University of Alberta

Biodegradation, Ozonation, and Characterization of Naphthenic Acids

by

Angela C. Scott

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The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I--
I took the one less traveled by,
And that has made all the difference.

Robert Frost

"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change."

~Charles Darwin

"Human beings, who are almost unique in having the ability to learn from the experience of others, are also remarkable for their apparent disinclination to do so."

~Douglas Adams

"Nothing in the world can take the place of persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education alone will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent."

~Calvin Coolidge

ABSTRACT

The Athabasca oil sands industry produces large volumes of process water during bitumen extraction and upgrading. This water contains naphthenic acids: a complex mixture of aliphatic and alicyclic carboxylic acids that are acutely toxic to aquatic organisms. Biodegradation and ozonation were used alone and in combination to evaluate the effectiveness of these treatments for reducing naphthenic acids concentrations and removing their associated toxicity. Experiments were conducted using actual process water as well as solutions of commercially-available naphthenic acids. Established analytical methods, including GC-MS and HPLC, were employed along with a novel HPLC/QTOF-MS method. Commercial preparations were more biodegradable than oil sands naphthenic acids but both mixtures were susceptible to chemical oxidation with ozone. Extensive ozonation of process water resulted in decreased naphthenic acids concentrations and complete removal of toxicity. Partial oxidation of commercial naphthenic acids also reduced their concentration and toxicity, but did not significantly change the rate of biodegradation.

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LIST OF ABBREVIATIONS

1MCHC	1-Methyl-1-cyclohexane carboxylic acid
2MCHC	2-Methyl-1-cyclohexane carboxylic acid
AOP	Advanced oxidation process
BOD	Biochemical oxygen demand
CHCA	Cyclohexane carboxylic acid
COD	Chemical oxygen demand
CPCA	Cyclopentane carboxylic acid
DHNA	Decahydro-2-naphthoic acid-8-^{14}C
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EI	Electron impact ionization, used in mass spectrometry
FT-IR	Fourier-transform infrared spectroscopy
GAC	Granular activated carbon
GC	Gas chromatography
GC-FID	Gas chromatography coupled to a flame ionization detector
GC-MS	Gas chromatography with mass spectrometry
HPLC	High performance liquid chromatography
HPLC/ QTOF-MS	High performance liquid chromatography coupled with quadrupole and time of flight mass spectrometry
IC_{20}	Concentration that decreases light output by 20% in Microtox™ assay
IC_{50}	Concentration that decreases light output by 50% in Microtox™ assay
IR	Infrared spectroscopy
LD_{50}	Concentration that results in 50% mortality of test organisms
m/z	Mass to charge ratio
MMBH	Modified Bushnell-Haas medium
MS	Mass spectrometry
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide
NPH	2-Nitrophenylhydrazine
OSPW	Oil sands process water
ODF	Ozone-demand free

PDI	Protein disulfide isomerase
RuBisCo	Ribulose-1,5-bisphosphate carboxylase
SCL WIP	Syncrude Canada Ltd., West In Pit storage pond
SPE	Solid-phase extraction
Suncor CT	Suncor Energy Inc., Consolidated Tailings pond
TAN	Total acid number
TEX	Tailings extract
TIC	Total ion current
TOC	Total organic carbon
TPW	Tailings pore water
UV	Ultraviolet detection

1 INTRODUCTION

1.1 Alberta's oil sands

Eight countries contain major accumulations of oil sands: Canada, Venezuela, the United States of America, Trinidad, Madagascar, Albania, Russia, and Romania (Chalaturnyk et al. 2002). Of these, Canada boasts the largest deposit, much of which is located in north-eastern Alberta (Figure 1.1). The Alberta Energy and Utilities Board (AEUB) estimates the total volume of in-place oil to be around 270 billion m^3, equivalent to 1.7 trillion barrels (AEUB 2006). This is more than ten times the size of the world's largest conventional oil field, located in Saudi Arabia, and more than 25 times greater than the combined volume of the conventional oil fields of Alberta (AEUB 2006).

The Alberta oil sands deposits, which occur over a total area of ~146 000 km^2, have been divided into three geographical regions: Athabasca, Cold Lake, and Peace River (Figure 1.1, Table 1.1). The Athabasca oil sands region is the largest (~100 000 km^2) and contains the majority (>80%) of the estimated in-place oil (AEUB 2006). Most of this volume is in the Wabiskaw-McMurray formation, which underlies about half of the Athabasca oil sands region (AEUB 2006).

The type of petroleum present in oil sands is a tar-like mixture called bitumen. Crude bitumen is denser (lower °API gravity) than conventional crude oil (Schramm et al. 2000), and, due to its high viscosity, it will not flow to a well (AEUB 2006). In Alberta, the bitumen deposits are primarily found in quartz sand, with some feldspar, mica flakes, and clays dispersed throughout (Chalaturnyk et al. 2002). Kaolinite and illite are the most commonly occurring clays, but chlorite, montmorillonite, calcite, and dolomite are also present (Camp 1977). It is generally accepted that water is associated with the oil sand grains, as depicted in Figure 1.2 (Camp 1977, Takamura 1982). This is referred to as a water-wet condition (Schramm et al. 2000, Chalaturnyk et al. 2002).

Typically, oil sands with a bitumen content of 12 to 19% (by weight) are considered high quality or "rich" (Schramm et al. 2000, AEUB 2006). Because much of the Athabasca oil sands region falls into this category (Strausz and Lown 2003, AUEB 2006), the majority of industrial enterprises have been concentrated there.

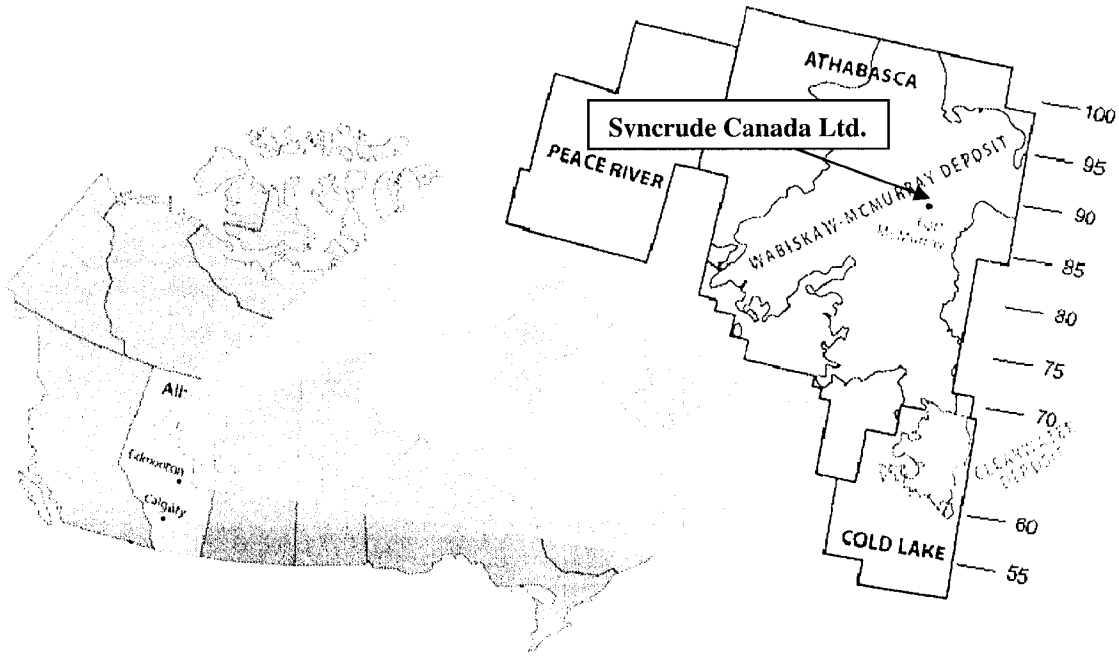

Figure 1.1 Map illustrating the location of Alberta's major oil sands deposits: Peace River, Athabasca, and Cold Lake. The combined area of the three deposits is about 146 000 km^2. The scale on the far right shows township markers that are about 50 km apart. Adapted from AEUB (2006).

Table 1.1 Initial in-place volumes and surface areas of the three major oil sands regions of Alberta. Adapted from AEUB (2006).

