stored in high-density polyethylene containers (Nero et al. 2006). Prior to use in the biodegradation tests, the oil sands NA extracts were filtered through a 47 mm, 0.45 μm mixed cellulose esters, hydrophilic Millipore membrane filter (Millipore Corporation, Billerica, MA, USA) and stored at room temperature in amber borosilicate glass bottles (Chase Scientific Glass Inc., Rockwood, TN, USA). To further reduce particulate organic carbon, NA extracts were also filtered through a 25 mm, 0.2 μm surfactant-free cellulose acetate Nalgene™ syringe filter (Nalge Nunc International, Rochester, NY, USA) for static tests.

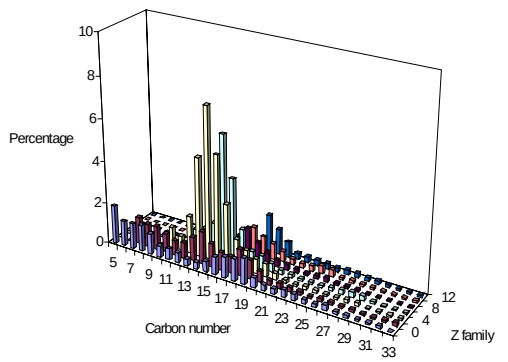
Although there have been recent advances in the analytical chemistry of NAs (Bataineh et al. 2006), at the time of this study analyses by gas chromatography-mass spectrometry provided partial information on the mass and relative distribution of NAs based on carbon numbers and Z families described by the general formula $C_nH_{2n+Z}O_2$ (Clemente et al. 2003). Figure 2.1 shows the relative distribution of the three naphthenic acids used in the tests. In general, Merichem Refined and Merichem Crude have a higher percentage of NAs with lower carbon number and Z families compared to the oil sands NA extract.



(a)



(b)



(c)

Figure 2.1. Distribution of naphthenic acids with the general formula, $C_nH_{2n+Z}O_2$ (n = carbon number; Z = amount of hydrogen deficiency produced from ring formation ie. Z=0, acyclic; Z=-2, one ring; Z=-4, two rings etc.). Sources of dissolved organic carbon used in biodegradation studies included Merichem Refined (a), Merichem Crude (b) and Mildred Lake Settling Basin (Syncrude) (c) (modified from Nero et al. 2006).

2.2.2 Mineral Media

Modified Bushnell-Haas mineral medium was used for all tests (Clemente et al. 2004). The mineral medium consisted of 1.0 g K_2HPO_4 (Sigma Chemicals Company, St. Louis, MO, USA), 1.0 g KH_2PO_4 (Fisher Chemical, Ottawa, ON, Canada), 0.2 g $MgSO_4 \cdot 7H_2O$ (Sigma Chemicals Company), 1 mL $CaCl_2 \cdot 2H_2O$ (0.02 g/mL) (BDH Chemicals Inc., Toronto, ON, Canada), 1 mL $FeCl_3 \cdot 6H_2O$ (0.005 g/mL) (BDH Chemicals Inc.) and 1.0 g NH_4NO_3 (BDH Chemicals Inc.) in 1 L of Milli-Q® ultra-pure water (Millipore Corporation, Billerica, MA, USA). To examine stable nitrogen isotope trends for biomass, the media was modified in some tests, such that quantities (0.4 g or 1.3 g) of NH_4Cl and (2.5 g) of KNO_3 (Mallinckrodt Baker Inc, Phillipsburg, NJ, USA) were used in place of 1.0 g NH_4NO_3. The source and quantity of the nitrogen source is specified for each test. After addition into the Milli-Q® ultra-pure water, the solution was stirred and pH adjusted to 7 with the addition of 1N NaOH and/or 1N HCL (Fisher Scientific, Nepean, ON, Canada).

2.2.3 Source of Bacteria and Microbial Culture

Bacteria used in this study originated from water samples taken from either an oil sands settling basin; MLSB or reclamation sites (Syncrude Canada Ltd, Alberta, Canada). Microbial cultures were grown at room temperature in modified Bushnell-Haas mineral medium with an NA mixture as the carbon source and transferred to fresh medium monthly. These microbial cultures were used for all tests with the exception of the short term static test (16 day) which used a direct inoculation of MLSB water.

2.2.4 Protocol for Static Test Incubations

Mineral medium (100 mL) was added into 250 mL TraceClean™ amber borosilicate glass bottles with a Teflon®-lined closure (Chase Scientific Glass Inc., Rockwood, TN, USA) and autoclaved at 120°C, 103 kPa for approximately 15 minutes in an American Cyclomatic Control (American Sterilizer Company of Canada, Brampton, ON, Canada) prior to usage. Next, either a commercial NA or oil sands NA extract in 0.1 N NaOH was added and then bottles were inoculated with 20 mL of a microbial culture or MLSB water sample (16-day test only). Bottles were maintained at room temperature and shaken every other day.

2.2.5 Protocol for Semi-Continuous Test Incubations

For the 3-day transfer semi-continuous test, the microcosms were prepared as described for the static tests using enriched microbial cultures that were grown on Merichem Refined NAs and 1.3 g/L NH_4Cl (Figure 2.2). To replenish nutrient and NA levels, 20 mL of the microbial suspension was transferred to 100 ml of fresh media every three days.

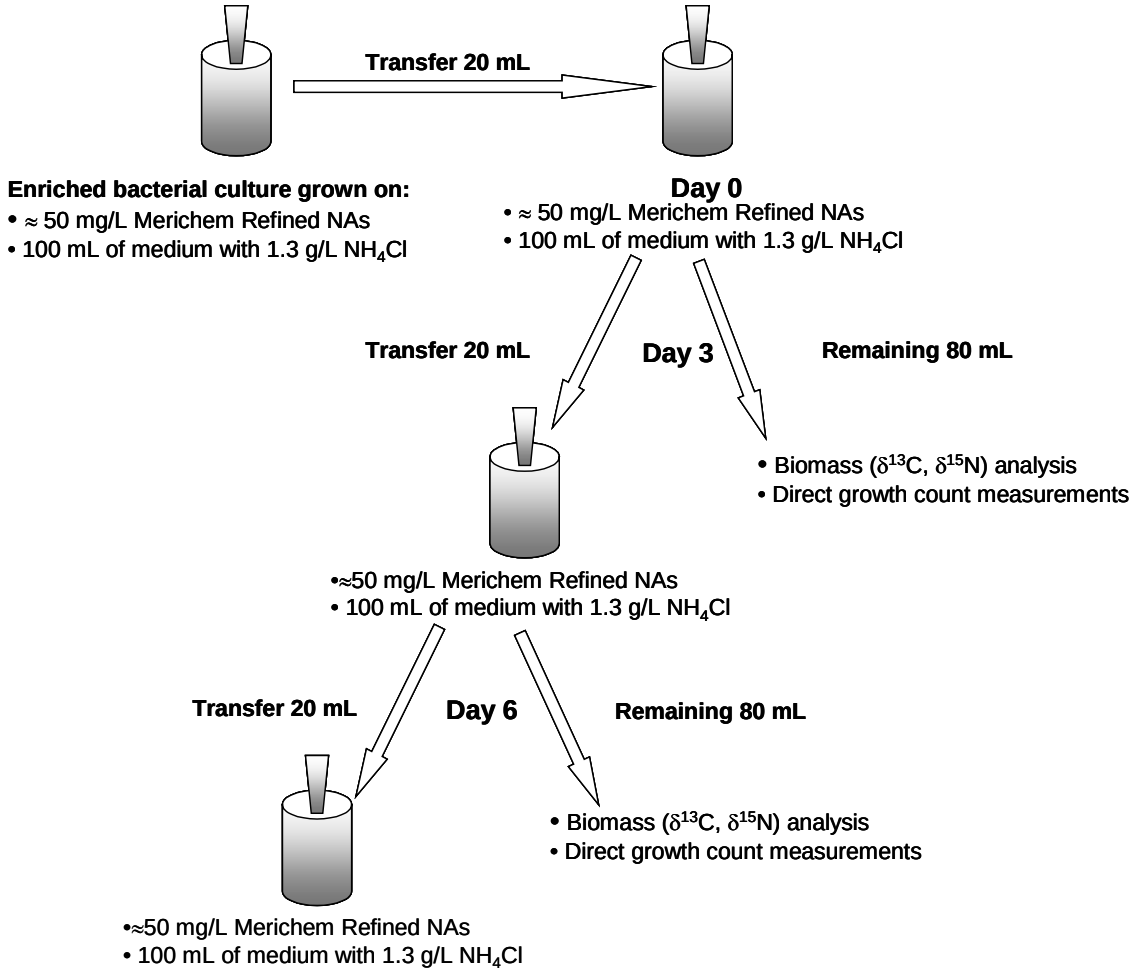


Figure 2.2. Description of the protocol for the 3 day transfer semi-continuous tests. Nominal NA concentrations are reported.

Modifications were made to the volume of media and method of transfer for the semi-continuous tests using either Merichem Refined NA or oil sands derived NA, with transfers every 13 days (Figure 2.3). For the 13 day transfer test, 400 mL of mineral medium with 1.3 g/L NH_4Cl was added to 1 L TraceClean™ amber borosilicate glass bottles (Chase Scientific Glass Inc., Rockwood, TN, USA), autoclaved and then supplied with either Merichem Refined and oil sands derived NA mixtures. The initial microbial culture used to inoculate the Merichem Refined NA test had previously been growing on 50 mg/L (nominal) Merichem Refined NA and 1.3 g/L NH_4Cl. The microbial culture used to inoculate the oil sands NA test had been growing on 50 mg/L (nominal) Merichem Refined NA and 2.5 g/L KNO_3. Cultures were centrifuged and resuspended in 100 mL of supernatant to provide the microbial suspension for inoculation. Every 13 days, 340 ml of the microcosm volume was centrifuged and the resulting microbial pellet was resuspended in 100 mL of supernatant and added to a bottle with fresh medium (400 mL) and NAs. The remaining volume (160 mL) was used for microbial counts and stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$).

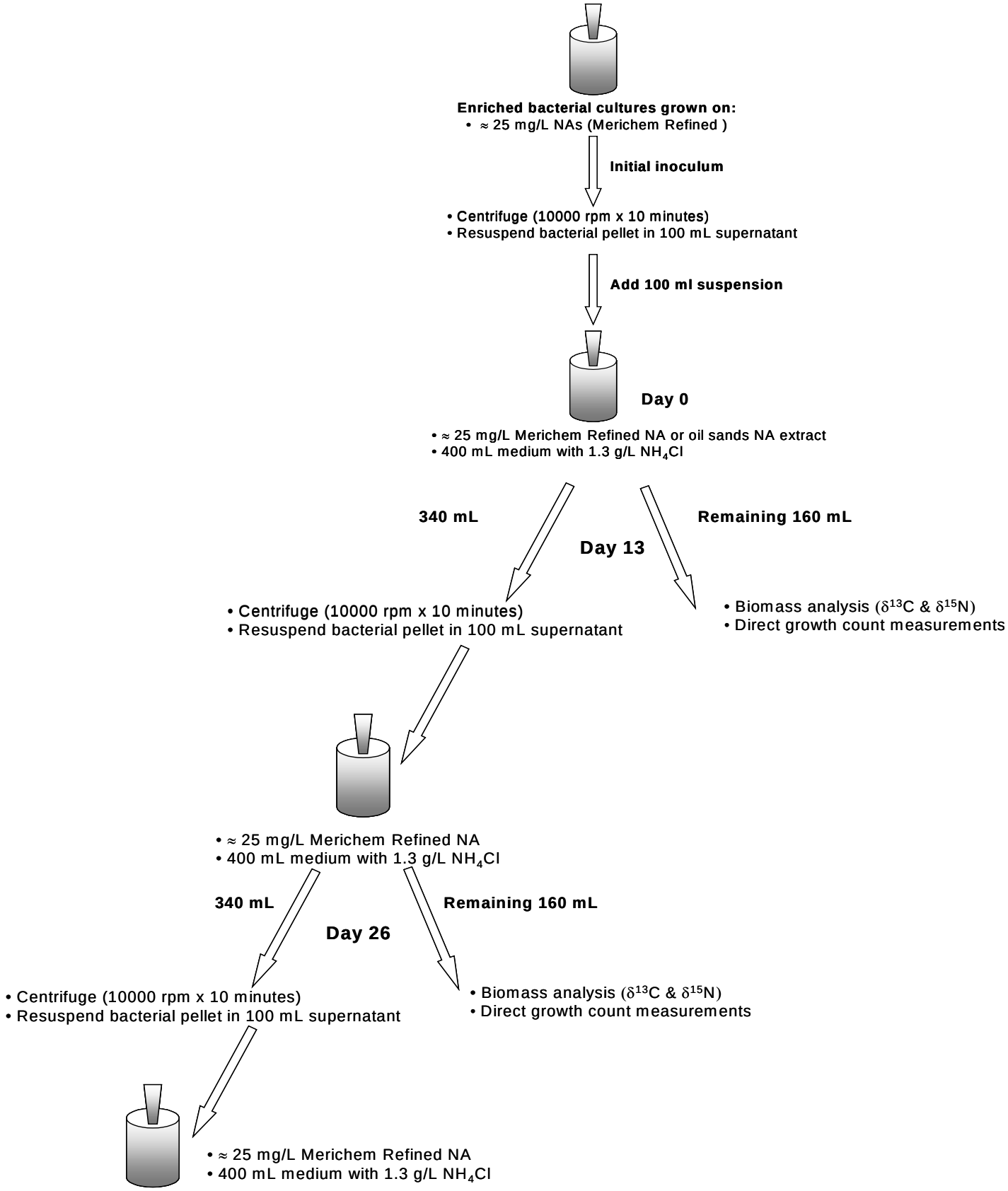


Figure 2.3. Description of the protocol for the 13 day transfer semi-continuous tests. Nominal NA concentrations are reported.

2.2.5 Measurement Endpoints for Tests

Microbial growth was monitored by direct counts using a Phase Contrast Nikon Labophot microscope (magnification 400x) (Nikon Corporation, Mississauga, ON, Canada) and a Petroff-Hausser counting chamber (cell depth = 1/50 mm) (Hausser Scientific, Horsham, PA, USA).

For the static tests, samples for measurements of DOC and DIC concentration and $\delta^{13}C$ values were preserved with 1 mL of 5% w/v $HgCl_2$ (Mallinckrodt Baker Inc, Phillipsburg, NJ, USA), and centrifuged at 10 000 rpm for 10 minutes using a Sorvall® RC-5B Refrigerated Superspeed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) with the Sorvall® SLA 1500 rotor (Thermo Fisher Scientific, Waltham, MA, USA) in 250 mL polypropylene copolymer centrifuge bottles (Nalge Nunc International, Rochester, NY, USA). The supernatant was filtered using a 25 mm, 0.45 μm polyethersulfone Nalgene® syringe filter (Nalge Nunc International, Rochester, NY, USA) into 40 mL TraceClean™ amber borosilicate glass vials (Chase Scientific Glass Inc., Rockwood, TN, USA) and sealed with open-top caps containing silicone-Teflon® septa (Chase Scientific Glass Inc., Rockwood, TN, USA). Samples were stored at 4°C and shipped to the G.G. Hatch Isotope Laboratory, University of Ottawa (Ottawa, ON, Canada) for analyses of concentration and stable isotopes for both DOC and DIC. Concentrations of DOC and DIC were determined using an OI high temperature persulfate wet-oxidation TIC-TOC Analyser Model 1010 (OI Analytical, College Station, TX, USA) with a precision of 0.002 ppm C. The $\delta^{13}C$ values for the DOC and DIC samples were analyzed with a continuous flow Thermo-Finnegan Mat DeltaPlus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). $\delta^{13}C$ values were

derived from the following formula, $(R_s/R_{st})–1) \times 10^3 = \delta^{13}C_{sample}$ (parts per thousand or per mil, ‰) where R_s is ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the ratio of the International Atomic Energy Agency (IAEA) and National Bureau of Standards (NBS) isotopic reference material; carbonate rock Vienna Pee Dee Belemnite (VPDB). All data is normalized to a precision of ± 0.2 ‰ using internal standards of sucrose, glycine and phthalate (St-Jean et al. 2003, personal communication with Paul Middlestead 2006).

Microbial biomass from static and semi-continuous tests were prepared for stable isotope analysis by preserving and centrifuging the sample as described for the DOC and DIC measurements. The particulate material containing the microbial cells was washed with 0.1 N NaOH to remove any residual NAs, centrifuged and the supernatant decanted. This process was repeated using Milli-Q® ultra-pure water to remove any residual salts. The microbial pellet was then transferred into 1.5 mL polypropylene microcentrifuge tubes (Fisherbrand, Ottawa, Canada) and centrifuged using a Thermo Electron IEC Micromax Centrifuge/RF (Thermo Fisher Scientific, Waltham, MA, USA) at 10 000 rpm for 10 minutes, the supernatant was removed and the pellet dried in a desiccator. Dried, homogenized material was weighed to approximately 1 mg into tin capsules (pressed, standard weight, 5 x 3.5 mm) (SerCon Ltd., Cheshire, United Kingdom) using a Mettler Toledo AT21 Comparator analytical balance (Mettler-Toledo Inc, Mississauga, ON, Canada). Samples and standard reference materials were analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios using the Thermo-Finnegan Delta-EA Isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Carlo Erba Elemental Analyzer (Thermo Fisher Scientific, Italy) at the Environmental Isotope Laboratory, Department of Earth Sciences, University of Waterloo (Waterloo, ON,

Canada). The following formula, $(R_s/R_{st})-1) \times 10^3 = \delta(^{13}$ or $^{15})$(C or N) (‰), where R = $^{13}C/^{12}C$ and/or $^{15}N/^{14}N$, R_s is the ratio for the sample and R_{st} is the ratio for the standard, was used to calculate isotope values. Standard reference materials included carbonate rock Vienna Pee Dee Belemnite (IAEA) for carbon and atmospheric air (National Institute of Standards and Technology) for nitrogen. Data is normalized to a precision of ± 0.2 ‰ for carbon analysis and ± 0.3 ‰ for nitrogen analysis using laboratory standards of sucrose, cellulose and graphite for carbon and ammonium sulphate for nitrogen analysis.