Oil sands region	Initial in-place volume 10^9 m^3	Area 10^3 km^2
Athabasca	218	103
Cold Lake	31	26
Peace River	21	17
Total	270	146

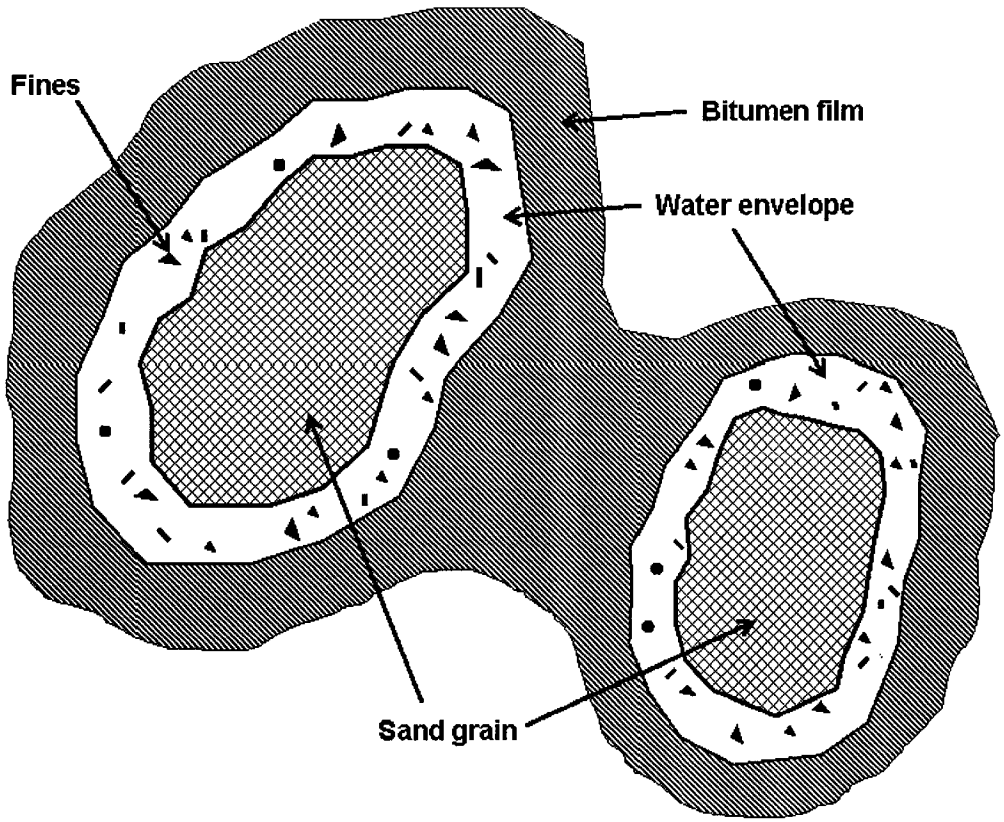

Figure 1.2 Schematic diagram of oil sands grains showing the association of water, bitumen, sand grains, and fine clay and sand particles (fines). Redrawn from Camp (1977).

1.1.1 Syncrude Canada Ltd. and Suncor Energy Inc.

Syncrude Canada Ltd. (Syncrude) and Suncor Energy Inc. (Suncor) are currently the largest Alberta oil sands companies. Both evolved from earlier ventures by oil sands entrepreneurs in the 1960s and 1970s: Syncrude took over the Cities Service Athabasca Inc. project and Suncor formed from the amalgamation of Great Canadian Oil Sands Ltd. and Sun Company Inc. (McKenzie-Brown et al. 1993). Located north of Fort McMurray, Syncrude and Suncor operate within the Athabasca oil sands region (see Figure 1.1).

Areas of the Suncor lease under active development (154 km^2) contain approximately 878 million m^3 of bitumen reserves, whereas those of the Syncrude lease

(217 km^2) contain just over 1.4 billion m^3 (AEUB 2006). Bitumen is upgraded onsite through complex and proprietary processes involving extreme thermal treatment and subsequent steps to remove organic sulfur and nitrogen (Syncrude 2003, Suncor 2007). The product is a synthetic crude oil, which Syncrude and Suncor market as a high quality refinery feedstock. In 2005, more than half of Canada's total domestic production of crude oil was contributed by the upgrading of bitumen by Alberta's oil sands refineries (AEUB 2006).

1.1.2 Environmental issues

Despite the positive effect the oil sands industry has had on crude oil availability and the Canadian economy, there are environmental issues associated with their operations. The impact of bitumen recovery on the local landscape, displacement of wildlife, emission of greenhouse gases and other pollutants, production of vast quantities of elemental sulfur and a byproduct solid waste called coke, as well as accumulation of huge volumes of process water are all major concerns from an environmental perspective. The oil sands companies have taken several initiatives to limit environmental disruption caused by their activities in Alberta's north (Syncrude 2003, 2007, Suncor 2007). Reclamation options have also been explored in preparation for the eventual shutdown of operations and lease closure, and some of these strategies have already been successfully implemented at Syncrude and Suncor. The ultimate goal asserted by both companies is to return all affected areas to a state equal to or better than their original condition (Syncrude 2003, 2007, Suncor 2007). This will include reestablishing stable, biologically self-sustaining ecosystems in all disturbed areas. In order to ensure that this is achieved, more research is needed to determine the long-term effects of potential reclamation efforts.

Water management has long been recognized as a particularly challenging aspect of the oil sands operations (Camp 1977). The following sections of this review will discuss the need for development of effective treatment options for oil sands process water (OSPW), and describe how some conventional refinery wastewater treatment methods may or may not fit into this scheme.

1.2 Athabasca oil sands operations

1.2.1 Recovery of bitumen

The viscosity of bitumen, as it occurs in Alberta oil sands, is high enough to permit mining of the ore (Schramm et al. 2000). However, overburden of muskeg, glacial till, and Cretaceous bedrock, which ranges in thickness from 50 to 825 m, must be removed before establishing mine operations (Chalaturnyk et al. 2002). As a general rule, areas with more than 75 m of overburden are not considered for surface mining (AEUB 2006). Instead, *in situ* methods for bitumen recovery are employed. These involve steam injection or combustion within the deposit to heat up the bitumen, lowering its viscosity and facilitating mobilization of the mixture to the surface via pump action (AEUB 2006). Syncrude tends to rely mostly on open-pit mining (Syncrude 2003, 2007), whereas Suncor uses both methods, with growing emphasis on *in situ* recovery (Suncor 2007). Based on existing recovery options, about 10% of the in-place bitumen is considered recoverable (AEUB 2006). However, the oil sands industry is constantly researching more effective ways to enhance the recovery potential of Alberta's oil sands deposit.

1.2.2 Extraction of bitumen

The problem of how to release bitumen from the oil-sand mixture was a major challenge faced by industry pioneers in the 1920s (McKenzie-Brown et al. 1993). Dr. Karl Clark, working in association with the Alberta Research Council, is credited with developing the fundamental hot-water extraction process that overcame this impediment and made the potential for an oil sands-based petroleum industry a reality (Clark and Pasternack 1932, McKenzie-Brown et al. 1993, Syncrude 2003). Although the method has undergone some modification, the basic principles of the Clark hot-water flotation are still employed today.

At Syncrude and Suncor, bitumen extraction involves mixing the oil sand with hot water, which causes expansion and release of air trapped in the sand particles (Schramm et al. 2000, Chalaturnyk et al. 2002). As the air bubbles are released they carry the bitumen with them as droplets, leaving the sand and other solids to settle out in a subsequent step. Sodium hydroxide (50 to 200 g/t of oil sand) is added during the

extraction to raise the pH to ~8: the optimal condition for stripping bitumen from the sand particles. Steam injection maintains a temperature of ~80 °C and aeration (~30% v/v gas) is employed to enhance flotation of the bitumen. Residence time in the extraction tumblers is variable depending on the properties of each oil sand load. The "digested" slurry is discharged onto vibrating screens to remove large solids. At this time the slurry is also sprayed with hot water to further break up remaining oil sand lumps.

After dilution with more water to enhance separation, the slurry is pumped to a large, cylindrical vessel where sand settles and bitumen floats. Residence time in the separation vessel is about 45 min. After this time, bitumen froth is recovered from the surface by skimming. Settled solids, which form a sludge-like suspension called tailings, are removed and transported to storage pits. The middle section of the vessel consists of water, suspended solids, and residual bitumen droplets that do not float. These "middlings" are drawn off and routed to a second separation vessel where more bitumen is recovered after longer residence time. Middlings from this vessel are pumped to a secondary extraction circuit consisting of more vigorous agitation and air sparging. Froth generated by these secondary efforts is combined with that from the initial extraction and sent on to upgrading processes. There are variations to the general process described here.

Of particular relevance to this thesis is the fact that naphthenic acids, which are present in bitumen (Clemente 2004), are released into the aqueous phase during caustic extraction (FTFC 1995, CONRAD 1998, Schramm et al. 2000). Naphthenic acids are discussed in detail in Section 1.3.

1.2.3 Management and general characteristics of oil sands process water

Extraction of bitumen from oil sands ore requires large volumes of water; approximately 3 m^3 of water are used to extract bitumen from each m^3 of oil sand. This water is generally referred to as oil sands process water (OSPW) (FTFC 1995, CONRAD 1998, Schramm et al. 2000). Some sources refer to OSPW as "tailings water" because of the presence of clay and other suspended particulates known as "fine tails" or "tailings." Within the oil sands industry, the terms OSPW and tailings water are used interchangeably.

The need for practical management strategies to deal with OSPW was recognized many years ago (Camp 1977), but few advances have been made in this area. In accordance with a zero-discharge policy, both Syncrude and Suncor store their OSPWs on-site in large storage ponds (FTFC 1995). The stored OSPW is continuously recycled back into the extraction process, meaning that no freshwater is currently used for this purpose (Warren Zubot, personal communication). However, freshwater is used by the oil sands companies for operating cooling towers, production of boiler feed water, and for potable water needs. For each barrel of synthetic crude oil produced, Syncrude utilizes approximately 0.3 to 0.4 m^3 of freshwater (Michael MacKinnon, personal communication). Much of this water ultimately ends up as OSPW. Storage of OSPW is necessary for tailings placement and water recycling. However, both Syncrude and Suncor will be required to implement appropriate water and land reclamation strategies prior to closure of their leases.

Syncrude is currently storing close to one billion m^3 of OSPW, with most of this volume being held in the Mildred Lake Settling Basin (MacKinnon et al. 2005). Current information regarding the storage ponds and OSPW volumes at Suncor was not available from the open literature or from Suncor personnel. Nix and Martin (1992) stated that Suncor had four major storage ponds: 1, 1A, 2, and 3. The surface areas of Ponds 1, 2, and 3 were approximately 2.4 km^2 and depth was between 2 and 10 m. The surface area of Pond 1A was about 6.4 km^2 and mean depth was similar to that of the other three ponds.

MacKinnon (1989), MacKinnon and Boerger (1986), the FTFC (1995), and Harris (2001) have published detailed descriptions of physical and chemical characteristics of Syncrude OSPW ponds, and Nix and Martin (1992) described the OSPW ponds at Suncor. The ponds are stratified, meaning that there are zones of differing composition and clarity: the tailings settle to the bottom as a bulky sludge and above this is a clarified zone, also referred to as the surface water zone or tailings water (MacKinnon and Boerger 1986, Nix and Martin 1992). Mean depth and total volume of each zone is dependent on the dimensions of the pond, concentration of solids in the original tailings slurry (from extraction), settling or dewatering rates of tailings sludge, volume of surface water being drawn off for recycle, and other environmental factors.

This review is concerned with the clarified zone of OSPW ponds, which is composed mainly of water with minor components of suspended solids, clays, and bitumen globules (MacKinnon and Boerger 1986, Nix and Martin 1992, FTFC 1995, Harris 2001). OSPW also contains low concentrations of dissolved organics (~100 mg/L), including toxic compounds like phenol, polycyclic aromatic hydrocarbons, naphthenic acids, and other priority pollutants. The concentration of ammonia in OSPW ponds tends to be substantially higher than in normal freshwater: 3.0 ± 0.5 mg/L in ponds versus 0.06 ± 0.09 mg/L in Athabasca River water (MacKinnon and Boerger 1986). Sodium, potassium, chloride, sulfate, sulfide, and bicarbonate are also found at higher concentrations in OSPW than in the local river water (MacKinnon and Boerger 1986).

The storage ponds at Syncrude and Suncor are reported to contain bacteria, phytoplankton and zooplankton, and benthic invertebrates (Harris 2001, Leung et al. 2001, 2003). However, the number of organisms belonging to higher trophic levels (eg. plants) and overall diversity are notably lower in OSPW ponds compared to reference lakes in northern Alberta (Harris 2001). These ecological differences are partly attributable to unfavorable characteristics and components of OSPW, including the presence of bitumen and other organic contaminants, high salt content, turbidity, and low dissolved oxygen. Besides these factors, one particular group of organic compounds, known as naphthenic acids, has been consistently implicated as the primary cause of OSPW toxicity (FTFC 1995, CONRAD 1998, Schramm et al. 2000, Madill et al. 2001, Harris 2001, Headley et al. 2004, Clemente and Fedorak 2005).

1.3 Naphthenic acids

Several reviews of topics related to naphthenic acids have been published recently (Headley and McMartin 2004, Quagraine et al. 2005a, b, Clemente and Fedorak 2005). These papers discuss sources, characteristics, quantification methods, as well as biodegradation of naphthenic acids. Burgeoning interest in naphthenic acids research is likely attributable to increasing environmental awareness and the realization that accumulation of these compounds poses a risk to aquatic ecosystems (Dokholyan and Magomedov 1984, MacKinnon and Boerger 1986, FTFC 1995, Leung et al. 2001, 2003). This issue is particularly relevant to the oil sands companies because the process water

from bitumen extraction is a reservoir of naphthenic acids. Because comprehensive reviews concerning naphthenic acids are available elsewhere, only details pertinent to this thesis will be given here.

1.3.1 Definition of naphthenic acids

Naphthenic acids are complex mixtures of alkyl-substituted aliphatic and alicyclic carboxylic acids (Brient et al. 1995, FTFC 1995, CONRAD 1998). This structurally diverse group of compounds is described by the chemical formula $C_nH_{2n+Z}O_2$, where n represents the number of carbon atoms and Z is a negative, even integer that corresponds to the number of hydrogen atoms lost due to ring formation. Some examples of structures that fit this description are shown in Figure 1.3. It should be noted that the carboxylic acid moiety typically occurs at the end of a substituent side-chain, rather than on the ring structure. Although only fused rings are shown in Figure 1.3, the rings can be bridged (Brient et al. 1995).

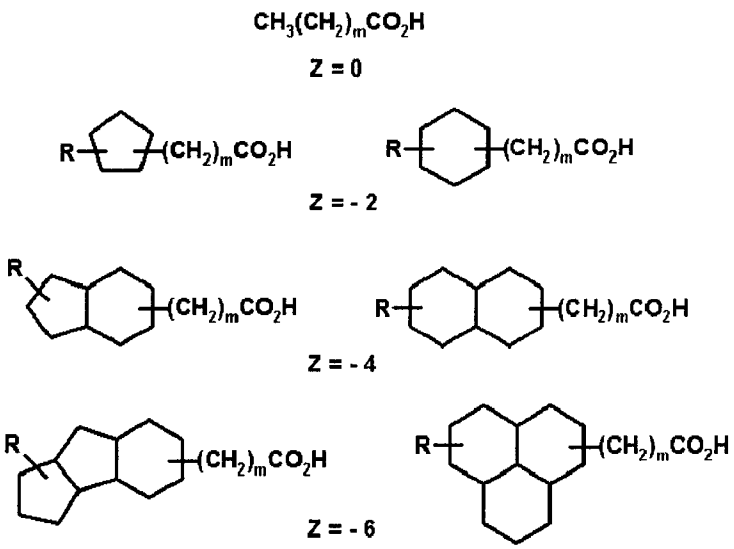

Figure 1.3 Some possible structures of naphthenic acids where R represents an alkyl substituent, m specifies the number of CH_2 units, and Z denotes the hydrogen deficiency resulting from ring formation. Naphthenic acids with $Z = 0$ are aliphatic, whereas those with $Z \leq -2$ have one or more saturated rings. Adapted from Holowenko et al. (2001).

The chemical formula given above is also applicable for fatty acids if Z is equal to zero. However, naphthenic acids can be differentiated from these biomolecules based on their highly branched alkyl groups, which are not characteristic of fatty acids (Rudzinski et al. 2002).