2.3 Results

2.3.1 Short Term Static Tests

Following a 10 day incubation period, bacteria in media containing the oil sands derived NA extract ($\delta^{13}C$, -28.9 ‰) and 1.0 g/L NH_4NO_3 mineral media ($\delta^{15}N$, 0.2 ‰) showed limited growth based on cell counts (day 0, 1.34 x 10^8 cells/mL; day 10, 3.29 x 10^8 cells/mL) and minimal changes in the concentrations of DOC (20 % decrease) and DIC (11 % increase) (Figure 2.4 a). As a result, there was little change in the $\delta^{13}C$ values of DOC (-0.1 ‰) and biomass (0.3 ‰), however there was slight ^{13}C depletion (-1.6 ‰) of DIC at day 10 relative to the initial DIC (Figure 2.4 b-d). The $\delta^{15}N$ of biomass varied throughout the exposure period (1.4 ‰) with mean values ranging from -3.1 ‰ to -4.5 ‰ between the six sampling dates (Figure 2.4 e).

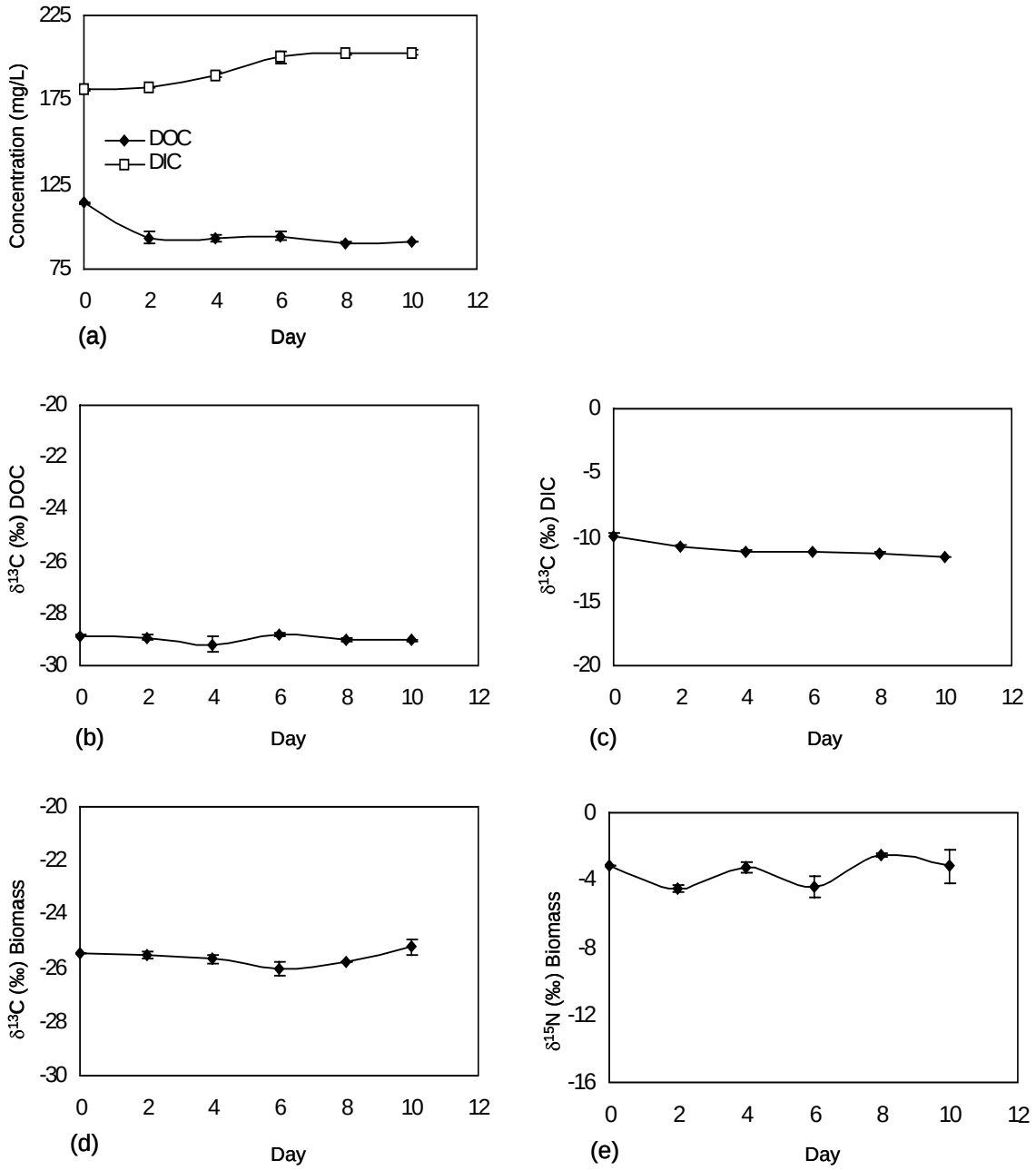


Figure 2.4. Changes in the concentrations of DOC and DIC (a) and the $\delta^{13}C$ values of DOC (b), DIC (c) and biomass $\delta^{13}C$ (d) and $\delta^{15}N$ (e) values over 10 days for cultures supplied oil sands derived NA extract (114.23 mg/L DOC) with 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

With a slightly longer incubation period (16 day), microcosms, inoculated with 20 mL of MLSB water and supplied with varying concentrations of Merichem Refined NAs ($\delta^{13}C$, -26.8 ‰) and 0.4 g/L NH_4Cl ($\delta^{15}N$, 1.6 ‰), showed moderate growth resulting in decreases in DOC concentrations between 47-67 % and increases in DIC between 7-24 % (Table 2.1). The highest concentration (45 mg/L DOC) had the greatest change in DOC and DIC concentration. At day 16, DIC was ^{13}C depleted (range of -2.8 to -5 ‰) relative to the initial $\delta^{13}C$ DIC values for all concentrations. While the $\delta^{13}C$ of DOC remained consistent (-0.1 to -0.4 ‰), there was ^{13}C depletion (-1.5 to -2.6 ‰) of biomass by day 16 indicating incorporation the ^{13}C depleted DOC. At day 16, the biomass was ^{13}C enriched by 1.6 to 2 ‰ relative to the initial DOC, independent of concentration. There was ^{15}N depletion of biomass (-3.4 to -5.8 ‰) for all concentrations.

Table 2.1. Endpoint measurements for the 16-day test using microbial cultures from MLSB cultured at different DOC concentrations of Merichem Refined NA with 0.4 g/L NH_4Cl[a].

Measurement	**Initial**	**Final**	**Δ [b]**
DOC (mg/L)	**44.45**	22.80	-21.65
Cell counts (cells/mL)	2.30×10^7	1.41×10^8	1.18×10^8
$\delta^{13}C$ DOC (‰)	-27.3	-27.7	-0.4
DIC (mg/L)	17.69	23.25	+5.56
$\delta^{13}C$ DIC (‰)	-9.4	-13.2	-3.8
$\delta^{13}C$ Biomass (‰)	-23.1	-25.7	-2.6
$\delta^{15}N$ Biomass (‰)	-9.7	-13.1	-3.4
DOC (mg/L)	**29.83**	16.09	-13.74
Cell counts (cells/mL)	3.45×10^7	1.61×10^8	1.26×10^8
$\delta^{13}C$ DOC (‰)	-27.3	-27.6	-0.3
DIC (mg/L)	14.32	17.13	+2.81
$\delta^{13}C$ DIC (‰)	-9.0	-11.8	-2.8
$\delta^{13}C$ Biomass (‰)	-23.9	-25.4	-1.5
$\delta^{15}N$ Biomass (‰)	-9.1	-12.6	-3.5
DOC (mg/L)	**23.08**	7.62	-15.46
Cell counts (cells/mL)	3.50×10^7	1.59×10^8	1.24×10^8
$\delta^{13}C$ DOC (‰)	-27.5	-27.6	-0.1
DIC (mg/L)	12.99	13.82	+0.83
$\delta^{13}C$ DIC (‰)	-6.6	-11.6	-5.0
$\delta^{13}C$ Biomass (‰)	na[c]	-25.7	na
$\delta^{15}N$ Biomass (‰)	-7.2	-13.0	-5.8

[a] Test used: Direct microbial inoculation from a water sample originating from MLSB.

[b] Δ = final (day 16)– initial (day 0)

[c] na = not available, due to low sample weight

Based on the short-term tests, longer incubation periods were used for subsequent exposures. In this case, Merichem Refined or Merichem Crude was used as a substrate since commercial NAs degrade more easily than oil sands NAs (Scott et al., 2005) and the DIC concentrations were far lower compared to the oil sands NA extract. Also, different sources of nitrogen were examined to further elucidate the $\delta^{15}N$ trends for biomass.

2.3.2 Long Term Static Tests

Microbial cultures were grown for 30 days with Merichem Refined NAs ($\delta^{13}C$, -26.8 ‰) as the carbon source and either 1.3 g/L NH_4Cl ($\delta^{15}N$, 2.0 ‰) or 1.0 g NH_4NO_3/L ($\delta^{15}N$, 0.24 ‰) as the nitrogen source (Figure 2.5). For both exposures, there were increased cell counts (NH_4Cl initial$_{day0}$ 7.07 x 10^7cells/mL, final$_{day30}$ 2.08 x 10^8 cells/mL; NH_4NO_3 initial$_{day0}$ 9.18 x 10^7cells/mL, final$_{day30}$ 2.70 x 10^8 cells/mL) and decreased DOC concentrations (> 75 %) indicating microbial growth (Figure 2.5 a). However, the concentration of DIC only increased slightly (Figure 2.5 a). Although the majority of the DOC had been utilized, there was little change by day 30 in the $\delta^{13}C$ of DOC (-0.5 ‰, NH_4Cl test; -0.3 ‰, NH_4NO_3 test) (Figure 2.5 b). The $\delta^{13}C$ of DIC was variable at day 3 but by the end of the incubation period, DIC was ^{13}C enriched by 5.5 ‰ and 3.8 ‰ for the NH_4Cl and NH_4NO_3 tests, respectively, relative to the initial $\delta^{13}C$ DIC values (Figure 2.5 c). Isotope values for biomass showed the greatest ^{13}C depletion by day 12 which suggests utilization of ^{13}C depleted DOC (Figure 2.5 d). At day 30, the biomass was ^{13}C enriched by 3.4 ‰ for the NH_4Cl test and 3.1 ‰ for the NH_4NO_3 test, relative to the initial DOC. The $\delta^{15}N$ of biomass was variable from day 0 to day 6 for the two tests, but

by day 30, there was ^{15}N depletion (-2.0 ‰, NH_4Cl test; -0.7 ‰, NH_4NO_3 test) relative to the $\delta^{15}N$ biomass values at day 0, despite the enriched nitrogen sources used (Figure 2.5 e).

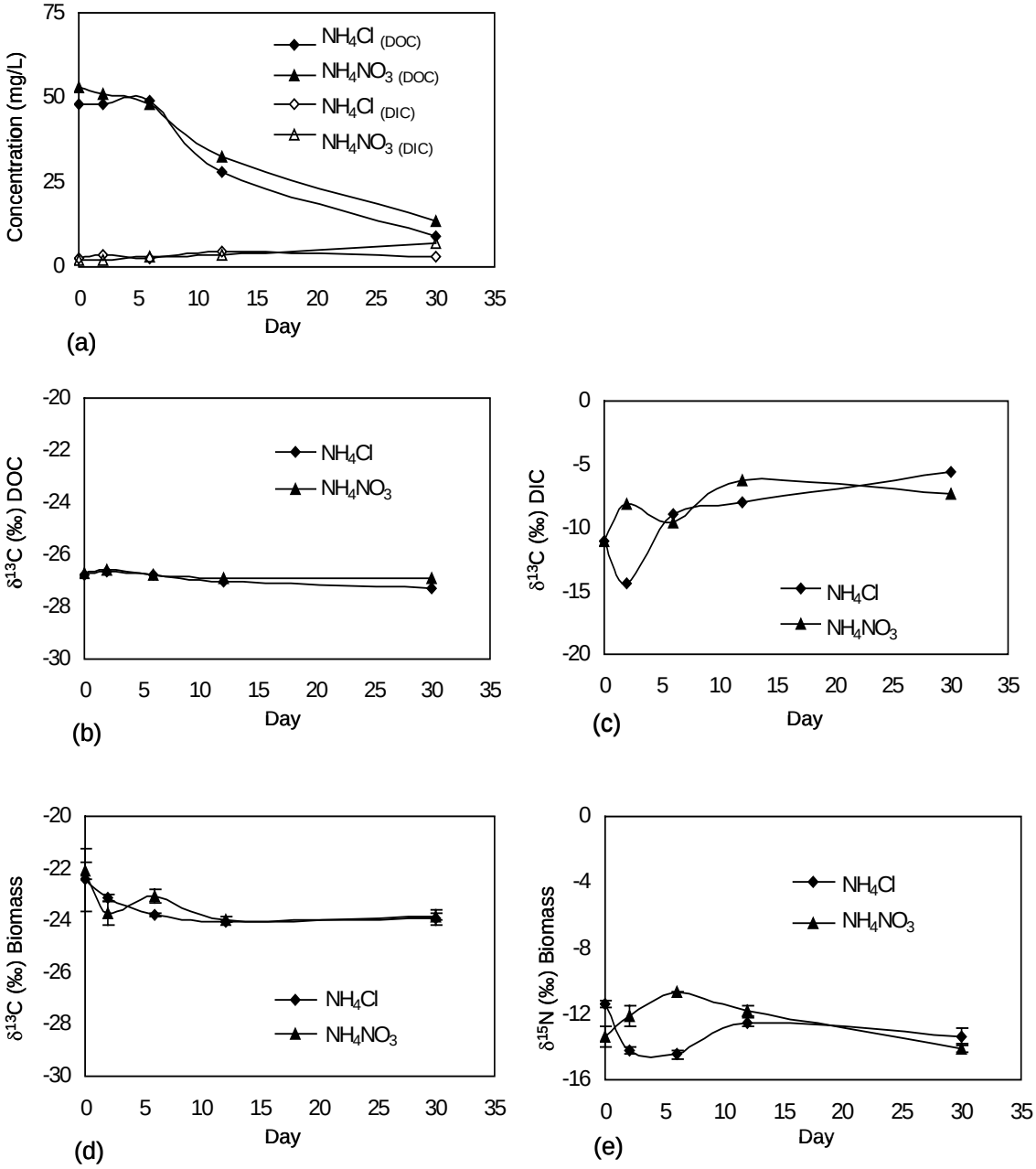


Figure 2.5. Changes in the concentrations of DOC and DIC (a) and the $\delta^{13}C$ values of DOC (b), DIC (c) and biomass $\delta^{13}C$ (d) and $\delta^{15}N$ (e) values following biodegradation of Merichem Refined NA (approximately 50 mg/L DOC) with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3 for 30 days. Mean ± standard error have been reported for biomass.

Microbial cultures were grown for 75 days in media containing Merichem Crude NAs ($\delta^{13}C$, -27.5 ‰) and 1.0 g/L NH_4NO_3 mineral media ($\delta^{15}N$, 0.2 ‰) (Table 2.2). For this exposure, the quantity of Merichem Crude was increased to 94 mg/L DOC to ensure adequate DOC for this incubation period. By day 75, DOC concentrations had decreased by 63 % and DIC concentrations had increased by 79 %. For DOC, there was little change in the $\delta^{13}C$ values over time (0.2 ‰). In this test, degradation of Merichem Crude NAs resulted in slight ^{13}C enrichment of the DIC by day 75 (1.3 ‰). The microbial culture used for this exposure had an initial $\delta^{13}C$ value similar to the $\delta^{13}C$ of the DOC thus there was little change in the $\delta^{13}C$ of the initial and final biomass. $\delta^{15}N$ values of the biomass were slightly ^{15}N enriched (0.9 ‰) by day 75 relative to day 0.

Table 2.2. Endpoint measurements for the 75-day test using bacteria cultured on Merichem Crude NA (93.86 mg/L DOC) and 1.0 g/L NH_4NO_3.

Measurement	**Day 0**	**Day 30**	**Day 75**	**Δ[a]**
Cell counts (cells/mL)	6.04×10^6	1.76×10^8	2.72×10^8	2.66×10^8
DOC (mg/L)	93.86	48.75	34.51	-59.35
$\delta^{13}C$ DOC (‰)	-26.9	-27.2	-26.7	+0.2
DIC (mg/L)	6.27	21.9	29.93	+23.66
$\delta^{13}C$ DIC (‰)	-22.8	-21.1	-21.5	+1.3
$\delta^{13}C$ Biomass (‰)	-26.2	-25.5	-26.6	-0.4
$\delta^{15}N$ Biomass (‰)	-14.8	-9.1	-13.9	+0.9

[a] Δ = final (day 75)– initial (day 0)

2.3.3 Semi-Continuous Tests

Given the complexity of the NA mixtures and the slow degradation as indicated by the static tests, semi-continuous cultures were used to examine changes in biomass $\delta^{13}C$ values following preferential degradation of readily degradable NAs within the mixture. Microbial cultures grown on Merichem Refined NAs ($\delta^{13}C$, -26.8 ‰) and 1.3 g/L NH_4Cl ($\delta^{15}N$, 2.0 ‰) were maintained at 8.50 x 10^7 to 3.00 x 10^8 cells/ml and transferred to fresh media every three days (Figure 2.6). Initially there was ^{13}C depletion of the biomass relative to day 0, but after day 12, $\delta^{13}C$ values remained constant, ranging between -25.3 ‰ to -24.8 ‰ (Figure 2.6 a). On average, biomass was 1.7 ‰ enriched relative to the source (-26.8 ‰). The $\delta^{15}N$ values of microbial biomass showed higher variability (2 ‰) throughout the incubation period (Figure 2.6 b).