Commercially available naphthenic acids mixtures are usually viscous and range in color from pale golden yellow to brown or amber (Brient et al. 1995). They act as surfactants and are non-volatile, with boiling points in the range of 250 to 350 °C (Brient et al. 1995, CONRAD 1998). The chemical behavior of naphthenic acids is similar to other carboxylic acids. For example, their dissociation constants range from 10^{-5} to 10^{-6} (Brient et al. 1995), whereas acetic acid, propionic acid, and palmitic acid have dissociation constants of $10^{-4.7}$, $10^{-4.9}$, and $10^{-8.7}$, respectively (Kanicky et al. 2000). In acid form, naphthenic acids are soluble in organic solvents and oils but have low solubility in water (Brient et al. 1995). Like most salts of organic acids, sodium naphthenates are water-soluble.

1.3.2 Sources of naphthenic acids

Naphthenic acids occur naturally in petroleums throughout the world (Seifert and Teeter 1969, Seifert et al. 1969, Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Jones et al. 2001, Tomczyk et al. 2001, Qi et al. 2004, Laredo et al. 2004a) and are thought to have originated from biodegradation of petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Tomczyk et al. 2001, Watson et al. 2002). Nascimento et al. (1999) suggested that naphthenic acids are biomarkers that can provide valuable information about the age of a reservoir and the extent of biodegradation that has occurred there over time.

Naphthenic acids are present in different concentrations in crude oil depending on the geographical location of the petroleum reservoir (Brient et al. 1995, CONRAD 1998). Reported values range from 0.0015 % w/w (Egyptian crude) up to 4 % w/w (California San Joaquin Valley crude). As mentioned previously, naphthenic acids are also present in Athabasca oil sands ore (Clemente 2004). Based on the ore samples tested by Clemente, naphthenic acids content appears to be quite variable depending on the sampling site

(Table 1.2). Interestingly, naphthenic acids concentration did not appear to be related to the bitumen content of the ore (Clemente 2004).

Table 1.2 Naphthenic acids content of 12 samples of oil sands ore. Adapted from Clemente (2004).

Company	Description of sample source	mg naphthenic acids / kg oil sand
Syncrude	Aurora Basal ore, poor processing	51
	Aurora Basal ore, good processing	149
	Aurora 1 km N. face F7x	74
	Aurora 1 km N. face F8	69
	Aurora transition ore F11	504
	North mine Upper bench	317
	North mine Lower bench	304
Suncor	Elevation 830	373
	Steepbank	396
True North Energy	#4C	123
	#5	122
	#28A	131

Suncor samples contained ~380 mg naphthenic acids/kg oil sand, whereas TrueNorth Energy samples contained ~125 mg naphthenic acids/kg oil sand (Table 1.2). Samples from Syncrude sites exhibited more variability than those from Suncor and TrueNorth Energy, ranging from 51 to 504 mg naphthenic acids/kg oil sand. However, this phenomenon may simply be due to variation in the number of samples analyzed from each company.

Naphthenic acids are also found in process waters from conventional petroleum refineries and oil sands processing (Dorn 1992, Wong et al. 1996, Dzidic et al. 1998, Schramm et al. 2000, Holowenko et al. 2002, Rogers et al. 2002a). They appear in these process waters as a result of alkaline conditions used during extraction (oil sands only) and upgrading, which cause the acids to become solubilized in the aqueous phase (FTFC 1995, CONRAD 1998, Schramm et al. 2000). Clemente and Fedorak (2005) estimated that up to 100 t of naphthenic acids could be generated every day from the extraction of

bitumen at Syncrude alone. This was based on an average naphthenic acids content of 200 mg/kg oil sand (Clemente 2004) and a processing rate of about 500 000 t of oil sands per day. Despite the magnitude of this estimate, the concentration of naphthenic acids in Syncrude OSPW holding ponds is less than 100 mg/L (Holowenko et al. 2002) because the volume of water is so immense. However, even at these concentrations there is concern about toxicity and the possibility of detrimental effects to aquatic ecosystems (Dokholyan and Magomedov 1984, MacKinnon and Boerger 1986, Madill et al. 2001). This issue is discussed in more detail in Section 1.6.

1.3.3 Corrosion caused by naphthenic acids in crude oil

The presence of naphthenic acids in petroleum initially became a concern in the 1920s when it was discovered that they caused corrosion of refinery equipment made from steel alloys (Derungs 1956). Since then many efforts have been made to characterize the specific mechanism of naphthenic acids corrosion so that the problem might be circumvented (reviewed by Slavcheva et al. 1999). Evidence from recent studies suggests that many factors affect the rate and extent of naphthenic acids corrosion, including naphthenic acids concentration, structural composition of the naphthenic acids mixture (i.e. alkyl chain length and saturation), operating temperature, steel alloy components, and the velocity of throughput (Turnbull et al. 1998, Kane and Cayard 1999, Laredo et al. 2004b). The exact mechanism of the process has never been conclusively defined, but reactions involving chelation of metal ions by the carboxylate undoubtedly play a key role (Slavcheva et al. 1999).

1.3.4 Commercial naphthenic acids and their applications

Despite the problems associated with naphthenic acids in petroleum, they also have properties that make them beneficial for commercial applications (Brient et al. 1995, CONRAD 1998). For example, naphthenic acids are surfactants and can therefore be used as emulsifiers or as antifoaming agents. Metal naphthenates have the greatest number of uses, including acting as antifungals in wood preservation, lubricants, antifoaming agents in jet fuels, preservatives and flame retardants in fabrics, and adhesion promoters in tire manufacture. Naphthenic acids are also added to oil-based

paints to promote rapid drying (Brient et al. 1995). More recently, commercial naphthenic acids preparations have been used as standards in analytical methods developed for quantification of naphthenic acids in environmental water samples (see Section 1.4).

Recovery of naphthenic acids from petroleum for commercial applications can be achieved by a variety of distillation and extraction processes (Brient et al. 1995). In general, the methods involve caustic extraction of petroleum distillates obtained between 200 and 370°C. This is followed by an ethanol extraction, which removes unsaponifiable material. Finally, the extract is acidified to return the recovered naphthenic acids to their acid form.

1.4 Quantification of naphthenic acids

The complexity of naphthenic acids mixtures makes their quantification particularly difficult. Current analytical methods do not permit separation and quantification of individual naphthenic acids. Instead, the compounds are quantified as a group, usually via techniques that exploit the carboxyl moiety. Clemente and Fedorak (2005) described various naphthenic acids quantification methods in their general review of naphthenic acids. Since the information in their review is both current and comprehensive, only quantification methods that are commonly employed by the oil sands companies and their collaborative research facilities will be described here.

1.4.1 Total acid number

The naphthenic acids content in petroleum is approximated by determining the total acid number (TAN) (Brient et al., 1995, Drews, 1998, Slavcheva et al., 1999, Meredith et al. 2000). Refineries use TAN as measure of the crude oil acidity and as a means to predict its corrosive potential. Although TAN can be affected by other factors, such as sulfur content, it is a rapid and simple technique for estimating naphthenic acids concentration. The analysis involves titrating samples with KOH, using either potentiometric (ASTM D664; American Standard Test Method 2001a) or colorimetric (ASTM D974; American Standard Test Method 2001b) detection. TAN is expressed as mg KOH/g sample.

1.4.2 Fourier transform infrared spectroscopy and high performance liquid chromatography

The usefulness of TAN is somewhat lessened when considering naphthenic acids in aqueous conditions (like refinery wastewaters) because it is not a true measure of naphthenic acids concentration. Two superior quantification methods are Fourier transform infrared (FT-IR) spectroscopy (Jivraj et al. 1995) and high performance liquid chromatography (HPLC) of derivatized naphthenic acids (Clemente et al. 2003, Yen et al. 2004).

The FT-IR method, developed by Jivraj et al. (1995), is the oil sands industry standard for determination of naphthenic acids. It involves acidifying a clarified sample to around pH 2.5 and then extracting the protonated acids into methylene chloride. The extraction solvent is evaporated and the naphthenic acids residue is dissolved in a known volume of methylene chloride. FT-IR spectroscopy is then used to measure the absorbance of samples at wave numbers of 1706 and 1743 cm^{-1}: the stretching frequencies of carbonyls. Naphthenic acids concentration is determined by comparing the absorbance values to a calibration curve generated from the analysis of standards.