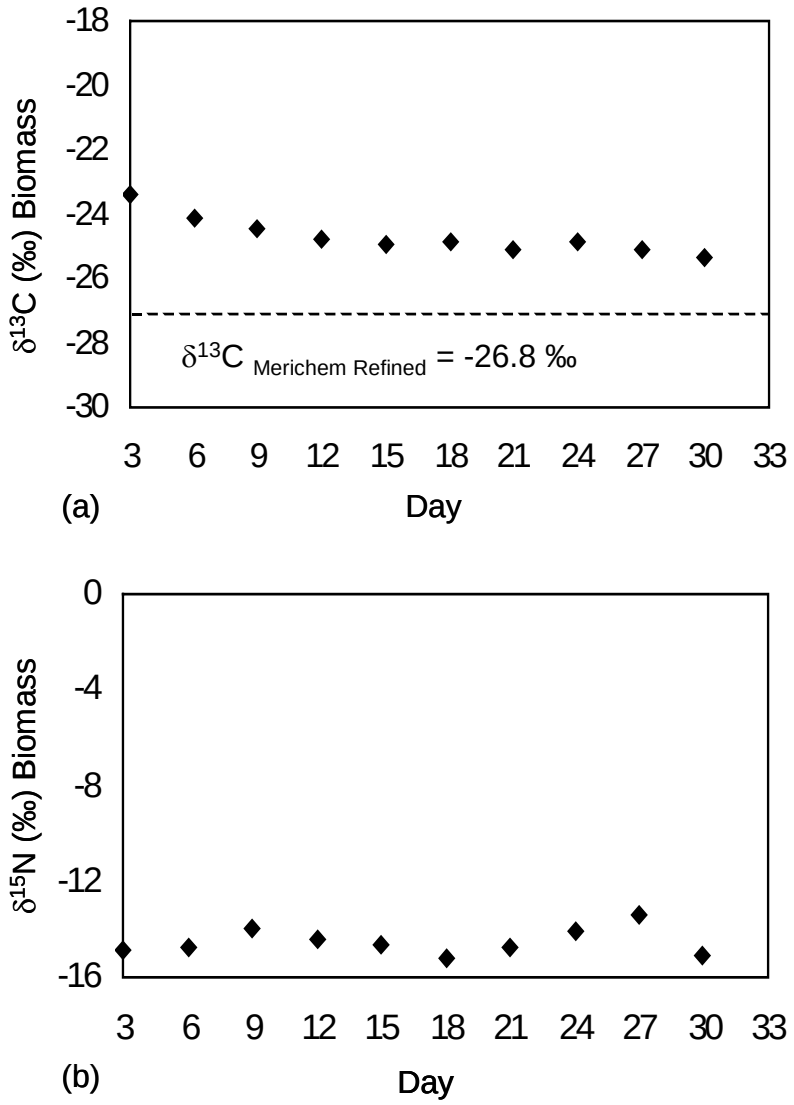


Figure 2.6. Changes in biomass $\delta^{13}C$ (a) and $\delta^{15}N$ (b) values following biodegradation of Merichem Refined NAs (50 mg/L, nominal) with 1.3 g/L NH_4Cl in a semi-continuous test with transfers to fresh NA and mineral medium every third day.

For the 13-day semi-continuous test, microbial cells grown on Merichem Refined NAs ($\delta^{13}C$, -26.8 ‰), (25 mg/L; nominal) and 1.3 g/L NH_4Cl were maintained at 1.04 x 10^8 to 2.88 x 10^8 cells/mL. The $\delta^{13}C$ values for the microbial biomass (Figure 2.7 a) showed a similar trend as biomass $\delta^{13}C$ values in the 3-day transfer test, with ^{13}C depletion (-1.5 ‰) of biomass by day 26 relative to the biomass at day 0. At day 90, the mean $\delta^{13}C$ of biomass was -25.4 ‰, which was 1.4 ‰ more ^{13}C enriched than the DOC source ($\delta^{13}C$, -26.8 ‰). The $\delta^{15}N$ values for the microbial biomass were ^{15}N enriched by 3.3 ‰ relative to the initial $\delta^{15}N$ biomass values by the end of the incubation (Figure 2.7 b).

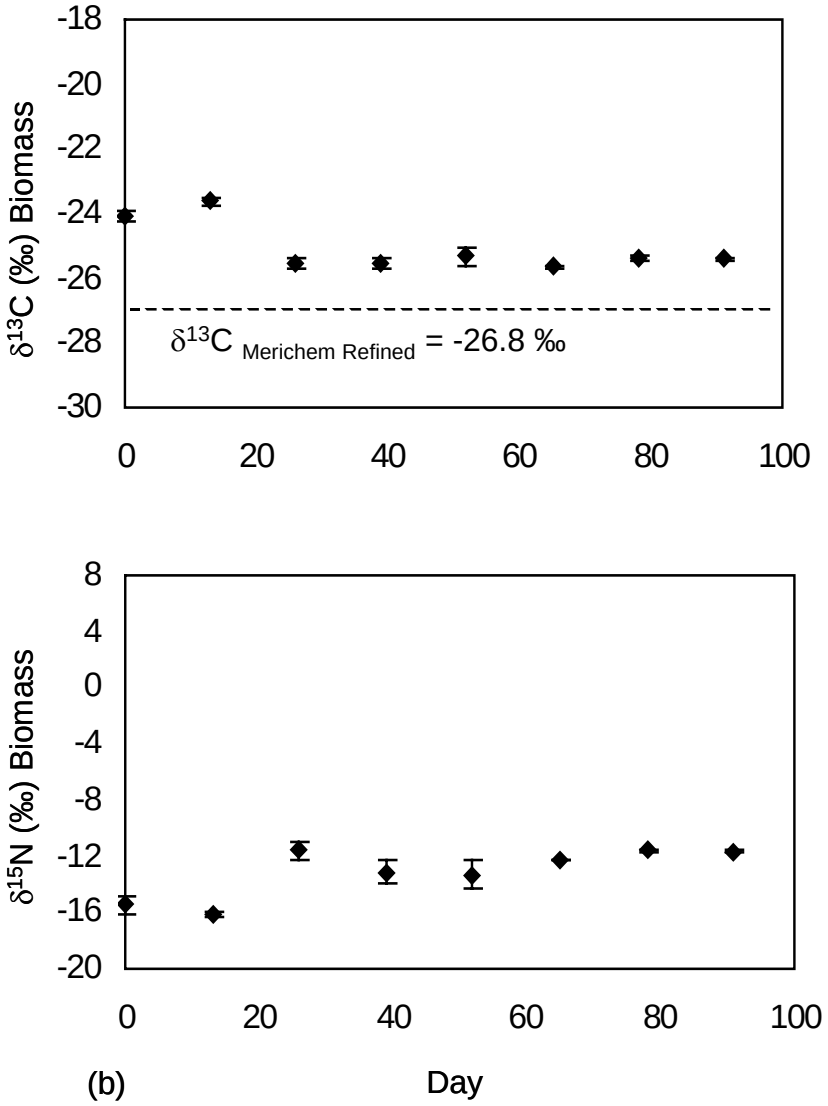


Figure 2.7. Changes in biomass $\delta^{13}C$ (a) and $\delta^{15}N$ (b) values following biodegradation of Merichem Refined NAs (25 mg/L, nominal) with 1.3 g/L NH_4Cl in a semi-continuous test with transfers to fresh NAs and mineral medium every 13 days. Mean ± standard error have been reported.

The semi-continuous 13-day transfer test with the oil sands NA extract ($\delta^{13}C$, -28.9 ‰) with a DOC concentration of 30.24 mg/L and 1.3 g/L NH_4Cl, was maintained at 1.12 x 10^8 to 1.94 x 10^8 cells/mL. In this test, the trend of ^{13}C enrichment for microbial biomass (Figure 2.8 a) was different from the $\delta^{13}C$ trend for biomass in Merichem Refined semi-continuous tests. At day 90, biomass was ^{13}C enriched by 2.3 ‰ relative to the initial biomass and 6.6 ‰ ^{13}C enriched relative to the DOC source. Microbial biomass was significantly ^{15}N enriched from -5.4 to 3 ‰ by the end of the incubation period (Figure 2.8 b).

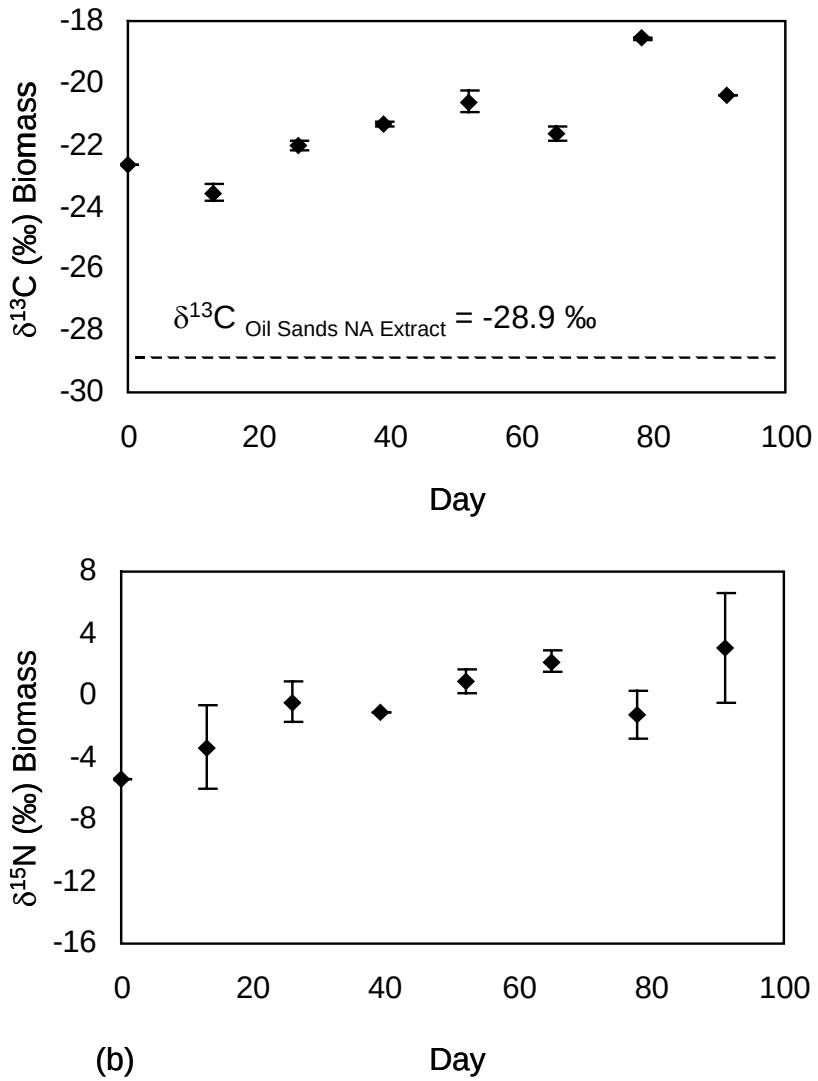


Figure 2.8. Changes in biomass $\delta^{13}C$ (a) and $\delta^{15}N$ (b) following biodegradation of MLSB NA extract; 30.24 mg/L DOC with 1.3 g/L NH_4Cl in a semi-continuous test with transfers to fresh NAs and mineral medium every 13 days. Mean ± standard error have been reported.

2.4 Discussion

The degradation of NAs in this study as well as other studies is a function of a variety of factors including the initial concentration, source of the NA mixture, exposure duration, as well as nutrients and dissolved oxygen levels. Preliminary degradation tests showed that with increasing nominal concentrations of Merichem Refined NAs from 5 to 100 mg/L DOC, degradation increased as indicated by direct cell counts. Herman et al. (1994) also demonstrated increased amounts of degradation occurring with increased amounts of NAs (21, 50 and 60 mg/L), as indicated by the amount of increasing CO_2 produced. In the current study, DOC concentrations tested were representative of average DOC concentrations found for a range of reclamation sites sampled in 2006 influenced by oil sands processed-affected materials (58.72 to 74.28 mg/L DOC) (chapter 3). NA concentration analysis from these sites, showed NAs concentrations between 42 to 57 mg/L, thereby contributing between 43 to 64 % to the DOC (Leonhardt 2003, Hayes 2005). Differences in NA concentrations from the different sites may affect the potential for degradation at each of these sites.

Biodegradation of NAs is strongly influenced by the source of the NAs. Scott et al. (2005) observed enhanced degradation of commercial NAs (Kodak, Merichem Refined, Fluka) relative to the oil sands tailings water sources (Syncrude West In Pit and Suncor Consolidated Tailings Wetland). Using the GC-MS technique to characterize the NAs, Scott et al. (2005) showed that commercial NAs, with a greater proportion of lower molecular weight NAs with shorter carbon chains and fewer rings, were more easily degraded than the oil sands derived NAs. Other studies have also documented structural differences between NA sources and impacts on degradation (Herman et al. 1993,

Herman et al. 1994, Lai et al. 1996, Clemente et al. 2004, Del Rio et al. 2006). Differences in the composition of the NA mixture may explain the minimal degradation of the oil sands NA extract (Figure 2.4) compared to Merichem Refined NAs (Table 2.1) in the short term static tests, although the Merichem Refined had a longer incubation period (+ 6 days).

Biodegradation of NAs is also influenced by nutrient and dissolved oxygen levels. Lai et al. (1996) found that the addition of phosphate (0.45 mg/L potassium phosphate) increased rates of degradation of surrogate NAs (palmitic and decahydro-2-naphthoic acid). In another study, degradation was significantly reduced as a function of dissolved oxygen levels. There was a 60 % decrease in oil sands derived NA concentration (30 mg/L) over a 141-day period under aerobic conditions but under anaerobic conditions there was no change in NA concentration for a 180-day incubation period (Gervais 2004).

In this study, regardless of the degree of degradation, the $\delta^{13}C$ of the DOC showed minimal change (-0.5 to 0.2 ‰) following substrate utilization. Similarly Mazeas et al. (2002) found no change in the $\delta^{13}C$ of the remaining hydrocarbon substrate at different time intervals after exposure to mixed microbial cultures in an aerobic environment. Hunkeler et al. (2001) also found no change in the $\delta^{13}C$ of benzene until 70 to 80 % was degraded, then the remaining benzene showed a ^{13}C enrichment averaging 3 to 5 ‰. Conversely, Hunkeler and Aravena (2000) found unusually high ^{13}C enrichment of 1,2-dichloroethane from -30 ‰ (initial substrate $\delta^{13}C$) to -10 ‰ (final substrate $\delta^{13}C$) following degradation. Differences in ^{13}C and ^{12}C utilization of substrate may be a function of the species of bacteria, (Hunkeler and Aravena 2000; Meckenstock et al. 2002; Wick et al. 2003), the type and amount of substrate degraded as well as whether the

environment is aerobic or anaerobic (Hunkeler and Aravena 2000, Morasch et al. 2004, Meckenstock et al. 2004).

In our tests, the lack of change of the $\delta^{13}C$ of the DOC could be a result of microbes consuming both ^{13}C and ^{12}C, however, since there is a greater proportion of ^{12}C in the environment (NIST 2005) and microbes preferentially metabolize the lighter isotope, then the heavier isotope will remain in their biomass thereby causing a ^{13}C depletion (DeNiro and Epstein 1978, Hall et al. 1999, Zyakun et al 2003, McCutchan Jr. et al. 2003). For the static tests using commercial NAs, final microbial biomass was ^{13}C enriched relative to the DOC source (range of ^{13}C enrichment, 1.6-3.4 ‰). The range of ^{13}C enrichment from substrate to biomass in these tests is similar to the ^{13}C enrichment of bacteria grown on different DOC leachates (0.4 to 2.8 ‰) (Hullar et al. 1996). In another study, microbial production was shown to be ^{13}C enriched relative to the source for *Pseudomonas* grown on phenol ($\delta^{13}C$ source -28.5 ‰; $\delta^{13}C$ final biomass -25.2 to -25.4 ‰) and benzoate ($\delta^{13}C$ source -26.2 ‰; $\delta^{13}C$ final biomass -24 to -25 ‰) (Hall et al. 1999). Coffin et al. (1990) cultured *Pseudomonas aeruginosa* on two different carbon sources (glucose $\delta^{13}C$, -13 ‰ and glutamate $\delta^{13}C$, -27 ‰) and found that the microbial cells reflected the $\delta^{13}C$ signature of the sole carbon source with an average ^{13}C enrichment of 2.3 ‰ ± 0.6. Similarly, Zyakun et al. (2003) found ^{13}C enrichment of *Burkholderia* sp. ($\delta^{13}C$ -19.2 ‰) grown on naphthalene ($\delta^{13}C$ -21.2 ‰).

In the semi-continuous test incubations, microbial cells grown on commercial NAs had ^{13}C enrichment between 1.5 to 1.7 ‰ relative to the DOC substrate which was similar to the ^{13}C enrichment of microbial cells in the static tests. However, bacteria grown on oil sand NA extracts were much more ^{13}C enriched (6.6 ‰) relative to the

DOC substrate, indicating preferential utilization of ^{13}C enriched material. The different trends may be a function of the complex composition of the oil sands NA extract. The oil sands NA extract has different percentages of NAs with specific carbon number and Z family (ring structure) compared to the commercial NAs. Also, the method used to extract NAs from oil sands process water will also extract higher molecular weight (800 to 100 000 amu) humic and fulvic acids which tend to be recalcitrant to degradation (Headley and McMartin 2004, Ressler et al. 1999, Ma et al. 2001, Choi et al. 2004, Young et al. 2005, Adani et al. 2006). As a result, the DOC extracted from oil sands process water will have a portion of recalcitrant acid substances, which may influence the $\delta^{13}C$ signature of the DOC. Recalcitrant acid substances have been reported to have $\delta^{13}C$ values of approximately -25.6 ‰ (St. Jean 2003). Utilization of ^{13}C enriched humic and fulvic acids would explain the ^{13}C enrichment of the bacteria biomass, however this is not likely due to the recalcitrant nature of these acids substances. Other possible types of carbon such as soluble oil sands hydrocarbons and PACs may be present in the extract, although only in trace amounts as these compounds would be removed during the dichloromethane clean up. Generally, raw bitumen from the oil sands, which includes oil sands hydrocarbons and PACs, tends to be $\delta^{13}C$ depleted (bitumen $\delta^{13}C$, -30.3 ‰) similar to the oil sands NA extract (Farwell et al. submitted); and therefore would not likely contribute to the observed $\delta^{13}C$ enrichment.

In addition to monitoring the DOC, respired CO_2 in the water as DIC (dissolved CO_2 present as H_2CO_3, HCO_3^- and CO_3^{2-}) was also monitored, as this may be an important carbon source for primary production in oil sands aquatic reclamation. At test pH in the range of 7 to 9, the predominant inorganic carbon species will be in the form of

HCO_3^- (Wetzel 1983). Generally, there were small increases in DIC concentrations ranging from 0.83 to 23.66 mg/L as the DIC represents a fraction of the evolved CO_2. Herman et al. (1994) found that degradation of 60 mg/L of NAs resulted in the evolution of 1.6 mg CO_2 whereby 48 % of the carbon from the NAs was converted to CO_2. Degradation of 21 and 50 mg/L of NAs produced 0.2 and 0.6 mg CO_2, an average of only 20 % of the carbon from the NAs was converted to CO_2 (Herman et al. 1994). In comparison, Clemente et al. (2004) found that 60 % of the carbon from the NAs were converted to CO_2 (approximately 1.9 mg CO_2) following degradation of NAs from 82 to < 3 mg/L and 109 to 8 mg/L.

The $\delta^{13}C$ of the DIC was monitored in the current study to determine changes in isotope values associated with microbial degradation. Generally, preferential metabolism of the lighter isotope will result in ^{13}C depleted by-products such as CO_2 or DIC in the water column that will be assimilated by primary producers (DeNiro and Epstein 1978, Lapham et al. 1999, Hall et al. 1999). The short term tests with Merichem Refined (Table 2.1) showed trends towards ^{13}C depletion of the DIC ranging from -5 ‰ to -2.8 ‰ indicating respiration of ^{12}C enriched CO_2. Other studies have also shown ^{13}C depletion of the inorganic by-product. Degradation of orimulsion fuel by a mixed microbial culture showed ^{13}C depletion of -11 ‰ for CO_2 (initial CO_2 $\delta^{13}C$ value, -11 ‰; final CO_2 $\delta^{13}C$ value, -22 ‰) whereas degradation of bitumen showed ^{13}C depletion of -7 ‰ for CO_2 (initial CO_2 $\delta^{13}C$ value, -15 ‰; final CO_2 $\delta^{13}C$, -22 ‰) (Lapham et al. 1999). Similarly, Hall et al. (1999) also observed ^{13}C depletion of -21 ‰ for CO_2 resulting from the complete degradation of phenol by *Pseudomonas putida*. Degradation of benzoate by *P. putida* resulted in similar ^{13}C depletion of the CO_2 (-20 ‰) (Hall et al. 1999).

The long term commercial NA tests showed trends toward ^{13}C enrichment rather than ^{13}C depletion of the DIC. The ^{13}C enrichment of DIC was more pronounced in the 30-day Merichem Refined test (+3.8 ‰ for the NH_4NO_3 treatment, +5.5 ‰ for the NH_4Cl treatment) than for the 75-day Merichem Crude test (+1.3 ‰). Some studies showed ^{13}C enrichment (2.6 to 30 ‰) of the DIC relative to the DIC signature at day 0 for degradation of 1,2 –dichloroethane (Hunkeler et al. 2001) and benzene (Hunkeler and Aravena 2000) by single microbial species. In another study, trends of ^{13}C enrichment of CO_2 following a 60-day degradation test using bitumen were attributed to methanogenesis in anaerobic microzones in the slurry of the microcosms (Lapham et al. 1999). Methanogens would preferentially use ^{12}C CO_2 and thereby leaving a ^{13}C enriched CO_2 pool and producing ^{13}C depleted CH_4 (Lapham et al. 1999).

The results of the current study support field based studies that have examined the stable isotopes of DOC, DIC (chapter 3) and microbial biomass (Daly 2007) in oil sands aquatic reclamation. The lack of isotope change in DOC in our degradation studies indicates a conservative value which is consistent with the narrow range of DOC $\delta^{13}C$ values (-28 ‰ to –26.3 ‰) observed in oil sands reclamation (chapter 3). Overall, microbial biomass was ^{13}C enriched (0.3 to 8.5 ‰) relative to the DOC source, particularly for the semi-continuous tests with bacteria grown on the oil sands derived NAs (^{13}C enrichment of 8.5 ‰). Using the range of ^{13}C enrichment observed in the current study, we can predict the $\delta^{13}C$ of microbial biomass under aerobic conditions based on the $\delta^{13}C$ DOC values ranging from -28 to -26.3 ‰ for oil sands reclamation (chapter 3). Predicted $\delta^{13}C$ values for microbial biomass under aerobic conditions in the range of -27.7 ‰ to -17.8 ‰ are slightly enriched compared to the measured microbial

biofilms (-32 ± 1.7 ‰ to –22.7 ± 2.9 ‰) (Daly 2007) for comparable oil sands reclamation sites. The more ^{13}C depleted values for biofilms ($\delta^{13}C$, -32 ‰; Daly 2007) may reflect other carbon sources or microzone effects such as increased methanogenesis and methane oxidation (Fry and Sherr 1984, Lapham et al. 1999, van Breukelen et al. 2003, Penning et al. 2005, Opsahl and Chanton 2006). In addition, or DOC limitation, thus reducing preferential utilization of specific isotopes.

The $\delta^{15}N$ values of biomass were often more variable between sampling intervals compared to the $\delta^{13}C$ values of biomass. In the static tests, the $\delta^{15}N$ values of biomass varied between 2.9 to 5.7 ‰ for tests with NH_4NO_3 and between 3.4 to 5.8 ‰ for tests with NH_4Cl between sampling intervals. This resulted in final biomass $\delta^{15}N$ values that were either ^{15}N enriched or depleted compared to the initial $\delta^{15}N$ values. In the semi-continuous tests, where the nitrogen source was NH_4Cl, replenished every 13 days, there were trends of ^{15}N enrichment between 3.3 ‰ to 8.4 ‰ for microbial biomass. Changes in nitrogen isotope values in biodegradation studies have been largely attributed to enzymatic action once the nitrogen substrates have been assimilated (Macko et al. 1987, Hoch et al. 1992, Wattiaux and Reed 1995).

The ^{15}N enrichment of microbial biomass from the semi-continuous 13-day transfer tests using NH_4Cl as a nitrogen source also contributes to our understanding of the ^{15}N enrichment of biota in oil sands aquatic reclamation. Microbial biofilms from different oil sands reclamation sites had $\delta^{15}N$ values ranged from -4.1 ± 1.4 ‰ to 11.6 ± 6.0 ‰ (Daly 2007). The more enriched $\delta^{15}N$ values were associated with the presence of oil sands processed material, which in this case refers to fine tailings and process water released through sedimentation of consolidated tailings and mature fine tailings (Daly,

2007). Similarly, ^{15}N enrichment has also been observed in selected benthic macroinvertebrates at sites with elevated levels of oil sands processed material (Farwell et al. submitted). The strong trend of ^{15}N enrichment in biota from reclamation sites with process material has been linked with elevated ammonium levels from various sources such as sewage, oil sands upgrading, addition of amines to the oil sands ore during bitumen extraction and leachates from peat amendments (Elshayeb 2006, Farwell et al. submitted, Daly 2007). Test results from the semi-continuous tests strongly suggest that with ample quantities of ammonium, microbes will preferentially excrete ^{14}N therefore resulting in ^{15}N enriched biomass. Tests by Hoch et al. (1992) demonstrated that with ample concentrations of NH_4^+ (23 mmoles) microbial cells showed ^{15}N enrichment of 13 ‰, whereas with much smaller quantities of NH_4^+(0.023 to 0.182 mmoles), there was ^{15}N depletion of -15 ‰. Changes in isotopes were attributed to changes in uptake pathways. When concentrations of NH_4^+ are high, uptake occurs through passive diffusion and is assimilated via glutamate dehydrogenase and at lower concentrations NH_4^+ uptake occurs through active transport and is assimilated via glutamine synthetase (Hoch et al. 1992).

2.5 Conclusions

Stable isotopes of carbon and nitrogen have been used in oil sands aquatic reclaimed sites to trace the flow and assimilation of carbon and nitrogen compounds. To improve upon their effectiveness, a greater understanding of microbial degradation of complex organic mixtures such as NAs and the changes in stable isotope values that occurs is needed. As such, this work provides further information into the correlation between microbial degradation of NAs and the changes that occur with stable isotope values.

Overall, utilization of the NAs (DOC source) by oil sands derived microbial cultures resulted in minimal change (-0.5 to +0.2 ‰) during the incubation period. Oppositely, change in the isotope values were seen with the microbial biomass in both static and semi-continuous tests. In static and semi-continuous tests, uptake of commercial NAs in laboratory microcosms resulted in microbial biomass that was similar or ^{13}C enriched (0.3 to 2.9 ‰) relative to the DOC source depending on the length of incubation. Greater ^{13}C enrichment of the microbial biomass (3.7 to 8.5 ‰) relative to the DOC source was seen during uptake of the oil sands NA extract. Oppositely, no clear trends could be distinguished from the $\delta^{13}C$ of the DIC, as it was more variable (-5 to +5.5 ‰). In some semi-continuous tests, the final $\delta^{15}N$ biomass signatures were ^{15}N enriched (3.8 to 8.4 ‰) relative to the initial biomass. The isotope trends established in this study enhance our interpretation of field-based data, particularly in terms of carbon source utilization and ^{15}N enrichment. Future field work examining carbon and nitrogen utilization may be improved upon based on the stable isotope trends correlated with microbial degradation of NAs established in this study.

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3 Chapter 3. Stable Isotope Analysis of Dissolved Carbon from Various Oil Sands Aquatic Reclaimed Sites

Abstract

The extraction of bitumen from oil sands produces large volumes of oil sands process material (OSPM) which must be reclaimed. Understanding carbon dynamics and assimilation of oil sands process material (OSPM) in aquatic ecosystems is essential for developing sustainable aquatic reclamation strategies. Previous studies have used stable isotopes analyses of aquatic organisms as a tool to better understand carbon cycling in these systems. However, baseline determinations for both the dissolved organic carbon (DOC); which is consumed by the microbial community and dissolved inorganic carbon (DIC) pools; which is consumed by the primary producers, in OSPM aquatic ecosystems are needed.

In this study, water samples were collected from 13 sites categorized by age of construction (young and mature), construction material and organic content level in August 2005 and June-September 2006 and analyzed for DOC and DIC concentration and $\delta^{13}C$ values. Examining 2006 data, OSPM contributed to elevated concentrations of DOC (+37.58 mg/L) and DIC (+83.37 mg/L), and elevated DOC concentrations were associated with sites categorized as high organic content. Results showed that $\delta^{13}C_{DOC}$ values from June to July had ^{13}C enrichment between 0.3 to 1.9 ‰. At mature sites, DOC values were ^{13}C depleted (+0.6 ‰) relative to the young sites. DIC values showed ^{13}C depletion (-0.3 to -8.8 ‰) for most sites from June to July. $\delta^{13}C$ DIC values were ^{13}C enriched in OSPM sites (-4 ‰) compared to no OSPM sites (-8.8 ‰).

3.1. Introduction

Oil sands mining in northeastern Alberta, Canada, has a total annual production of over 120 million barrels of sweet crude oil (Holowenko et al. 2002). For every 1 m^3 of bitumen that is produced, 3 m^3 of water is required, generating 4 m^3 of tailings/OSPM (solids/fluid) (Mackinnon and Boerger 1986, Holowenko et al. 2002). Oil sands process material (OSPM) is a mixture of sand, silt, clays and organic materials such as naphthenic acids (NAs), polycyclic aromatic compounds (PACs), unrecovered bitumen, humic and fulvic acids, phenols, cresols, and mercaptans (Mackinnon 1989, Madill et al. 2001, Holowenko et al. 2002, Quagraine et al. 2005). Under provincial law, this material will be reclaimed. There are numerous strategies involving the use of OSPM to assist in producing healthy sustainable aquatic ecosystems. For example, using stockpiled muskeg peat from oil sands mining may be added to an OSPM substrate to increase nutrient levels to promote the growth of microbial and plant communities (Renault et al. 2003, Bois et al. 2005).

Stable isotopes have been employed to determine carbon dynamics and trophic structure within oil sands food webs. Preliminary research documented trends of ^{13}C depletion and ^{15}N enrichment in damselflies and dragonflies along a gradient of increasing OSPM in a series of 4 ha experimental ponds constructed in 1989, suggesting incorporation of oil sands material (Farwell et al. submitted). Detailed examination of a variety of reclaimed systems of differing age (>10 years old), size, and OSPM content identified similar ranges of $\delta^{13}C$ values for plankton (- 30.42 to –26.99 ‰), periphytic material (-25.49 to -18.65 ‰), and particulate organic matter (- 28.28 to –27.79 ‰) (Elshayeb 2006). Also, a wide range of $\delta^{13}C$ values of microbial biofilms (-32 ± 1.7 ‰

to -22.7 ± 2.9 ‰) from different reclaimed wetlands indicated assimilation of many different sources of carbon (Daly 2007).

This study was initiated to gain a better understanding of the dissolved carbon pools in oil sands reclamation. DOC and DIC concentrations and stable isotope values were determined for aquatic reclaimed sites selected based on age (young < 7 years old; mature > 7 years old), construction material (OSPM; no OSPM) and organic material content to establish spatial trends. Temporal trends for the growing season from June to September were also determined. Information on dissolved carbon concentrations and stable isotopes will add to our understanding of carbon dynamics in oil sands reclamation as part of a larger project involving the assessment of microbial and primary production.

3.2. Materials and Methods

3.2.1 Site Classifications

The wetlands are classified by age of construction: young (< 7 years old as of 2006) and mature (> 7 years old as of 2006); construction material: OSPM (present, not present) and organic content based on the addition of peat amendments. The age of a wetland was determined by the year of construction or appearance (through flooding/dyke seepage) (Table 3.1).

Table 3.1. Classification of young (< 7 years old as of 2006) and mature (> 7 years old as of 2006) oil sands reclamation sites (personal communication with Carla Wytrykush 2006).

Status	**Organic Level**	**Wetland Age**	
		Young	**Mature**
No Oil Sands Process Material	Low	• Bill's Lake (BL) • CNRL (CNRL)	• Shallow Wetland (SW) • Northwest Intercept Ditch (NWID)
	High	• Peat Pond (PP) • Golden Pond (GP)	• High Sulphate Pond (HS) • Southwest Sands Beaver Pond (SSBP)
Oil Sands Process Material	Low	• Syncrude Consolidated Tailings Pond (SCT) • CTW-No Peat (CTW-NP)	• Test Pond 9 (TP9)
	High	• CTW-Peat (CTW-P)	• Natural Wetland (NW)

The OSPM-affected wetlands may contain mature fine tailings (MFT) which consists of water, clays, silts, residual bitumen, PACs, as well as NAs and other organic acids such as humic and fulvic acids. Generally, MFT contains 80 % water by volume, 70-100 mg/L of organics and has a total ion content greater than 2000 mg/L indicative of high salinity levels (Leung et al. 2001, Salloum et al. 2002). Tailings mixed with gypsum ($CaSO_4$) to coagulate clays and silts are referred to as consolidated/composite tailings (CT) (Leung et al. 2001, Renault et al. 2003). Deposition of CT released waters contains pH levels greater than 7.6, high concentrations of NAs and high concentrations of ions such as SO_4^{2-} (1300 to 8000 mg/L), Na^+, Cl^- and HCO_3^- (Leung et al. 2001, Salloum et al. 2002, Renault et al. 2003).

Organic content refers to the presence of muskeg peat (personal communication with Carla Wytrykush 2006). Muskeg peat, derived from the removal of the organic layer during open-pit mining of bitumen enhances the soil/MFT for revegetation purposes by improving the texture of the sediment/soil as well as and providing nutrients (Renault et al. 2003, Boils et al. 2005, Reid and Naeth 2005).