A review paper by Miwa (2000) described the development of an HPLC method for determination of carboxylic acids as their 2-nitrophenyl hydrazides (2-NPHs). Miwa and collaborators applied this method primarily for quantification of fatty acids in biological materials and foods. Clemente et al. (2003) adapted the protocol of Miwa (2000) for quantifying naphthenic acids in aqueous samples. Their derivatization method involved mixing an alkaline solution of naphthenic acids with acidic 2-NPH in the presence of a catalyst: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). After incubation at 60°C for 20 min, potassium hydroxide (in methanol:water, 80:20) was added to bring up the pH and quench the reaction. This mixture was incubated for another 15 min at 60°C and then cooled in ice-water. The derivatized naphthenic acids were then analyzed by HPLC with detection by a UV-Visible diode array detector set at 400 nm. The mobile phase was a gradient, from 70:30 methanol:water with phosphoric acid to 100% methanol with phosphoric acid. Derivatized naphthenic acids eluted from the column as an unresolved hump and the area under this hump was then

compared to a calibration curve of derivatized standards to determine the naphthenic acids concentration. The minimum detection limit of this method was ~15 mg/L.

Yen et al. (2004) improved the derivatization and HPLC methods such that the minimum detection limit became ~5 mg/L. This was achieved by changing the final concentrations of some reagents, increasing the total reaction volume, and simplifying the composition of the mobile phase to include only methanol and water. Further improvements to the HPLC analysis program have since been made to ensure consistent integration of only the naphthenic acids hump (Marsh, Scott, and Fedorak, unpublished results).

Yen et al. (2004) compared the FT-IR and HPLC methods for determination of naphthenic acids in oil sands tailings waters. From their analyses of 58 samples, there was a strong correlation between the measurements ($P<0.01$) and the FT-IR results were on average 11% higher than those obtained by their HPLC method. Despite the superior sensitivity of the FT-IR method, it has the disadvantages of requiring larger sample volumes and substantially greater preparation time than the HPLC method. The researchers also noted that both methods are subject to certain biases and limitations. The FT-IR method assumes that only naphthenic acids are extracted into the methylene chloride and that they are extracted quantitatively. The HPLC method assumes that the reaction of carboxylic acids with 2-NPH is complete. Divergence from any of these assumptions would result in erroneous quantification. The HPLC method also assumes that only naphthenic acids react with the 2-NPH but aldehydes and ketones have been shown to react with this particular derivatizing agent (Peters et al. 2004). Therefore, the presence of aldehydes or ketones would also impact quantification by HPLC. For these reasons it is difficult to ascertain which method is truly more accurate. Furthermore, both methods assume that a particular naphthenic acids preparation (Kodak Naphthenic Acids, The Eastman Kodak Company, Rochester, NY) is an appropriate standard for comparison to samples containing oil sands naphthenic acids. This assumption has never been proven accurate. It would be interesting to generate calibration curves for several commercially available preparations and examine what effect they have on the apparent concentration of naphthenic acids in oil sands water samples.

1.4.3 Alternative quantification methods

Other methods of quantification include IR spectroscopy of fluoroesters (Yu and Green 1989), gas chromatography-negative-ion electrospray mass-spectrometry (Headley et al. 2004), and nonaqueous ion exchange solid-phase extraction followed by gas chromatography-flame ionization detection (Jones et al. 2001). Costliness, lengthy preparation times, and lack of availability of specialized equipment have limited widespread use of these alternative methods.

1.5 Characterization of naphthenic acids by gas chromatography-mass spectrometry

The complexity of naphthenic acids mixtures has precluded development of a method for resolving individual naphthenic acids, although electrospray ionization high-field asymmetric waveform ion mobility spectrometry (ESI-FAIMS) has shown some promise (Gabryelski and Froese 2003). Unfortunately, the specialized instruments involved in that type of MS are not readily available to most researchers. Various alternative methods involving mass spectrometry (MS) have been developed for qualitative characterization of naphthenic acids profiles or fingerprints (reviewed by Clemente and Fedorak 2005). Typically, MS data are used to classify naphthenic acids into groups according to their carbon number and Z value (St. John et al. 1998, Holowenko et al. 2002), although other presentation methods have been used (reviewed by Clemente and Fedorak 2005).

Originally developed for quality control of commercial naphthenic acids, the type of information provided by MS analyses has since become useful for explaining or predicting differences in the behavior of naphthenic acids mixtures. For example, Holowenko et al. (2002) observed greater toxicity in oil sands water samples that contained a high proportion of naphthenic acids with carbon numbers ≤ 21. Qualitative MS data have also been used to show changes in naphthenic acids mixtures during biodegradation (Clemente et al. 2004), and may eventually prove useful in predicting the biodegradability of naphthenic acids from different sources.

Although several MS methods have been developed for naphthenic acids characterization, the majority require expensive, specialized equipment that is not readily available to most laboratories (Clemente and Fedorak 2005). To circumvent this problem, St. John et al. (1998) developed a method for analyzing the *tert*-butyldimethylsilyl derivatives of naphthenic acids using gas chromatography coupled with electron impact mass spectrometry (GC-EIMS). This type of equipment is considerably more common and, therefore, accessible to a broader base of researchers.

The method of St. John et al. (1998) requires extraction of naphthenic acids into methylene chloride prior to derivatization with N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). MTBSTFA was selected as the derivatizing agent because there is minimal fragmentation of the major ion formed in the electron impact ionization step. This ion was denoted $[M + 57]^+$, where M is the molecular mass of the underivatized naphthenic acids and 57 is the mass contributed by the dimethylsilyl (C_2H_5Si) group. As with the HPLC method described above, derivatized naphthenic acids were not resolved by the gas chromatography (GC) step, causing the total ion current chromatogram from the MS to appear as hump. The average mass spectrum of the hump was determined and the $[M + 57]^+$ ions were then assigned to carbon number and Z value groups. Using 1-methyl-1-cyclohexanecarboxylic acid and decanoic acid as model naphthenic acids, St. John et al. (1998) showed that their method yielded results (ion profiles) comparable to other accepted MS methods. Using commercial naphthenic acids preparations, they also showed that the method could be used to determine the profile of highly complex mixtures.

Holowenko et al. (2002) used the GC-MS method of St. John et al. (1998) to characterize naphthenic acids in OSPW. In this case, average mass spectrum data were exported into a matrix created using Microsoft© Excel. The matrix excluded the majority of *m/z* (mass to charge ratio) values that were not plausible naphthenic acids structures. This was an improvement over the method of St. John et al. (1998) who did not attempt to correct for non-naphthenic acids. Holowenko et al. (2002) presented their data as three-dimensional plots of relative intensity (from the average mass spectrum of the total ion current) as a function of carbon number and Z value. This presentation provides a fingerprint of the naphthenic acids profile (Figure 1.4).

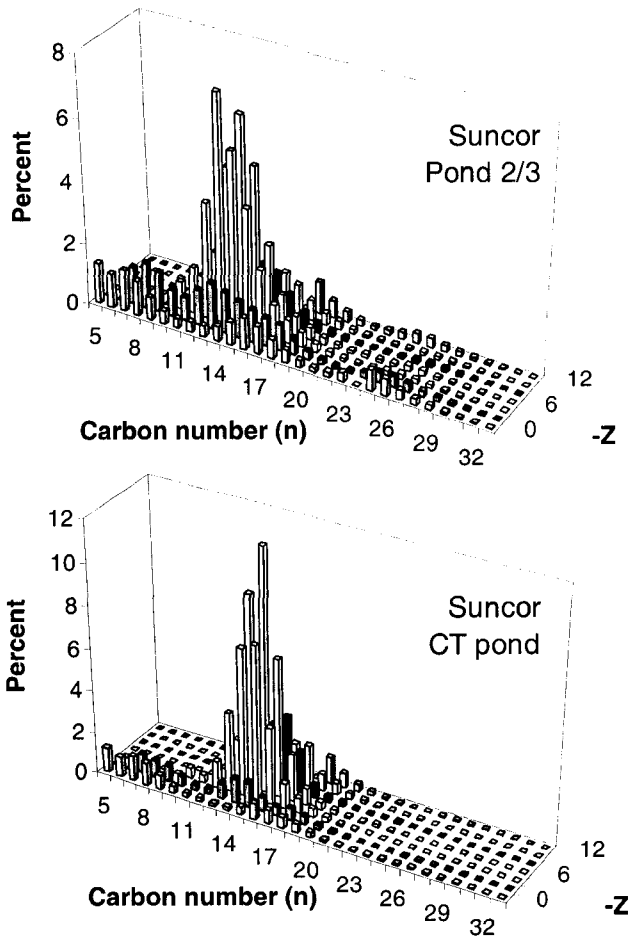

Figure 1.4 Examples of the type of three-dimensional plots used to illustrate the naphthenic acid fingerprint or profile of water samples. Data were generated from GC-MS analyses of naphthenic acids that were extracted from Suncor Pond 2/3 and the Suncor Consolidated Tailings (CT) pond and were plotted using the method of Holowenko et al. (2002). The bars represent the percentage of ions in the mixture that have been assigned to a given carbon number of a given Z family, on the basis on *m/z* ratio.[1] The sum of all the bars in each panel equals 100%.