3.2.2 Young Sites (less than 7 years old as of 2006)

Bill's Lake (BL) is situated on the Syncrude Canada lease and is located at latitude 56°59'925" and longitude 111°56'697". It was built in 1996 overtop of 20 to 50 cm of saline sodic overburden and capped with surface water (Golder 2002, Leonhardt 2003). Overburden is composed of surface (< 1m) topsoil, clay and some vegetation and is removed to expose the oil sands for mining. Once removed, it is stockpiled for use in reclamation (Daly 2007). The sodic overburden material contributes to the high conductivity of the surface water (Golder 2002, Leonhardt 2003). NA concentrations have been reported at 1.4 mg/L, which is relatively low with respect to other reclaimed sites (Leonhardt 2003). Canadian Natural Resource Limited East Access Road Ditch (CNRL) is located on the CNRL lease site and was created in 2004 following excavation. This depression has a sand substrate which receives runoff from both a forest ecosystem and logged area. To date, CNRL has a surface area of 7500 m^2 with a water depth of 0.7 m (Daly 2007). Peat Pond (PP) is located at 56°59'611" and 111°56'697" on the Syncrude Canada lease and was constructed in 2001 (Leonhardt 2003). The surface water contains high conductivity with NA concentrations of 8.7 mg/L and the substrate contains additions of peat (Leonhardt 2003, Daly 2007). Approximate water depth is 0.7 m (Daly 2007). Golden Pond (GP) is situated on the Syncrude Canada lease at

56°99’72” and 111°62’38”and contains 2 mg/L of NAs (Mackinnon unpublished 2005). Syncrude Consolidated Tailings Pond (SCT) was constructed in 1997 with a water depth of 3.5 m and is located on the Syncrude Canada lease site (57°06’734” and 111°40’874”). As a result of containing CT process waters, elevated levels of NAs as high as 42 mg/L are found (Golder 2002, Leonhardt 2003, Hayes 2005, Daly 2007). Consolidated Tailings Wetland (no peat and peat zones) (CTW-NP, CTW-P) was constructed in 1998 on the Suncor lease site (latitude, 56°98’49”and longitude, 111°530’11”) (Leonhardt 2003, Daly 2007). It contains OSPM including: CT, tailings and dyke drainage water resulting in a total NA content of 52 mg/L (Leonhardt 2003, Mackinnon unpublished 2006). Approximate water depth of this wetland is 0.5 m (Daly 2007). (Table 3.2. for water chemistry parameters for young sites

Table 3.2. Water chemistry parameters for young wetland sites (< 7 years old as of 2006): Bill's Lake (BL), CNRL East Access Road Ditch (CNRL), Peat Pond (PP), Golden Pond (GP), Syncrude Consolidated Tailings Pond (SCT) and Consolidated Tailings Wetland (CTW).

Wetland	**pH**	**Cond (μS/cm)**	**PO_4 (mg/L)**	**NH_4 (mg/L)**	**NH_3 (mg/L)**	**NO_3 (mg/L)**	**NO_2 (mg/L)**	**SO_4 (mg/L)**	**CO_3^{2-} (mg/L)**	**HCO_3^- (mg/L)**	**DOC (mg/L)**	**NAs (mg/L)**	**Chl a (μg/L)**
BL	7.86 [a]	768	BDL	BDL	BDL	BDL	BDL	240	0	281	ND	1.4	ND
CNRL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP	7.01 [a]	3750	BDL	BDL	BDL	BDL	BDL	2800	0	53.7	ND	8.7	ND
GP	8.20 [a]	936.37 [a]	BDL [a]	0.360 [a]	ND	BDL [a]	BDL [a]	305.11[a]	0 [a]	227 [a]	26.1 [a]	2.0 [a]	ND
SCT	8.55	4950	BDL	< 0.01	ND	BDL	BDL	1200	40.2	627.65	36 [b]	42.1 [b]	0.65 [b]
CTW	8.11 [c]	2453 [c]	BDL	1.71 [c]	<0.01	BDL	BDL	578 [c]	16.3 [c]	675 [c]	53.6 [c]	32.8 [c]	ND

ND: No data available
All data are from Leonhardt (2003) unless otherwise stated.
[a] Mackinnon (2005 unpublished)
[b] Hayes (2005)
[c] Mackinnon (2006 unpublished)

3.2.3 Mature Sites (> 7 years old)

Shallow Wetland (SW) is a natural depression located (57°04'899" and 111°41'427") on the Syncrude Canada lease (Leonhardt 2003). It was formed in1993 with non-process-affected water containing a low NA content (1.2 mg/L) on top of a large volume of clay material with a fine detritus surface layer (Ganshorn 2002, Leonhardt 2003, Mackinnon unpublished 2006, Daly 2007). Shallow Wetland has a depth of 0.6 m, with a volume of 418 m^3 and a surface area of 722 m^2 (Ganshorn 2002, Daly 2007). In early spring, *Typha* (cattails) and *Chara* are predominant and cover approximately 50 % of the wetland. However, by midsummer, 75 % of the wetland is covered by dense beds of *Chara* (Ganshorn 2002). Northwest Intercept Ditch (NWID) is located on the Syncrude lease at 57°06'705" and 111°41'467" (Leonhardt 2003). It was formed from a mined out pit with surface water in 1970 (Ganshorn 2002, Leonhardt 2003). The surface water has both low conductivity and NA concentration (1.4 mg/L). (Leonhardt 2003). High Sulphate Pond (HS) is a natural wetland formed in 1985 and is located on the Suncor lease, at 56°59'837" and 111°33'291" (Leonhardt 2003). This wetland is 75 cm deep with a volume of 669 m^3 covering an area of 1208 m^2. The substrate consists of lean oil sands mixed with overburden material such as stockpiled peat and the water cap contains surface water which has high levels of conductivity with NA concentrations of approximately 11.3 mg/L (Ganshorn 2002, Leonhardt 2003, Hayes 2005, Mackinnon unpublished 2006). This wetland has also received additional inputs of water from runoff and seepage from overburden (Daly 2007). *Typha, Equisetum* (horsetails) and *Chara* are the predominant macrophytes in the wetland (Ganshorn 2002). Southwest Sands Beaver Pond (SSBP) was naturally formed in a stream channel prior to

mining on the lease site (Daly 2007). The substrate consists of clay with a detrital component and the water contains no OSPM and, as such, minimal levels of NAs have been found (2.1 mg/L) (Leonhardt 2003, Hayes 2005, Mackinnon unpublished 2006, Daly 2007). It is located on the Syncrude Canada lease at 56°43'997" and 111°31'446" and covers an area of 220 m^2 and has a depth of 0.7 m with a volume of 3.3 x 10^3 m^3 (Ganshorn 2002, Leonhardt 2003, Daly 2007). Natural Wetland (NW) is on the Suncor lease, at 56°58'892" and 111°30'642" (Leonhardt 2003). In 1984 this area was to be reclaimed with a forest ecosystem, and as such was amended with muskeg peat overtop of a sand substrate. However, in 1987 NW formed naturally through dyke seepage of process waters (Ganshorn 2002, Leonhardt 2003, Daly 2007). The water cap consisted of 80 % of dyke drainage water with 20 % surface water, however, currently, the wetland continuously receives inputs of dyke seepage and CT water which have elevated the salinity of the surface water and increased the levels of NAs to approximately 32.9 mg/L (Ganshorn 2002, Leonhardt 2003, Mackinnon unpublished 2006). NW covers an area of 1.27 x 10^4 m^2 with a depth of 90 cm. The dominant vegetation consists of *Typha* and *Chara,* but by July there is substantial die off of the plants which corresponds with increased water turbidity (Ganshorn 2002). Test Pond 9 (TP 9) was constructed in 1993 with OSPM water originating from Mildred Lake Settling Basin and CT-process overtop of a clay substrate (Golder 2002, Leonhardt 2003, Daly 2007). Located 57°05'05" and 111°41'505" on the Syncrude lease, TP 9 has a total volume of 6000 m^3 with a depth of 0.6 m (Golder 2002, Leonhardt 2003, Daly 2007). NA concentration is relatively high at a level of 22.3 mg/L (Mackinnon unpublished 2006). (Table 3.3 for water chemistry parameters for mature sites).

Table 3.3. Water chemistry parameters for mature wetland sites (> 7 years old as of 2006): Shallow Wetland (SW), Northwest Intercept Ditch (NWID), High Sulphate Pond (HS), Southwest Sands Beaver Pond (SSBP), Test Pond 9 (TP9) and Natural Wetland (NW).

Wetland	**pH**	**Cond (μS/cm)**	**PO_4 (mg/L)**	**NH_4 (mg/L)**	**NH_3 (mg/L)**	**NO_3 (mg/L)**	**NO_2 (mg/L)**	**SO_4 (mg/L)**	**CO_3^{2-} (mg/L)**	**HCO_3^- (mg/L)**	**DOC (mg/L)**	**NAs (mg/L)**	**Chl a (μg/L)**
SW	7.76 [a]	427 [a]	BDL	0.35 [a]	0.2 [c]	BDL	BDL	10 [a]	0.68 [a]	253 [a]	20.1 [a]	1.2 [a]	ND
NWID	9.1	378	BDL	BDL	BDL	BDL	BDL	21	10.2	148	ND	1.40	ND
HS	7.71 [a]	2885 [a]	BDL	1.39 [a]	ND	BDL	BDL	1588 [a]	0 [a]	247 [a]	60.5 [a]	11.3 [a]	4.51 [b]
SSBP	7.47 [a]	260 [a]	BDL	1.4 [a]	BDL	BDL	BDL	12 [a]	0 [a]	188 [a]	23.2 [a]	2.1 [a]	16.55 [b]
TP9	8.87 [a]	2168 [a]	BDL	0.34 [a]	BDL	BDL	BDL	162 [a]	52.38 [a]	668 [a]	58.2 [a]	22.3 [a]	ND
NW	8.26 [a]	1232 [a]	BDL	1.17 [a]	0.30	BDL	BDL	176 [a]	29.2 [a]	531 [a]	53 [a]	32.9 [a]	0.58 [b]

ND: No data available
All data from Leonhardt (2003) unless otherwise stated.
[a] Mackinnon (2006 unpublished), data from 2006
[b] Hayes (2005)
[c] Smits et al. (2000), data from 1998

3.2.4 Sample Collection

Water samples for the analysis of DOC and DIC concentration and $\delta^{13}C$ values were collected from the surface layer (< 1 m) of 13 wetland sites on August 3 of 2005, June 26-27, July 27, August 27 and September 12, 2006. Samples from the 2005 field season were collected into 40 mL TraceClean™ amber borosilicate glass vials (Chase Scientific Glass Inc., Rockwood, TN, USA) with silicone-Teflon® septa (Chase Scientific Glass Inc., Rockwood, TN, USA). In 2006, samples were collected in 250 mL TraceClean™ amber borosilicate glass bottles with a Teflon®-lined closure (Chase Scientific Glass Inc., Rockwood, TN, USA). Samples were preserved with 5 % W/V $HgCl_2$ and the remaining headspace was topped off with water from the site. Samples were kept cool at approximately 4°C and shipped to the University of Waterloo, Waterloo, Ontario.

3.2.5 Sample Preparation and Analysis

Samples were filtered using a 0.45 μm polyethersulfone Nalgene® syringe filter (Nalge Nunc International, Rochester, NY, USA) into 40 mL TraceClean™ amber borosilicate glass vials (Chase Scientific Glass Inc., Rockwood, TN, USA) and sealed with open-top caps containing silicone-Teflon® septa (Chase Scientific Glass Inc., Rockwood, TN, USA). For 2006 samples, glass vials had open-top caps with polytetrafluroethylene (PTFE)/rubber septa (flexseal disc, 22 mm, 5/50 mL) (Chromatographic Specialties Inc, Brockville, ON, Canada) to decrease the potential for

loss of inorganic carbon as CO_2 (g) (personal communication with Paul Middlestead 2006).

Samples were stored 4°C and shipped to G.G. Hatch Isotope Laboratory, University of Ottawa (Ottawa, ON, Canada) for analyses of concentration and stable isotopes for DOC and DIC. Concentrations of DOC and DIC were determined using an OI high temperature persulfate wet-oxidation TIC-TOC Analyser Model 1010 (OI Analytical, College Station, TX, USA) with a precision of 0.002 mg/L C. $\delta^{13}C$ values for the DOC and DIC samples were determined using a continuous flow Thermo-Finnegan Mat DeltaPlus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Isotope values were derived from the following formula $((R_s/R_{st})-1) \times 10^3 = \delta^{13}C_{sample}$ (parts per thousand or per mil, ‰) where R_s is ratio of $^{13}C/^{12}C$ in the sample and R_{st} is the ratio of the International Atomic Energy Agency (IAEA) and National Bureau of Standards (NBS) isotopic reference material; carbonate rock Vienna Pee Dee Belemnite. Internal standards such as sucrose, glycine and phthalate were used to normalize the data to a precision of ± 0.2 ‰ (St-Jean et al. 2003, personal communication with Paul Middlestead 2006).

3.2.6 Statistical Analysis

For the 2006 samples, differences in concentration and $\delta^{13}C$ values for DOC and DIC in each of the following categories: age of construction, construction material used, and organic content were evaluated. Data collected from June to September for each of the sites was pooled and then each of the sites was grouped by category. Differences between the two groupings (young vs. mature; OSPM vs no OSPM; high vs low organic

content) were compared using a paired sample T-test at 95 % confidence level (SYSTAT® version 10 for Windows).

3.3. Results

3.3.1 Temporal Variation

For each site, DOC and DIC concentrations varied over the sampling periods (2005 and 2006) but no trends could be established (Figures 3.1 and 3.2; a and b). DOC mean concentrations for all 13 sites from 2005 and 2006 ranged from 19.79 to 72.62 mg/L. Within sites, DOC concentrations varied with changes in concentrations from as low as 5.29 mg/L (16 %) to as high as 57.21 mg/L (68 %) for the 2005 and 2006 sampling periods. DIC mean concentrations for all 13 sites from 2005 and 2006 ranged from 12.81 to 155.69 mg/L. Changes in the DIC concentrations over the 2005 and 2006 sampling periods ranged from 10.87 mg/L (22 %) to 81.85 mg/L (76 %).

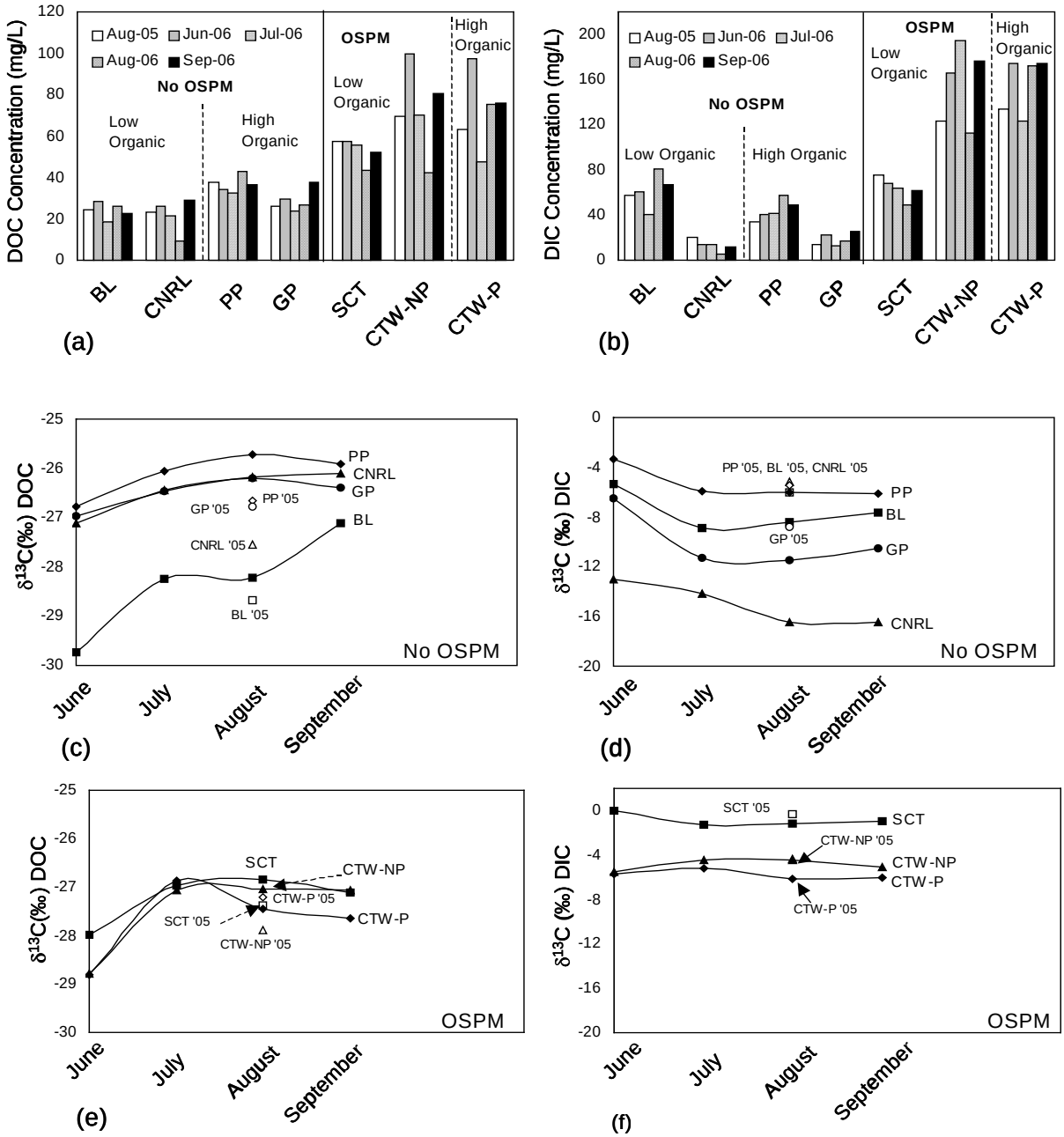

Figure 3.1. Young (< 7 years old) reclamation sites with no OSPM/low organic: BL, CNRL; no OSPM/high organic: PP, GP; OSPM/low organic: SCT, CTW-NP; OSPM/high organic: CTW-P (see Table 1 for abbreviations). DOC concentrations (a); DIC concentrations (b); δ^{13}C DOC (c) and δ^{13}C DIC (d) for sites with no OSPM; δ^{13}C DOC (e) and δ^{13}C DIC (f) for sites with OSPM.

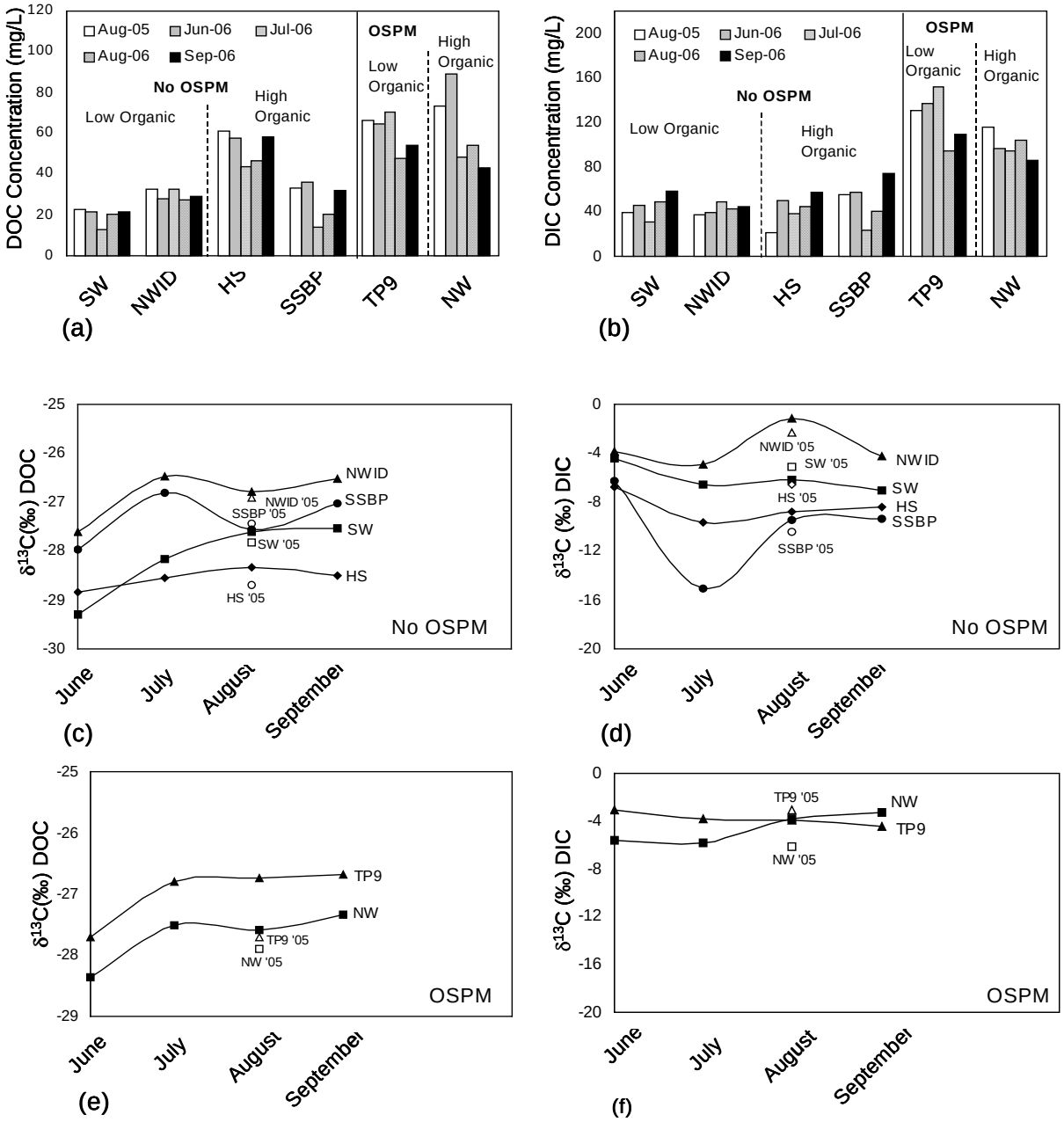

Figure 3.2. Mature (> 7 years old) reclamation sites with no OSPM/low organic: SW, NWID; no OSPM/high organic: HS, SSBP; OSPM/low organic: TP9; OSPM/high organic: NW (see Table 1 for abbreviations). DOC concentrations (a); DIC concentrations (b); δ^{13}C DOC (c) and δ^{13}C DIC (d) for sites with no OSPM; δ^{13}C DOC (e) and δ^{13}C DIC (f) for sites with OSPM.

Overall $\delta^{13}C$ values of DOC ranged from -28.6 to -26.1 ‰ over the 2006 sampling period. There was a trend of ^{13}C enrichment of the DOC (0.2 to 1.9 ‰) from June (-29.7 to -26.8 ‰) to July (-28.6 to -26 ‰) which was evident at all sites. Generally, the DOC remained ^{13}C enriched from July to September (-28.5 to -25.9 ‰) relative to June (Figures 3.1 and 3.2; c and e). The $\delta^{13}C$ values of DIC ranged by -15 to -0.9 ‰ over the 2006 sampling period (Figures 3.1. and 3.2; d and f). The $\delta^{13}C$ DIC values were ^{13}C depleted (-8.8 to -0.3 ‰) from June (-13 to -0.0 ‰) to July (-15.1 to -1.2 ‰). These values remained ^{13}C depleted in September (-16.5 to -1 ‰) relative to the June values. Oppositely, CTW-NP and CTW-P sites had ^{13}C enrichment (+1 and + 0.5 ‰) from June to July. However, September $\delta^{13}C$ DIC values relative to June, showed that CTW-NP had ^{13}C enrichment (+0.4 ‰) and CTW-P had ^{13}C depletion (-0.4 ‰).

3.3.2 Spatial Variation

To examine spatial differences, data from the 2006 sampling periods were averaged for each of the sites (Table 3.4).

Table 3.4. Mean ± standard error for DOC and DIC concentrations and stable isotope values for 2006 samples for oil sands reclamation sites.

Age	Status	Organic Level	Sites	Mean ± standard error			
				[DOC] (mg/L)	[DIC] (mg/L)	δ^{13}C DOC (‰)	δ^{13}C DIC (‰)
Young	No OSPM	Low	BL	23.76 ± 2.158	62.48 ± 8.430	-28.3 ± 0.54	-7.6 ± 0.79
			CNRL	21.37 ± 4.135	10.84 ± 2.028	-26.5 ± 0.23	-15 ± 0.86
		High	PP	36.50 ± 2.325	47.07 ± 3.986	-26.1 ± 0.23	-5.4 ± 0.69
			GP	29.22 ± 3.011	19.62 ± 2.909	-26.5 ± 0.17	-9.9 ± 1.17
	OSPM	Low	SCT	52.06 ± 3.106	60.52 ± 4.101	-27.2 ± 0.26	-0.9 ± 0.28
			CTW-NP	73.35 ± 11.930	162.17 ± 17.658	-27.5 ± 0.43	-4.9 ± 0.25
		High	CTW-P	74.28 ± 10.209	161.18 ± 12.565	-27.7 ± 0.41	-5.8 ± 0.22
Mature	No OSPM	Low	SW	18.99 ± 2.181	45.82 ± 5.720	-28.2 ± 0.40	-6.1 ± 0.57
			NWID	29.49 ± 1.194	43.87 ± 1.979	-26.8 ± 0.27	-3.6 ± 0.81
		High	HS	51.46 ± 3.751	47.29 ± 4.094	-28.6 ± 0.10	-8.4 ± 0.61
			SSBP	25.64 ± 5.221	48.69 ± 11.068	-27.3 ± 0.26	-10 ± 1.83
	OSPM	Low	TP9	59.32 ± 5.039	123.20 ± 12.935	-27 ± 0.24	-3.8 ± 0.27
		High	NW	58.72 ± 10.345	95.66 ± 3.733	-27.7 ± 0.23	-4.6 ± 0.64

Sites were then grouped based on age of construction, construction material used, and organic content and data was averaged (Table 3.5).

Table 3.5. Mean ± standard error for DOC and DIC concentrations and isotope values for 2006 samples for site groupings (young, no OSPM, low organic); (young, no OSPM, high organic); (young, OSPM, low organic); (young, OSPM, high organic); (mature, no OSPM, low organic); (mature, no OSPM, high organic); (mature, OSPM, low organic); (mature, OSPM, high organic).

Age	**Status**	**Organic Level**	**Mean ± standard error**			
			[DOC] (mg/L)	**[DIC] (mg/L)**	**δ^{13}C DOC (‰)**	**δ^{13}C DIC (‰)**
Young	No OSPM	Low	22.57 ± 2.279	36.66 ± 10.552	-27.4 ± 0.45	-11.3 ± 1.51
		High	32.86 ± 2.235	33.35 ± 5.667	-26.3 ± 0.15	-7.7 ± 1.07
	OSPM	Low	62.70 ± 6.982	111.34 ± 20.962	-27.4 ± 0.24	-2.9 ± 0.78
		High	74.28 ± 10.209	161.18 ± 12.565	-27.7 ± 0.41	-5.8 ± 0.22
Mature	No OSPM	Low	24.24 ± 2.294	44.84 ± 2.826	-27.5 ± 0.33	-4.8 ± 0.66
		High	38.55 ± 5.715	47.99 ± 5.469	-28 ± 0.27	-9.2 ± 0.94
	OSPM	Low	59.32 ± 5.039	123.20 ± 12.935	-27 ± 0.24	-3.8 ± 0.27
		High	58.72 ± 10.345	95.66 ± 3.733	-27.7 ± 0.23	-4.6 ± 0.64

There were no significant differences in DOC ($p = 0.665$) and DIC ($p = 0.467$) concentrations and δ^{13}C for the DIC ($p = 0.278$) between young and mature sites. However, the δ^{13}C for the DOC at young sites was significantly ^{13}C enriched (-27 ‰) compared to mature sites (-27.6 ‰).

Significant differences in DOC concentrations ($p < 0.001$), DIC concentrations ($p < 0.001$) and $\delta^{13}C$ DIC ($p < 0.001$) were found between sites constructed with OSPM compared to those without OSPM. Both DOC and DIC concentrations were significantly elevated in OSPM sites (mean values: 63.54 mg/L (DOC), 120.54 mg/L (DIC)) compared to no OSPM sites (mean values: 25.96 mg/L (DOC), 37.17 mg/L (DIC)). The $\delta^{13}C$ DIC values were significantly more ^{13}C enriched in OSPM sites with a mean value of -4 ‰ compared to -8.8 ‰ in no OSPM sites. The $\delta^{13}C$ for the DOC did not show significant differences between sites containing different construction material ($p = 0.216$).

Significant differences between low and high organic sites were found for the DOC concentration values ($p = 0.040$) whereby low organic sites on average had lower DOC concentrations (mean= 36.5 mg/L) as compared to high organic sites (45.97 mg/L). DIC concentration ($p = 0.715$), $\delta^{13}C$ DOC ($p = 0.733$) and $\delta^{13}C$ DIC ($p = 0.452$) did not show significant differences.

3.4. Discussion

DOC concentrations were elevated at OSPM sites (mean DOC, 63.54 mg/L) compared to sites with no OSPM sites (mean DOC, 25.96 mg/L) and at sites classified as high organic (mean DOC, 45.97 mg/L) compared to low organic sites (mean DOC, 36.5 mg/L). Similarly, there were greater temporal changes in DOC concentration at OSPM sites (42.56 to 99.77 mg/L) vs. no OSPM sites (9.23 to 58.27 mg/L) from June to September, 2006. Also, high organic sites (13.71 to 97.64 mg/L) had greater temporal changes in DOC concentration than low organic sites (9.23 to 99.77 mg/L). With respect to other water bodies in Alberta, DOC concentrations in OSPM and high organic content sites are elevated. Generally, lakes in the Athabasca River basin, have DOC

concentrations in the range of approximately 10 to 20 mg/L, similar to other regions in Alberta (Michell and Prepas 1990). In peatlands (high organic content), DOC concentrations in surface waters are elevated with a range of 46 to 87 mg/L compared to sites with lower amounts of peat which range between 23 to 35 mg/L (Kalbitz and Geyer 2002).

Typically, DOC is complex and is comprised of a wide range of organic sources originating from allochthonous inputs associated with leaf litter, soil and autochthonous inputs from macrophytes, phytoplankton, and microbial biomass. Commonly, allochthonous organics tend to be more recalcitrant to degradation compared to organics of autochthonous origin (Waichman 1996, Choi et al. 2004, Young et al. 2005). For example, humic and fulvic acids are recalcitrant allochthonous organic compounds with molecular weights ranging from 800 to 100 000 amu. These compounds are hydrophilic, heterogeneous mixtures of aromatic and non-aromatic compounds bound with carbohydrates, fatty acids and amino acids (Ressler et al. 1999, Ma et al. 2001, Choi et al. 2004, Young et al. 2005, Adani et al. 2006, Wallage et al. 2006). In general, humic acids are the dominant fraction in the DOC and contribute between one third to one half of the DOC in natural waters (Ma et al. 2001, Wallage et al. 2006). In peatlands, humic acid concentrations are also variable and may contribute between 30 to 90 % of the DOC (Wallage et al. 2006). Although humic acid concentrations were not measured in this study, humic acids likely contributed to the higher DOC concentrations at high organic content sites. However, sites with peat additions, which create more favourable environments for primary producers such as periphyton and macrophytes, may also have increased levels of autochthonous DOC (Renault et al. 2003, Reid and Naeth 2005).

In waters influenced by OSPM, elevated levels of naturally occurring compounds such as NAs, humic and fulvic acids, phenols, cresols, mercaptans, PACs and unrecovered bitumen (Mackinnon 1989, Holowenko et al. 2002, Quagraine et al. 2005) likely contribute to higher DOC concentrations. Although, concentration data for many of these groups of compounds are not available, concentrations of total NAs are well documented. In general, sites with no OSPM have low NA concentrations (1.2 to 11.3 mg NA/L) (Leonhardt 2003, Mackinnon unpublished 2006), contributing less than 10% of the total DOC concentrations (20.2 to 60.5 mg DOC/L) (Leonhardt 2003, Mackinnon unpublished 2006). At OSPM sites, NA concentrations were higher (22.3 to 42.1 mg NA/L), contributing between 40 to 60 % of the total DOC concentrations (53.6 to 58.2 mg DOC/L) (Mackinnon unpublished 2006).

While DOC concentrations are elevated at OSPM sites and at sites with high organic content, it is the biodegradable fraction that will influence the $\delta^{13}C$ values (or signatures) of the DOC, DIC and microbial biomass (Daly, 2007). Generally, the biodegradation of organic acid compounds (NAs, humic and fulvic acids) will be influenced by their chemical structure and molecular weight. For example, fulvic acids are slightly more biodegradable than humic acids due to their lower molecular weight (Wallage et al. 2006). Similarly, NAs with molecular weights ranging from 140 to 450 amu (Headley and McMartin 2004) are likely more biodegradable than humic and fulvic acids. Studies of NA biodegradation have shown that NA mixtures with greater quantities of low molecular weight NAs with fewer ring structures were more easily degraded than mixtures with high molecular weight NAs with multiple ring structures (Herman et al. 1993, Lai et al. 1996, Del Rio et al. 2006).

Changes in the $\delta^{13}C$ DOC values, particularly the ^{13}C enrichment of DOC from June to July at all sites, may be indicative to utilization of readily biodegradable DOC from aquatic plant decomposition over the previous winter months. From June to July, DOC values became ^{13}C enriched by 0.2 to 1.9 ‰ indicating utilization of ^{12}C enriched DOC prior to the July sampling period. Field studies examining DOC degradation have also demonstrated ^{13}C enrichment of the remaining DOC (Kalbitz et al. 2000, Van Breuklen et al. 2003). Also, decreases in DOC concentrations from June to July, particularly at sites with OSPM and sites with high organic content, may indicate increased microbial production at the beginning of the growing season. Increases in microbial degradation in mid to late June could account for the ^{13}C enrichment of DOC from June to July. Del Rio (2004) and Hadwin et al. (2006) have shown higher metabolic potential of microbial communities to degrade NAs in June compared to August. Also, although the temporal change in ^{13}C enrichment of the DOC is minimal (< 2 ‰), it is greater than the changes in the $\delta^{13}C$ values of DOC (-0.5 to 0.2 ‰) following microbial degradation of commercial NAs in laboratory microcosms (chapter 2). This may suggest that fractions other than NAs, particularly autochthonous DOC, may influence the temporal trends in DOC $\delta^{13}C$ values at oil sands reclamation sites.

Differences in the $\delta^{13}C$ values of DOC between young (-27 ‰) and mature (-27.6 ‰) sites, with slight ^{13}C depletion of the DOC at mature sites compared to young sites, may also be a function of bioavailable DOC. Although there were no significant differences in DOC concentrations between young and mature sites, the bioavailable fraction of the DOC may be different. Mature reclaimed sites with well established macrophyte and periphyton communities likely have greater autochthonous DOC that is

bioavailable. If this bioavailable DOC is ^{12}C enriched (^{13}C depleted), as suggested by the ^{13}C depletion of DOC in June for all sites, it would explain the ^{13}C depletion of the DOC at the mature sites. Daly (2007) collected macrophytes from a series of reclaimed wetlands and found that they varied in their $\delta^{13}C$ values (-33.2 to -12.7 ‰). In a mature OSPM site (TP 9), $\delta^{13}C$ values for macrophytes ranged between -28.1 to -20.1 ‰ and for algae and seston, values ranged from -30 to -26 ‰ (Daly 2007). Eventually, depending on the rate of decomposition, DOC such as carbohydrates and amino acids from autochthonous matter would enter the DOC pool thereby altering the $\delta^{13}C$ values of DOC.