Clemente and Fedorak (2004) described limitations of using GC-MS analyses to characterize naphthenic acids. Their evaluation of the method was based on analyses of six model naphthenic acids and five extracts of naphthenic acids from oil sand ore samples and OSPW. They determined that naphthenic acids profiles generated by this

[1] As discussed in Chapter 6, it is now known that GC-MS analyses of oil sands naphthenic acids (i.e. extracted from OSPW) do not generate accurate profiles due to the misassignment of derivatized hydroxylated naphthenic acids to high carbon numbers (n-values) and incorrect Z-values.

technique, and the graphing method used by Holowenko et al. (2002), show a falsely high abundance of low molecular-weight compounds due to secondary fragmentation of the $[M + 57]^+$ ion. However, Clemente and Fedorak (2004) conceded that the GC-MS method is still useful for qualitative analyses of naphthenic acids, provided that researchers acknowledge its limitations when interpreting experimental data.

1.6 Biological effects of naphthenic acids and oil sands tailings water

Understanding the potential biological effects of naphthenic acids is essential for determining what precautions should be taken in dealing with products and waters (eg. refinery wastewater, OSPW) that contain naphthenic acids. Unfortunately, this aspect of their character has not been well studied. There is limited information about the consequences of exposure to commercial naphthenic acids on mammals (using rat and mouse models) (Khanna et al. 1971, Pennisi and Lynch 1977) and fish (Dokholyan and Magomedov 1984, Dorn 1992). Studies with plants have generated conflicting results depending on test species, naphthenic acids source, and methodology (Wort and Patel 1970, Wort et al. 1973, Kamaluddin and Zwiazek 2002, Apostol et al. 2004).

Studies focusing on the biological effects of naphthenic acids from oil sands tailings waters are also scarce; only one study directly investigated exposure to oil sands naphthenic acids, using solutions of an extract of OSPW (Rogers et al. 2002b). Greater interest lies in evaluating large-scale reclamation options, which requires characterizing the biological effects of whole OSPW, rather than just naphthenic acids (MacKinnon and Boerger 1986, Nix and Martin 1992, van den Heuvel et al. 1999a, b, 2000, Crowe et al. 2001, Harris 2001, Leung et al. 2001, 2003, Farrell et al. 2004). Nevertheless, general consensus within the field is that the toxic character of OSPW is almost entirely attributable to naphthenic acids (FTFC 1995, CONRAD 1998, Schramm et al. 2000, Madill et al. 2001, Headley et al. 2004).

1.6.1 Studies with mammals

Studies of the physiological effects and toxicity of naphthenic acids on mammals are surprisingly scarce considering the number of commercial products that contain these compounds (see Brient et al. 1995). The reported lethal dose for humans is 1 L (Rockhold

1955) but this value was obtained using a particular naphthenic acids preparation and may not be valid for all naphthenic acids mixtures. Furthermore, it was not clear from the publication how this value was determined. Information on the effects of chronic human exposure to naphthenic acids could not be found in the literature.

Khanna et al. (1971) investigated the effects of chronic exposure to potassium naphthenates using rats. Forty male rats were used in the study: 20 served as controls and 20 were used as test subjects. Test rats were injected intraperitoneally with 1-mL doses of 1% potassium naphthenates every second day for 21 d. The researchers observed that weight gain and liver mass was similar for control and test groups. However, there were significant increases in red blood cell counts ($P<0.01$), keto-steroid levels ($P<0.05$), and liver glycogen levels ($P<0.01$) in treated rats compared to controls. Most notably, histopathology showed a substantial increase in cell growth within the testes of rats exposed to potassium naphthenates. This result was considered evidence that potassium naphthenates may mimic hormones. From their findings, Khanna et al. (1971) suggested that exposure to naphthenic acids might influence maturation and reproductive potential of mammals.

Acute and sub-acute toxicity of naphthenic acids has also been studied in mice (Pennisi and Lynch 1977). In that investigation, naphthenic acids were administered both orally and intraperitoneally to young, white male mice. The total number of mice tested was not specified. In the acute exposure tests, observed effects included depression of the central nervous system (without analgesia or loss of the corneal reflex), corneal eye opacity, dry-mouth, convulsions, diarrhea, and asphyxia. The LD_{50} (LD = lethal dose), which is defined as the amount or concentration of a substance that will result in 50% mortality of test subjects, was determined for both methods of naphthenic acids delivery. LD_{50} for oral administration was 3550 mg/kg body weight and 860 mg/kg body weight for intraperitoneal administration. The discrepancy of these values illustrates the importance of considering different modes of delivery when testing the biological effects of chemicals on mammals. Moreover, the fact that the LD_{50} for oral delivery was higher suggests that naphthenic acids are not readily absorbed by the gut. It is arguable that knowing the potential consequences of naphthenic acids consumption is more relevant in cases like that of the oil sands, where exposure of wildlife is the primary concern,

animals are most likely to encounter chemicals through their food or water. Investigation of sub-acute (chronic) exposure is important for the same reason. Pennisi and Lynch (1977) examined this by administering oral (1000 mg/kg body weight) and intraperitoneal (250 mg/kg body weight) doses of naphthenic acids daily for a total of 30 d. The observed effects were similar to those seen with acute exposure but fewer mice were affected in the chronic tests.

More recently, Rogers et al. (2002b) conducted acute and sub-chronic toxicity tests on male and female Wistar rats to determine the effects of exposure to oil sands naphthenic acids. An extract from Mildred Lake Settling Basin (Syncrude) served as the source of naphthenic acids. For the acute test, dosages corresponding to 0.5, 5, and 50 times the predicted "worst-case" for environmental exposure (i.e. 3, 30, and 300 mg naphthenic acids per kg body weight, respectively), were given orally to previously starved (12 h fast) rats. Control rats were dosed with tap water. The test group that received the highest dose displayed lethargy and mild ataxia for 1 or 2 h following treatment. They also consumed less food over the first 4 d, after which time their consumption returned to about the same level as control rats. Except for these differences, the behavior of rats in the test groups was virtually identical to that of control rats. There were, however, some significant differences ($P<0.05$) between test and control groups when organ weights were measured after euthanization. Specifically, female rats that received the highest dose had heavier ovaries and spleens, and male rats in the high-dose group had heavier testes and hearts. Histological analyses of select organs from high-dosed rats showed hepatic lesions, mild to moderate eosinophilic pericholangitis, mild myocardial necrosis with periarteriolar fibrosis, and some incidences of cerebral hemorrhaging. The enlargement of rat reproductive organs seems to indicate that naphthenic acids have a hormonal effect, as proposed by Khanna et al. (1971). However, Rogers et al. (2002b) suggested instead that naphthenic acids might be targeted to the liver where induction of detoxifying enzymes inadvertently causes the observed physiological responses. More research is needed to determine which explanation is correct.

To test the effects of sub-chronic exposure to the same extract of oil sands naphthenic acids, female mice were dosed 5 d per week for 90 d with 0.6, 6, and 60

mg/kg body weight naphthenic acids per day. These dosages corresponded to 0.1, 1, and 10 times the predicted "worst-case" for environmental exposure (i.e., Rogers et al. 2002b). As before, oral delivery was used and control rats were dosed with tap water. A number of rats from all groups (including controls) experienced seizures when doses were being administered. One rat from the high-dose group died as a result but all others recovered rapidly. Rats in the high-dose group tended to consume more water and less food than rats in the lower-dose test groups, whose consumption patterns resembled those of the control group. Average body weight of high-dosed rats was significantly ($P<0.05$) lower than rats in the other groups and significant differences ($P<0.05$) were found when liver, kidney, and brain weights of high-dosed rats were compared to control rats. Most notably, the livers of high-dosed rats were 36% heavier than those of control rats. Livers of test rats also showed dose-related histological changes such as glycogen accumulation, but, overall, organ damage and histological effects were less than those observed in the acute exposure experiment.