To assess DOC utilization in oil sands reclamation, the $\delta^{13}C$ values of DOC were compared with the $\delta^{13}C$ values of microbial biomass (Daly, 2007). $\delta^{13}C$ DOC values for these sites ranged from -28.6 to -26.1 ‰. Daly (2007) found that microbial biofilms had $\delta^{13}C$ values in the range of -32.0 ±1.7 ‰ to -22.7 ±2.9‰. Based on laboratory tests using oil sands derived bacterial cultures, aerobic degradation of a DOC source (either commercial NA or oil sands derived NA) resulted in ^{13}C enrichment of microbial biomass (0.3 to 8.5 ‰) relative to the DOC source (chapter 2). Based on the $\delta^{13}C$ values of DOC at oil sands reclaimed sites and the ^{13}C enrichment of microbial biomass from aerobic laboratory tests, the biofilms at oil sands reclaimed sites would be expected to have a range of $\delta^{13}C$ values between -28 ‰ to -18 ‰. The more ^{13}C depleted biofilms from oil sands reclaimed sites (Daly, 2007) may indicate utilization of more ^{13}C depleted autochthonous DOC as suggested based on the spatial and temporal trends of DOC $\delta^{13}C$ values in this study. Microbial communities assimilating other forms of carbon such as carbon dioxide (CO_2) and methane (CH_4), may also contribute to the ^{13}C depletion of

microbial biofilms at oil sands reclaimed sites. Methanogenic bacteria, use CO_2 to produce CH_4 (Lapham et al. 1999), and methanotrophic bacteria, which oxidize methane to CO_2 have been examined under various conditions in oil sands tailings and reclamation (Holowenko et al. 2000, Holowenko et al. 2001, Fedorak et al. 2002, Fedorak et al. 2003, Siddique et al. 2006, Siddique et al. 2007), in addition, CO_2 and CH_4 fluxes have been measured in reclaimed systems (Daly 2007).

Concentrations of DIC were significantly higher in OSPM sites (mean DIC, 120.55 mg/L) compared to no OSPM sites (mean DIC, 40.71 mg/L). Temporal changes in DIC concentrations from June to September were greater at OSPM (48.87 to 194.03 mg/L) compared to no OSPM sites (4.93 to 74.53 mg/L). DIC originates from bacterial and plant respiration, dissolution of atmospheric CO_2 (gas exchange) and carbonate rocks (Kendall et al. 2001, Parker et al. 2005). Generally, 60 to 70 % of DIC in the water column can be attributed to the breakdown of DOC (Brown 1995). The DIC created is then assimilated by methanogenic bacteria (Wetzel 1983) and primary producers such as phytoplankton, periphyton and submerged macrophytes which use the inorganic carbon as their principle carbon source (Wetzel 1983, Kendall et al. 2001). Ultimately, respiration and photosynthesis are the two main factors controlling DIC in an aquatic environment (Wetzel 1983).

The DIC encompasses the following species; CO_2 dissolved in the water column as H_2CO_3, HCO_3^- and CO_3^2. The pH level of the aquatic environment controls the proportions of the species present. At a pH between 7 to 9, HCO_3^- will be the predominant species whereas at a pH below 5, the predominant anion will be H_2CO_3. At pH levels greater than 9.5, CO_3^- will predominate (Wetzel 1983, St. Jean 2003). On

average, pH values in reclamation site samples were greater than 7 but less than 9, with the exception of SW (pH of 10), thus the predominant DIC species in these systems is HCO_3^-. Concentrations of HCO_3^- are elevated in OSPM sites (Tables 3.1 and 3.2). Other studies have also reported high concentrations of HCO_3^-, particularly in CT released waters containing elevated levels of NAs (Leung et al. 2001, Salloum et al. 2002, Renault et al. 2003).

Since respiration and photosynthesis are the two principle factors controlling levels of DIC, it is important to characterize the primary productivity. Hornung (2007) examined primary production of macrophytes and found that *Typha* plants (cattails), which are emergent macrophytes, grown in OSPM sites contained a greater leaf area (higher density) than those grown in no OSPM sites indicating greater carbon accrual in OSPM sites. This is important, in that, as these plants complete their lifecycle, they will be recycled back into the environment and provide a source of labile autochthonous DOC for microbes, thereby enhancing this population. Further work analyzed community composition of macrophytes in these systems and found that the diversity of the macrophytes was lower in OSPM sites (Ciborowski et al. 2007). This may have implications on the DIC demand depending on the proportion of submergent vs emergent macrophytes. If there is a greater proportion of emergent macrophytes using atmospheric CO_2 (g) vs.submerged macrophytes using DIC, this will influence the DIC concentration and $\delta^{13}C$ values.

To assist in the understanding of the demands on the DIC and what is being produced, the levels of submerged primary production including phytoplankton, periphyton and submerged macrophytes to respiration need to be characterized;

Wytrykush 2007 is currently conducting such work. Preliminary work (2005) showed that in young, OSPM sites, production was greater than in young, no OSPM sites. In 2006, a more extensive study was conducted by Wytrykush, but showed that independently, age of construction, material of construction and organic content did not affect rates of production (Ciborowski et al. 2007). Among various sites, it was found that young, high organic content, no OSPM sites had high rates of primary production compared to mature sites with the same parameters, and in young, low organic content, no OSPM sites, production was lower than in mature. Analysis of temporal trends of ratios of primary production to respiration is currently being examined (Ciborowski et al. 2007).

Generally, $\delta^{13}C$ values of DIC were ^{13}C depletion (-0.3 to -8.8 ‰) from June to July. The trend in ^{13}C depletion of the DIC early in the season (July) is consistent with the timing of ^{13}C enrichment of the DOC at all sites. This may be as a result of spring thaw which releases readily degradable organics which stimulates the microbial community, creating ^{13}C enrichment of the DOC as they preferentially utilize ^{12}C within the DOC pool. With increased respiration of ^{12}C in June, the DIC pool becomes enriched in ^{12}C, resulting in DIC that is ^{13}C depleted for the July sampling period compared to the June sampling period. Short term laboratory tests (chapter 2) showed that aerobic microbial degradation of NAs resulted in trends towards ^{13}C depletion of the DIC.

OSPM sites were found to contain $\delta^{13}C$ DIC values that were significantly ^{13}C enriched (-3.9 ‰) compared to the DIC at sites with no OSPM (-8.8 ‰). At sites where microbial production is high, the DIC that is produced would likely be ^{13}C depleted (^{12}C enriched) due to respiration of ^{12}C rich CO_2 as suggested based on short term laboratory

tests (chapter 2). However, the DIC would not remain depleted because of the preferential uptake of ^{12}C by the primary producers during photosynthesis thereby leaving a ^{13}C enriched DIC pool (Leggett et al. 1999, Parker et al. 2005). Since levels of production in OSPM and no OSPM sites have not been fully assessed at this point, it is difficult to determine the influence of microbial respiration and primary production on the $\delta^{13}C$ values of DIC at OSPM vs. no OSPM sites. The ^{13}C enrichment of the DIC at OSPM sites may also be attributed to microbial biofilms oxidizing methane and producing ^{13}C enriched CO_2 (Opsahl and Chanton 2006). Increases in DIC isotope signatures may also occur where methanogenesis is dominant. During methanogenesis, methane is produced via the consumption of CO_2 (preferential selection for ^{12}C) resulting in ^{13}C enriched CO_2/DIC (Lapham et al. 1999, van Breukelen et al. 2003, Penning et al. 2005). The presence of ^{13}C depleted microbial biofilms (Daly 2007) beyond the range of isotope signatures expected based on the $\delta^{13}C$ values of DOC (this chapter) and isotope changes following aeorobic microbial degradation (chapter 2) suggests that methanogenesis may also influence the $\delta^{13}C$ values of DIC.

3.5. Conclusions

To summarize, OSPM and additions of organic material were found to elevate DOC concentrations. DIC concentrations were found to be elevated only at OSPM vs. no OSPM sites. $\delta^{13}C$ DOC values showed ^{13}C enrichment from June to July for all sites. This change is likely due to utilization of readily available autochthonous DOC following spring thaw. DOC compositions were not characterized, but this information may be useful in determining portion of the DOC which is bioavailable. $\delta^{13}C$ DOC values were also found to be ^{13}C depleted at mature sites compared to young sites. Similar to the ^{13}C

depletion of DOC in June relative to the remainder of the growing season, differences between sites may be a function of greater availability of more easily degradable organic compounds at mature sites with well developed macrophyte and periphyton communities. Lastly, $\delta^{13}C$ DIC values were found to be ^{13}C depleted from June to July suggesting enhanced microbial degradation of available autochthonous DOC. At sites impacted by OSPM, DIC values were found to be ^{13}C enriched compared to no OSPM sites which will be a function of respiration to primary production.

The results provide baselines for DOC and DIC concentrations and isotopes values that are available for assimilation at the base of the food web in various aquatic reclaimed sites. These results can be used to further assist in understanding carbon flow and dynamics in these systems. DOC and DIC are not static and are influenced by many variables. Levels of DOC may be affected by autochthonous and allochthonous sources as well as degradation by microbial communities. DIC levels are affected by populations of primary producers which in turn are influenced by factors such as turbidity levels and depth which influence sunlight penetration. As well, microbial respiration and anaerobic activity will affect DIC values. This data will be used to support detailed analysis of the microbial and primary production to further aid in the understanding of carbon flow and dynamics in oil sands reclaimed systems.

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4 Chapter 4. Conclusions

Previous field studies in oil sands aquatic reclamation sites had found a trend towards ^{13}C depletion and ^{15}N enrichment in benthic organisms present in sites of increasing amounts of oil sands process material (OSPM). To better understand these trends laboratory degradation tests were initiated. The primary objective of these tests was to attempt to correlate changes in the stable isotope values occurring as a result of microbial degradation of complex organic carbon mixtures such as naphthenic acids (NAs), which may comprise of a significant portion of the dissolved organic carbon (DOC) within reclamation sites and ammonium sources which are also elevated at sites with OSPM.

One of the principle findings found from the laboratory study was the ^{15}N enrichment between 3.8 to 8.4 ‰ of the biomass in the semi-continuous tests that occurred with microbial utilization of ammonium chloride. It was evident from the laboratory study that ample quantities of ammonium led microbes to preferentially excrete ^{15}N. These findings correlate with increasing ^{15}N enrichment seen in invertebrates in aquatic reclaimed sites increasing in amounts of OSPM and ammonium levels (Elshayeb 2006, Farwell et al. submitted). $\delta^{15}N$ values for microbial biofilms from reclaimed sites with increasing amounts of OSPM also showed ^{15}N enrichment (Daly 2007). Increased levels of ammonium, lead to increase utilization of the ammonium, therefore causing ^{15}N enrichment of the microbial biomass, which is incorporated into the food web and seen at higher trophic levels such as benthic invertebrates.

In the field DOC concentration values differ between sites and are significantly higher in OSPM sites by approximately 40 mg/L compared to no OSPM sites and greater

in sites with high organic content by approximately 10 mg/L compared to low organic sites. $\delta^{13}C$ DOC values are quite similar between sites although there is slight ^{13}C enrichment (0.6 ‰) is seen in young sites compared to mature sites. Laboratory studies also showed minimal change in the $\delta^{13}C$ DOC values (-0.5 to + 0.2 ‰) despite varying levels of microbial utilization of the DOC, as seen with decreases in DOC concentration and ^{13}C enrichment of the biomass for both the commercial NAs (0.3 to 2.9 ‰) and oil sands derived NAs (3.7 to 8.5 ‰) relative to the DOC source. Temporal analysis in the field survey showed that between June to July there was a trend of ^{13}C enrichment (0.3 to 1.9 ‰) of the DOC. Spatial and temporal changes in $\delta^{13}C$ seen in the field may be due to greater amounts of autochothonous DOC available for uptake. The DOC is complex in its composition and this will impact its bioavailability in a food web. Future goals will characterize the composition of the DOC to predict its potential for biodegradation. As well, future studies will use whole water samples as the DOC source rather than solely the NAs extract to examine the whole spectrum of DOC that may be available for assimilation.

Concentrations of dissolved inorganic carbon (DIC) are largely regulated by respiration and photosynthesis in aquatic systems (Wetzel 1983). In the aquatic reclaimed sites sampled, DIC concentrations were variable but on average were significantly higher at OSPM sites by approximately 80 mg/L compared to no OSPM sites. Examining the $\delta^{13}C$ DIC values at OSPM sites, ^{13}C enrichment of the DIC (-3.9 ‰) compared to no OSPM sites (-8.8 ‰) was seen. However, a trend of ^{13}C depletion from June to July was seen for most sites, correlating with the ^{13}C enrichment that was seen with the DOC. Spatial and temporal trends in $\delta^{13}C$ may also be due to

autochthonous DOC from spring thaw. The respired DIC is generally ^{12}C enriched (^{13}C depleted), as the aerobic microbial community preferentially uptakes the lighter isotope (^{12}C). Short term degradation tests showed the production of ^{13}C depleted DIC, which correlates with the trends seen in the DIC stable values between June to July. However, DIC levels would likely not remain ^{12}C enriched (^{13}C depleted) as these systems also have a primary producer community which would likely preferentially uptake the ^{12}C thereby leaving a ^{13}C enriched DIC pool. In addition, methanogenic and methanotrophic communities, also present in these systems, will influence DIC concentration and stable isotope values by consuming and releasing CO_2. Since levels of production and methanogenic activity in reclaimed systems have not yet been assessed it is difficult to determine their influence on the DIC. Future research goals will examine the influence of primary production and methanogenic activity on the stable isotope values of the biomass and the DIC.

4.2. References

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Appendix 1. 10-Day Short Term Static Test

Table A1. Direct cell counts following biodegradation of an oil sands derived NA extract for 10 days with 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) (x 10^8) ± std. error
0	1.34 ± 4.202
2	1.69 ± 4.933
4	2.46 ± 5.015
6	2.82 ± 5.207
8	2.60 ± 10.661
10	3.29 ± 4.572

Table A2. Changes in the concentrations of DOC and DIC following biodegradation of an oil sands derived NA extract for 10 days with 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	DOC Concentration (mg/L)		Mean DOC Concentration (mg/L) ± std. error	DIC Concentration (mg/L)		Mean DIC Concentration (mg/L) ± std. error
	#1	#2		#1	#2	
0	115.03	113.43	114.23 ± 0.800	181.91	180.76	181.34 ± 0.575
2	89.98	97.11	93.55 ± 3.565	181.97	182.71	182.34 ± 0.370
4	91.29	95.06	93.18 ± 1.885	188.47	191.27	189.87 ± 1.400
6	91.94	97.35	94.65 ± 2.705	204.14	197.11	200.63 ± 3.515
8	91.01	89.96	90.49 ± 0.525	203.12	202.01	202.57 ± 0.555
10	91.02	91.11	91.07 ± 0.045	205.01	201.39	203.20 ± 1.810

Table A3. Changes in the $\delta^{13}C$ values of the DOC and DIC following biodegradation of an oil sands derived NA extract for 10 days with 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	$\delta^{13}C$ DOC (‰)		Mean $\delta^{13}C$ DOC (‰) ± std. error	$\delta^{13}C$ DIC (‰)		Mean $\delta^{13}C$ DIC (‰) ± std. error
	#1	#2		#1	#2	
0	-28.9	-28.8	-28.8 ± 0.015	-9.7	-10.2	-9.9 ± 0.245
2	-28.8	-29.0	-28.9 ± 0.085	-10.5	-10.8	-10.7 ± 0.145
4	-28.9	-29.5	-29.2 ± 0.295	-11.1	-11.1	-11.1 ± 0.040
6	-28.7	-28.9	-28.8 ± 0.065	-11.1	-11.1	-11.1 ± 0.010
8	-29.1	-28.9	-29 ± 0.060	-11.1	-11.4	-11.3 ± 0.130
10	-29	-29	-29 ± 0.035	-11.6	-11.5	-11.6 ± 0.065

Table A4. Changes in the $\delta^{13}C$ and $\delta^{15}N$ values of the biomass following biodegradation of an oil sands derived NA extract for 10 days with 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	**$\delta^{13}C$ Biomass (‰)**		**Mean $\delta^{13}C$ Biomass (‰) ± std. error**	**$\delta^{15}N$ Biomass (‰)**		**Mean $\delta^{15}N$ Biomass (‰) ± std. error**
	#1	**#2**		**#1**	**#2**	
0	-25.5	No value	-25.5	-3.1	No value	-3.1
2	-25.7	-25.3	-25.5 ± 0.121	-4.3	-4.8	-4.5 ± 0.237
4	-25.9	-25.4	-25.6 ± 0.081	-3.6	-2.9	-2.9 ± 0.401
6	-26.4	-25.7	-26 ± 0.259	-3.7	-5.1	-4.4 ± 0.667
8	-25.7	-25.8	-25.8 ± 0.021	-2.6	-2.4	-2.5 ± 0.075
10	-25.6	-24.8	-25.2 ± 0.294	-4.1	-2.2	-3.2 ± 0.951

Appendix 2. 16-Day Short Term Static Test

Table A5. Direct cell counts following biodegradation of Merichem Refined NAs for 16 days using microbial cultures from MLSB with 0.4 g/L NH_4Cl. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) ± std. error		
	44.45 mg/L DOC	29.83 mg/L DOC	23.08 mg/L DOC
0	$2.30 \times 10^7 \pm 46.259$	$3.45 \times 10^7 \pm 50.461$	$3.50 \times 10^7 \pm 45.981$
16	$1.41 \times 10^8 \pm 14.638$	$1.61 \times 10^8 \pm 18.006$	$1.59 \times 10^8 \pm 16.557$

Table A6. Changes in the DOC and DIC concentrations following biodegradation of Merichem Refined NAs for 16 days using microbial cultures from MLSB with 0.4 g/L NH_4Cl.

Day	44.45 mg/L DOC		29.83 mg/L DOC		23.08 mg/L DOC	
	DOC (mg/L)	DIC (mg/L)	DOC (mg/L)	DIC (mg/L)	DOC (mg/L)	DIC (mg/L)
0	44.45	17.69	29.83	14.32	23.08	12.99
16	22.8	23.25	16.09	17.13	7.62	13.82

Table A7. Changes in the $\delta^{13}C$ DOC and DIC values following biodegradation of Merichem Refined NAs for 16 days using microbial cultures from MLSB with 0.4 g/L NH_4Cl.

Day	44.45 mg/L DOC		29.83 mg/L DOC		23.08 mg/L DOC	
	$\delta^{13}C$ DOC (‰)	$\delta^{13}C$ DIC (‰)	$\delta^{13}C$ DOC (‰)	$\delta^{13}C$ DIC (‰)	$\delta^{13}C$ DOC (‰)	$\delta^{13}C$ DIC (‰)
0	-27.3	-9.4	-27.3	-9	-27.5	-6.6
16	-27.7	-13.2	-27.6	-11.8	-27.6	-11.6

Table A8. Changes in the $\delta^{13}C$ and $\delta^{15}N$ biomass values following biodegradation of Merichem Refined NAs for 16 days using microbial cultures from MLSB with 0.4 g/L NH_4Cl.