Based on the results obtained in the studies using rat and mouse models, naphthenic acids were found to have low toxicity in acute and chronic testing. However, as mentioned by Rogers et al. (2002b), the exact chemical composition of naphthenic acids mixtures varies with the source, meaning that the toxicity test results described here may not be accurate for making broad predictions regarding naphthenic acids toxicity. Furthermore, the exposure of mammals to naphthenic acids is still a concern due to the potential for causing adverse health effects such as those discussed above.

1.6.2 Studies with fish

Dokholyan and Magomedov (1984) assessed the effects of sodium naphthenates on several species of fish native to the Caspian Sea. These included juvenile and adult sturgeon (*Acipenser gueldenstaedti*), Caspian round goby (*Neogobius melanostomus affinus*), roach (*Rutilus rutilus casicus*), kutum (*Rutilus frisii kutum*), and chum salmon (*Oncorhynchus keta*). Survival and various hematological and biochemical responses were monitored in fish exposed to sodium naphthenate concentrations ranging from 0.05 to 100 mg/L. A difference in mortality was observed among fish of the same species depending on their age, with older fish displaying greater resistance to the effects of

exposure to sodium naphthenates. Species-specific responses were also evident: adult roach and Caspian round goby were the most resistant to exposure to sodium naphthenates (LD_{50}= 75 mg/L). Biochemical and hematological analyses showed irregularity in glycogen and leukocyte production. Chronic exposure to sodium naphthenates was also tested with these fish species over a period of 60 d at sodium naphthenate concentrations ranging from 0.05 to 25 mg/L. Similar age- and species-dependent trends were observed. Overall, the results of this study led Dokholyan and Magomedov (1984) to suggest the maximum permissible limit of sodium naphthenates be kept at 0.15 mg/L in order to ensure the safety of fish and other aquatic species. The average concentration of naphthenic acids in oil sands tailings waters is well above this value (Schramm et al. 2000, CONRAD 1998, Holowenko et al. 2002).

Dorn (1992) conducted 96-h static bioassays with three-spine stickleback (*Gasterosteus aculeatus*) using solutions of a commercial naphthenic acids preparation (Eastman Chemical Company, Kingsport, TN). They observed 100% fish mortality with naphthenic acids concentrations of about 10 mg/L and established the LD_{50} of this preparation to be approximately 5 mg/L, based on a 96-h exposure.

Nix and Martin (1992) documented a decrease in trout (*Oncorhynchus mykiss*) egg hatchability and hatchling survival as a result of exposure to Suncor tailings water. Mean body weight of the hatchlings was also significantly ($P<0.01$) less than that of hatchlings maintained in control water from Ruth Lake, AB. These effects were attributed to the combined toxicity of naphthenic acids, phenolic compounds, ammonia, and metals present in the tailings water.

Three publications (van den Heuvel et al. 1999a, b, 2000) and a report published by Harris (2001) described studies conducted by researchers at the University of Waterloo on the health of adult yellow perch (*Perca flavescens*) and growth and development of their embryos when exposed to Syncrude OSPW. The adult fish were stocked in an experimental pond at Syncrude and comparisons were made between these fish and fish in reference lakes. Some signs of chronic stress, such as elevated levels of liver detoxifying enzymes, were observed in the perch exposed to oil sands chemicals (hereafter referred to as experimental perch). The researchers also observed changes in the gill structure of experimental perch, thought to aid in preventing the loss of ions from

body tissues. An increased incidence of bacterial and viral infections suggested that immune system depression was occurring in the experimental perch population. Interestingly, the researchers found evidence that the fish were taking up and metabolizing some oil sands chemicals. However, they were not able to determine whether naphthenic acids were included in this group because there was no standard method for detecting or quantifying naphthenic acids in tissues. Overall, the study showed that adult perch were able to survive and spawn in a pond containing OSPW. In fact, spawning rates were actually higher in the experimental pond than in reference lakes. Exposure to OSPW had a much less positive effect on perch embryos (Harris 2001). Teratogenic effects, including deformities of the eyes, head, spine, and tail, were observed in embryos exposed to different dilutions of OSPW. Naphthenic acids were thought to be the major cause of these phenomena. To confirm this suspicion, solutions of an unspecified commercial naphthenic acids preparation were also tested for their effect on perch embryos and larvae. As expected, the same teratogenic effects were observed, even at concentrations of only 2 mg/L (much lower than naphthenic acids concentrations in OSPW). Japanese medaka (*Oryzias latipes*), a more robust species of fish commonly used in laboratory studies, displayed the same embryological malformations (to a slightly lesser degree) when exposed to the commercial naphthenic acids solutions. In summary, the research conducted by University of Waterloo personnel suggested that, although adult perch were able to survive in OSPW, there might be difficulty in producing subsequent generations of healthy, viable perch. A long-term study could answer this question.

Farrell et al. (2004) examined the effects of exposure to two OSPW samples from Suncor on various characteristics of fathead minnows (*Pimephales promelas*). They found that fish mortality was 100% within 28 d of exposure to the Suncor process waters, whereas no fish died over the 28-d period in a reference wetland. Fathead minnows that survived the initial 96 h of exposure to OSPW had suppressed lymphocyte counts and elevated red blood cell counts compared to fish from the reference wetland. Fish exposed to the OSPW also displayed evidence of gill cellular hyperplasia and hypertrophy, which are common responses to toxicants and irritants. The results reported by Farrell et al.

(2004) indicated that Suncor OSPW could not sustain fathead minnow populations without treatment to mitigate toxicity.

1.6.3 Studies with plants

Wort and Patel (1970) studied the effects of exposing plants to Eastman potassium naphthenates and surrogate naphthenic acids (pure compounds with naphthenic acids-like structures). Plant species included bush bean (*Phaseolus vulgaris*), wheat (*Triticum aestivum*), maize (*Zea mays*), sugar beet (*Beta vulgaris*), and radish (*Raphanus sativus*). A concentrated solution of potassium naphthenates (11.5 g/L) was sprayed onto the leaves of 14-d old plants and this caused an 8 to 12% increase in the growth of foliage. Other species-specific responses were observed, including increases in the fresh weight of radish taproots and green pods on bush bean plants. It should be noted that the naphthenic acids concentration tested by Wort and Patel (1970) was about 100-fold higher than the concentration of oil sands naphthenic acids in OSPW (<0.1 g/L) (Schramm et al. 2000, CONRAD 1998, Holowenko et al. 2002). Of three surrogate naphthenic acids tested, only cyclohexanecarboxylic acid enhanced the growth and development of all species tested. This was determined by measuring a variety of species-appropriate factors such as foliage, fresh weight of taproots, and pod production.

In a later study, Wort et al. (1973) attempted to find the mechanism by which potassium naphthenates were stimulating plant growth and development. This time they focused their research on bush bean plants but expanded their evaluation to include several indicators of cellular activity. They monitored DNA and RNA synthesis, intracellular concentrations of protein and free amino acids, and activity of enzymes involved in metabolism. They also used a lower concentration (4.6 g/L) of potassium naphthenates than Wort and Patel (1970). After spraying the plants with this solution, measurements were made at weekly intervals for a total of 3 weeks. As expected, plants treated with potassium naphthenates produced more green pods and pods of greater weight than control plants. Treated plants also had higher concentrations of RNA and protein, as well as enhanced activity of enzymes involved in nitrogen metabolism. Moreover, there were differences in the amino acid compositions of total proteins harvested from treated plants compared to controls. Specifically, arginine and lysine

levels were lower in proteins from treated plants, whereas glutamic acid, serine, and proline levels were higher. Taken together, these results suggested that potassium naphthenates were functioning at the metabolic level.