Day	44.45 mg/L DOC		29.83 mg/L DOC		23.08 mg/L DOC	
	$\delta^{13}C$ Biomass (‰)	$\delta^{15}N$ Biomass (‰)	$\delta^{13}C$ Biomass (‰)	$\delta^{15}N$ Biomass (‰)	$\delta^{13}C$ Biomass (‰)	$\delta^{15}N$ Biomass (‰)
0	-23	-9.7	-23.9	-9.1	na[a]	-7.2
16	-25.7	-13.1	-25.4	-12.6	-25.7	-13

[a] na = not available, due to low sample weight

Appendix 3. 30-Day Long Term Static Test

Table A9. Direct cell counts following biodegradation of Merichem Refined NAs for 30 days with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) ± std. error	
	1.3 g/L NH_4Cl	**1.0 g/L NH_4NO_3**
0	$7.07 \times 10^7 \pm 7.834$	$9.18 \times 10^7 \pm 3.351$
2	$1.52 \times 10^8 \pm 8.966$	$1.32 \times 10^8 \pm 3.119$
6	$1.65 \times 10^8 \pm 11.605$	$2.24 \times 10^8 \pm 13.768$
12	$2.08 \times 10^8 \pm 7.973$	$2.56 \times 10^8 \pm 12.809$
30	$2.08 \times 10^8 \pm 10.774$	$2.70 \times 10^8 \pm 11.707$

Table A10. Changes in the DOC and DIC concentrations following biodegradation of Merichem Refined NAs for 30 days with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3.

Day	1.3 g/L NH_4Cl		1.0 g/L NH_4NO_3	
	DOC (mg/L)	**DIC (mg/L)**	**DOC (mg/L)**	**DIC (mg/L)**
0	48.23	2.58	52.76	1.9
2	47.87	3.57	51.02	1.81
6	49.14	2.31	48.08	2.96
12	28.01	4.63	32.27	3.52
30	8.91	2.8	13.29	7.24

Table A11. Changes in the $\delta^{13}C$ DOC and DIC values following biodegradation of Merichem Refined NAs for 30 days with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3.

Day	1.3 g/L NH_4Cl		1.0 g/L NH_4NO_3	
	$\delta^{13}C$ DOC (‰)	**$\delta^{13}C$ DIC (‰)**	**$\delta^{13}C$ DOC (‰)**	**$\delta^{13}C$ DIC (‰)**
0	-26.8	-11	-26.7	-11.1
2	-26.7	-14.4	-26.6	-8.1
6	-26.8	-8.9	-26.8	-9.6
12	-27	-8	-26.9	-6.2
30	-27.3	-5.6	-26.9	-7.4

Table A12. Changes in the $\delta^{13}C$ biomass values following biodegradation of Merichem Refined NAs for 30 days with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	1.3 g/L NH_4Cl		Mean $\delta^{13}C$ Biomass (‰) ± std. error	1.0 g/L NH_4NO_3		Mean $\delta^{13}C$ Biomass (‰) ± std. error
	#1	#2		#1	#2	
0	-21.2	-23.7	-22.4 ± 1.23	-22.7	-22.1	-22.1 ± 0.33
2	-23	-23.2	-23.1 ± 0.14	-23.3	-24.2	-23.7 ± 0.44
6	-23.8	-23.7	-23.8 ± 0.07	-23.3	-22.8	-23.1 ± 0.26
12	-24.1	-24	-24 ± 0.02	-23.8	-24.1	-24 ± 0.11
30	-24.2	-23.6	-23.9 ± 0.30	-23.7	-24	-23.8 ± 0.13

Table A13. Changes in the $\delta^{15}N$ biomass values following biodegradation of Merichem Refined NAs for 30 days with 1.3 g/L NH_4Cl or 1.0 g/L NH_4NO_3. Mean ± standard error have been reported.

Day	1.3 g/L NH_4Cl		Mean $\delta^{15}N$ Biomass (‰) ± std. error	1.0 g/L NH_4NO_3		Mean $\delta^{15}N$ Biomass (‰) ± std. error
	#1	#2		#1	#2	
0	-11.2	-11.6	-11.4 ± 0.20	-12.8	-14	-13.4 ± 0.37
2	-14	-14.4	-14.2 ± 0.22	-11.7	-12.6	-12.1 ± 0.45
6	-14.7	-14.2	-14.5 ± 0.28	-10.7	-10.7	-10.7 ± 0.01
12	-12.8	-12.2	-12.5 ± 0.26	-11.6	-12	-11.8 ± 0.21
30	-13.9	-12.9	-13.4 ± 0.49	-14.3	-13.9	-14.1 ± 0.19

Appendix 4. 3-Day Semi-Continuous Test

Table A14. Direct cell counts following biodegradation of Merichem Refined NAs with 1.3 g/L NH_4Cl transferred every third day onto fresh NAs and mineral medium. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) ± std. error
0	$8.50 \times 10^7 \pm 5.273$
3	$1.64 \times 10^8 \pm 8.954$
6	$1.48 \times 10^8 \pm 11.511$
9	$1.81 \times 10^8 \pm 9.959$
12	$2.59 \times 10^8 \pm 12.233$
15	$3.00 \times 10^8 \pm 7.320$
18	$2.00 \times 10^8 \pm 13.421$
21	$1.85 \times 10^8 \pm 7.373$
24	$1.80 \times 10^8 \pm 12.134$
27	$2.30 \times 10^8 \pm 9.342$
30	$2.43 \times 10^8 \pm 9.283$

Table A15. Changes in the $\delta^{13}C$ and $\delta^{15}N$ biomass values following biodegradation of Merichem Refined NAs with 1.3 g/L NH_4Cl transferred every third day onto fresh NAs and mineral medium.

Day	$\delta^{13}C$ Biomass (‰)	$\delta^{15}N$ Biomass (‰)
0	-24.6	-13.5
3	-23.4	-14.9
6	-24.1	-14.8
9	-24.4	-14
12	-24.8	-14.4
15	-25	-14.6
18	-24.9	-15.2
21	-25.1	-14.7
24	-24.8	-14.1
27	-25.1	-13.4
30	-25.3	-15.1

Appendix 5. 13-Day Semi-Continuous Test with Commercial NAs

Table A16. Direct cell counts following biodegradation of Merichem Refined NAs with 1.3 g/L NH_4Cl transferred every 13 days onto fresh NAs and mineral medium. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) ± std. error	
	Bottle #1	Bottle #2
0	$1.04 \times 10^8 \pm 5.331$	$1.10 \times 10^8 \pm 5.123$
13	$1.44 \times 10^8 \pm 8.377$	$1.34 \times 10^8 \pm 4.820$
26	$2.07 \times 10^8 \pm 20.915$	$1.72 \times 10^8 \pm 5.568$
39	$2.35 \times 10^8 \pm 8.026$	$2.23 \times 10^8 \pm 16.708$
52	$2.28 \times 10^8 \pm 17.324$	$2.19 \times 10^8 \pm 14.496$
65	$2.01 \times 10^8 \pm 2.828$	$2.13 \times 10^8 \pm 3.530$
78	$2.22 \times 10^8 \pm 8.132$	$2.51 \times 10^8 \pm 20.860$
91	$2.46 \times 10^8 \pm 6.364$	$2.08 \times 10^8 \pm 14.849$
104	$2.88 \times 10^8 \pm 4.596$	$2.65 \times 10^8 \pm 22.627$
117	$2.38 \times 10^8 \pm 17.324$	$1.67 \times 10^8 \pm 10.607$

Table A17. Changes in the $\delta^{13}C$ and $\delta^{15}N$ values of the biomass following biodegradation of Merichem Refined NAs with 1.3 g/L NH_4NO_3 transferred every 13 days onto fresh NAs and mineral medium. Mean ± standard error have been reported.

Day	$\delta^{13}C$ Biomass (‰)		Mean $\delta^{13}C$ Biomass (‰) ± std. error	$\delta^{15}N$ Biomass (‰)		Mean $\delta^{15}N$ Biomass (‰) ± std. error
	#1	#2		#1	#2	
0	-24.2	-23.9	-24.1 ± 0.16	-14.9	-16.1	-15.5 ± 0.60
13	-23.7	-23.5	-23.6 ± 0.11	-16.2	-16	-16.1 ± 0.11
26	-25.7	-25.3	-25.5 ± 0.17	-12.2	-11	-11.6 ± 0.62
39	-25.7	-25.4	-25.5 ± 0.16	-12.3	-13.9	-13.1 ± 0.80
52	-25.6	-25	-25.3 ± 0.29	-12.2	-14.3	-13.3 ± 1.05
65	-25.7	-25.6	-25.7 ± 0.02	-12.2	-12.2	-12.2 ± 0.03
78	-25.4	-25.3	-25.4 ± 0.07	-11.7	-11.5	-11.6 ± 0.11
91	-25.3	-25.5	-25.4 ± 0.07	-11.8	-11.6	-11.7 ± 0.11

Appendix 6. 13-Day Semi-Continuous Test with Oil Sands NA Extract

Table A18. Direct cell counts following biodegradation of an oil sands NA extract with 1.3 g/L NH_4Cl transferred every 13 days onto fresh NAs and mineral medium. Mean ± standard error have been reported.

Day	Mean Cell Counts (cells/mL) ± std. error	
	Bottle #1	Bottle #2
0	$1.51 \times 10^8 \pm 9.499$	$1.66 \times 10^8 \pm 5.515$
13	$1.36 \times 10^8 \pm 6.513$	$1.17 \times 10^8 \pm 3.301$
26	$1.74 \times 10^8 \pm 4.882$	$1.63 \times 10^8 \pm 7.969$
39	$1.91 \times 10^8 \pm 1.958$	$1.70 \times 10^8 \pm 9.911$
52	$1.80 \times 10^8 \pm 9.546$	$1.75 \times 10^8 \pm 3.889$
65	$1.03 \times 10^8 \pm 8.839$	$1.18 \times 10^8 \pm 2.475$
78	$1.40 \times 10^8 \pm 5.657$	$1.40 \times 10^8 \pm 11.314$
91	$1.14 \times 10^8 \pm 3.536$	$8.10 \times 10^7 \pm 0.707$
104	$1.15 \times 10^8 \pm 7.425$	$1.13 \times 10^8 \pm 2.828$
117	$1.11 \times 10^8 \pm 7.778$	$1.19 \times 10^8 \pm 8.839$

Table A19. Changes in the $\delta^{13}C$ and $\delta^{15}N$ values of the biomass following biodegradation of an oil sands NA extract with 1.3 g/L NH_4NO_3 transferred every 13 days onto fresh NAs and mineral medium. Mean ± standard error have been reported.

Day	$\delta^{13}C$ Biomass (‰)		Mean $\delta^{13}C$ Biomass (‰) ± std. error	$\delta^{15}N$ Biomass (‰)		Mean $\delta^{15}N$ Biomass (‰) ± std. error
	#1	#2		#1	#2	
0	-22.6	-22.7	-22.7 ± 0.02	-5.4	-5.4	-5.4 ± 0.00
13	-23.8	-23.3	-23.6 ± 0.29	-6.1	-0.6	-3.4 ± 2.71
26	-21.9	-22.2	-22 ± 0.15	0.92	-1.7	-0.4 ± 1.31
39	-21.4	-21.2	-21.3 ± 0.09	-1	-1.1	-1.1 ± 0.05
52	-20.3	-21	-20.6 ± 0.35	0.18	1.7	0.9 ± 0.77
65	-21.9	-21.4	-21.6 ± 0.22	2.9	1.5	2.2 ± 0.74
78	-18.6	-18.6	-18.6 ± 0.01	-2.8	0.25	-1.3 ± 1.52
91	-20.4	-20.4	-20.4 ± 0.02	-0.5	6.6	3 ± 3.51

Appendix 7: Field Survey

Table A20. DOC and DIC concentrations and $\delta^{13}C$ values for water samples collected August 3, 2005 from 13 sites divided according to age of construction, construction material and organic content level (see Table 3.1 for abbreviation names).

Age	Status	Organic Level	Sites	August 2005 Data			
				[DOC] (mg/L)	[DIC] (mg/L)	$\delta^{13}C$ DOC (‰)	$\delta^{13}C$ DIC (‰)
Young	No OSPM	Low	BL	24.14	57.07	-28.7	-6
			CNRL	22.98	20.67	-27.5	-5.2
		High	PP	37.63	34.49	-26.6	-5.5
			GP	26.05	13.89	-26.8	-8.8
	OSPM	Low	SCT	57.52	75.06	-27.4	-0.3
			CTW-NP	69.72	123.03	-27.9	-4.4
		High	CTW-P	63.09	133.76	-27.2	-6.5
Mature	No OSPM	Low	SW	22.99	39.76	-27.8	-5.1
			NWID	32.45	37.69	-26.9	-2.3
		High	HS	60.99	21.3	-28.7	-6.6
			SSBP	33.2	55.16	-27.4	-10.4
	OSPM	Low	TP9	66.45	131.14	-26.7	-3.0
		High	NW	73.15	115.58	-27.9	-6.2

Table A21. DOC concentrations for water samples collected in the 2006 summer season from 13 sites divided according to age of construction, construction material and organic content level (see Table 3.1 for abbreviation names).

Age	Status	Organic Level	Sites	DOC Concentration Data (2006 Season)			
				June 26-27	July 27	August 27	September 12
Young	No OSPM	Low	BL	28.31	18.42	25.94	22.38
			CNRL	25.82	21.55	9.23	28.87
		High	PP	34.09	32.52	43.07	36.3
			GP	29.31	23.58	26.43	37.54
	OSPM	Low	SCT	57.19	55.72	43.34	51.97
			CTW-NP	99.77	70.41	42.56	80.64
		High	CTW-P	97.64	47.82	75.6	76.04
Mature	No OSPM	Low	SW	21.68	12.53	20.14	21.6
			NWID	28.1	32.9	27.61	29.35
		High	HS	57.46	43.55	46.57	58.27
			SSBP	36.21	13.71	20.32	32.33
	OSPM	Low	TP9	64.84	70.21	47.89	54.32
		High	NW	89.04	48.58	54.01	43.23

Table A22. DIC concentrations for water samples collected in the 2006 summer season from 13 sites divided according to age of construction, construction material and organic content level (see Table 3.1 for abbreviation names).

Age	Status	Organic Level	Sites	DIC Concentration Data (2006 Season)			
				June 26-27	July 27	August 27	September 12
Young	No OSPM	Low	BL	60.86	40.69	81.3	67.07
			CNRL	13.53	13.45	4.93	11.45
		High	PP	40.59	41.14	57.55	48.99
			GP	22.62	13.01	16.83	26.03
	OSPM	Low	SCT	67.99	63.49	48.87	61.73
			CTW-NP	165.6	194.03	112.18	176.86
		High	CTW-P	174.69	123.52	172.17	174.33
Mature	No OSPM	Low	SW	45.19	30.66	49.37	58.06
			NWID	39.21	48.56	42.56	45.13
		High	HS	50.01	37.73	44.41	56.99
			SSBP	57.08	23.04	40.09	74.53
	OSPM	Low	TP9	136.97	151.76	94.75	109.31
		High	NW	97.08	95.1	104.3	86.16

Table A23. $\delta^{13}C$ DOC values concentrations for water samples collected in the 2006 summer season from 13 sites divided according to age of construction, construction material and organic content level (see Table 3.1 for abbreviation names).

Age	Status	Organic Level	Sites	$\delta^{13}C$ DOC Values (2006 Season)			
				June 26-27	July 27	August 27	September 12
Young	No OSPM	Low	BL	-29.7	-28.2	-28.2	-27.1
			CNRL	-27.1	-26.4	-26.2	-26.1
		High	PP	-26.8	-26.0	-25.7	-25.9
			GP	-27	-26.5	-26.2	-26.4
	OSPM	Low	SCT	-28	-27	-26.8	-27.1
			CTW-NP	-28.8	-27.1	-27	-27.1
		High	CTW-P	-28.8	-26.9	-27.7	-27.6
Mature	No OSPM	Low	SW	-29.3	-28.2	-27.6	-27.5
			NWID	-27.6	-26.5	-26.8	-26.5
		High	HS	-28.8	-28.6	-28.3	-28.5
			SSBP	-28	-26.8	-27.6	-27
	OSPM	Low	TP9	-27.7	-27	-26.7	-26.7
		High	NW	-28.4	-27.5	-28	-27.3

Table A24. $\delta^{13}C$ DIC values concentrations for water samples collected in the 2006 summer season from 13 sites divided according to age of construction, construction material and organic content level (see Table 3.1 for abbreviation names).

Age	Status	Organic Level	Sites	$\delta^{13}C$ DIC Values (2006 Season)			
				June 26-27	July 27	August 27	September 12
Young	No OSPM	Low	BL	-5.3	-8.9	-8.4	-7.6
			CNRL	-13	-14.2	-16.4	-16.5
		High	PP	-3.3	-6	-6.1	-6.2
			GP	-6.5	-11.3	-11.5	-10.5
	OSPM	Low	SCT	-0.0	-1.2	-1.2	-1
			CTW-NP	-5.5	-4.5	-4.4	-5.1
		High	CTW-P	-5.7	-5.2	-6.1	-6.1
Mature	No OSPM	Low	SW	-4.5	-6.6	-6.2	-7.1
			NWID	-3.9	-4.9	-1.2	-4.2
		High	HS	-6.8	-9.7	-8.8	-8.4
			SSBP	-6.3	-15.1	-9.5	-9.4
	OSPM	Low	TP9	-3.1	-3.8	-3.9	-4.4
		High	NW	-5.6	-5.9	-3.8	-3.3