Contrary to the stimulatory effects observed by Wort and Patel (1970) and Wort et al. (1973), more recent investigations showed inhibitory effects in aspen (*Populus tremuloides*) (Kamaluddin and Zwiazek 2002) and jack pine (*Pinus banksiana*) (Apostol et al. 2004) exposed to Acros Organics sodium naphthenates. Decreases in leaf growth, photosynthetic activity, root and leaf hydraulic conductivity, root respiration, and leaf chlorophyll concentrations occurred when aspen seedlings were grown in medium containing 75, 150, or 300 mg sodium naphthenates/L (Kamaluddin and Zwiazek 2002). Jack pine seedlings were grown in medium with 150 mg sodium naphthenates/L and 45 mM NaCl, conditions thought to be similar to what plants would experience if grown in regions affected by the oil sands operations (Apostol et al. 2004). This level of exposure caused a reduction in the fresh weight of shoots and inhibition of stomatal conductance, root hydraulic conductance, and root respiration. Furthermore, electrolyte leakage from needles and roots was exacerbated in plants exposed to sodium naphthenates + NaCl compared to plants treated with NaCl alone. Although these results were drastically different from those of earlier studies (Wort and Patel 1970, Wort et al. 1973), the test species, naphthenic acids preparation, naphthenic acids concentration, and manner of exposure were also different. It is very possible that naphthenic acids could have variable, species-dependent effects on plants.

One study looked at the effects of exposure to OSPW on some plant species native to the wetlands of Northern Alberta (Crowe et al. 2001). The researchers compared cattail (*Typha latifolia*) and alsike clover (*Trifolium hybridum*) found on the Suncor site to specimens of the same species obtained from an off-site, reference wetland. Analysis of the waters showed that those from Suncor were contaminated with naphthenic acids and had higher ion concentrations than water from the reference wetland. Based on the fact that cattail and clover were found growing in or near these waters, it was evident that the plants could at least tolerate short-term exposure to oil sands effluents containing naphthenic acids. To further investigate this, laboratory microcosms were constructed using sediments and waters from several wetland sites on the Suncor lease and from the

reference wetland. The laboratory study enabled the researchers to control temperature, light exposure, and precipitation, while taking measurements of apparent photosynthesis and cellular levels of ribulose-1,5-bisphosphate carboxylase (RuBisCo), dehydrin-related proteins, and protein disulfide isomerase (PDI). RuBisCo is directly involved in carbon fixation and, therefore, depressed levels or lowered activity of this enzyme indicates osmotic stress. Conversely, osmotic stress causes upregulation of dehydrin-related proteins and PDIs (involved in protein folding). Apparent photosynthesis was consistently higher in plants from the Suncor wetlands compared to reference plants. The researchers did not think that this effect was attributable to the presence of naphthenic acids, but, rather, that it was an adaptive response to the high ionic strength of the waters. RuBisCo, dehydrin-related protein, and PDI concentrations were similar in all plants of the same species. However, the profile of dehydrin-related proteins was more diverse in cattail from the Suncor sites. This result was also attributed to osmotic stress arising from ion concentrations rather than the presence of naphthenic acids. Overall, this study actually did little to enhance the understanding of how naphthenic acids affect plant species, but it did provide data illustrating that some plants can survive in areas contaminated by OSPW.

1.6.4 Studies with zooplankton and phytoplankton

Zooplankton and phytoplankton are essential components of stable, self-sustaining aquatic ecosystems. Thus, understanding how these organisms respond to OSPW is no less important than the studies conducted on higher organisms.

To this end, researchers from the University of Waterloo designed a set of experiments that enabled them to study the effects of Syncrude OSPW on zooplankton and phytoplankton communities (Harris 2001, Leung et al. 2001, 2003). Several environmental factors, including the presence of predators, initially precluded their ability to correlate any observed differences with the composition of the tailings water (i.e. ion and naphthenic acids concentrations). However, by setting up a system of enclosures (mesocosms) they were able to circumvent most of these problems. The mesocosms were filled with OSPW that was filtered to remove all microbiota, including native bacteria, zooplankton, and phytoplankton. Samples of phytoplankton or zooplankton from a

reference lake were then added such that the initial number and types of organisms were known. The mesocosms were then submerged in OSPW ponds and assessed for various parameters over the following weeks. The researchers found that the total biomass of zooplankton remained lower in OSPW ponds than in the reference lake water. Furthermore, analysis of community structures showed several differences between test and reference groups. Most notable was the low proportion of rotifers (one type of zooplankton) in the OSPW. These results were statistically (Canonical Correspondence Analysis) associated with naphthenic acids and ion concentrations, as well as sediment suspensions. The biomass of phytoplankton also remained lower in OSPW than in the reference lake water. Community structure was substantially different in each water source, with the reference lake being dominated by cyanophyta and OSPW being considerably more diverse and dominated by chlorophyta. As with the zooplankton study, these community effects were statistically (Canonical Correspondence Analysis) correlated with naphthenic acids and ion concentrations (Leung et al. 2003).

Exposure to OSPW clearly has a detrimental affect on zooplankton and phytoplankton communities. This is a concern at the ecosystem level because there are many species that feed on zooplankton and phytoplankton. More research would have to be conducted before any conclusions could be drawn regarding the broader ecological implications of exposure to OSPW.

1.7 Petroleum refinery wastewater treatment

Petroleum refineries are obligated to ensure that all wastewater generated during processing is sufficiently treated to meet applicable regulatory standards for discharge. This includes removal of chemical contaminants and solids that may have a negative impact on receiving waters. There are industry standards for wastewater treatment, but most refineries have individually-tailored treatment protocols that allow them to meet their specific needs and environmental objectives. Reviewing all of these methods is beyond the scope of this thesis. Moreover, much of the information on refinery wastewater treatment exists in proprietary reports and is, therefore, not available to the public. Based on information that can be obtained from the open literature, petroleum

refinery wastewater treatment involves a combination of various physical, chemical, and microbiological processes.

1.7.1 Physical and chemical processes

Physical and chemical processes will be considered together because they are often applied in combination. Unless otherwise indicated, the following information is a synopsis of details from papers by MacKinnon and Boerger (1986), Baker and Dold (1992), Chin (1994), and Chalaturnyk et al. (2002), a wastewater engineering handbook (Stephenson and Blackburn 1998), and two wastewater engineering textbooks, (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003).

Typically, the first target of petroleum refinery wastewater treatment is recovery of any oil associated with the water. This is achieved in an oil-water separation unit, commonly referred to as an API separator because the original design was put forth by the American Petroleum Institute (API). Oil and grease is skimmed from the surface of the water and sent back to refining processes. Sometimes air is bubbled through the wastewater to draw up smaller, finely dispersed oil droplets. This is referred to as air flotation. In the oil sands industry, air sparging and flotation is carried out as part of the secondary extraction of bitumen (Schramm et al. 2000).

Removal of settleable solids is usually achieved by gravity settling in clarifier tanks, although filtration and centrifugation can also be used for smaller volumes of wastewater. Chemical additives, such as lime, alum, gypsum, and polyelectrolyte, can enhance the rate and extent of settling by promoting coagulation and flocculation of suspended solids. This method is also used to improve the dewatering efficiency of oil sands tailings, which contain fines and clay particulates.

Metals and other inorganic ions are precipitated out of wastewater using appropriate chemical additives. Volatile organic compounds are removed by adsorption techniques, typically using activated carbon, or by gas stripping. Dissolved organic carbon (DOC) can be removed by adsorption techniques, chemical oxidation, or microbiological processes.

Adsorption, using granular activated carbon (GAC), has been pilot-scale tested for removing naphthenic acids from a biotreated refinery wastewater (Wong et al. 1996). The

results of that study showed the disappearance of naphthenic acids post-GAC based on analysis of the effluent by fast ion bombardment MS. Despite this success, the open literature contains no further reports of GAC being used for the removal of naphthenic acids. Therefore, it is not known whether this treatment method was ever implemented.

1.7.2 Microbiological processes and bioremediation

Microorganisms often play a significant role in the treatment of wastewaters due to their proficiency in degrading organic compounds as part of their normal metabolic activities. In engineered treatment systems, biodegradation is referred as a biological process (Stephenson and Blackburn 1998, Metcalf & Eddy, Inc. 2003), although it is actually *micro*biological (i.e. involving bacterial processes). When microbiological processes are exploited *in situ*, such as at the site of an oil spill or in contaminated groundwater, it is called bioremediation (Bouwer and Zehnder 1993). In some cases biostimulation (addition of nutrients) or bioaugmentation (addition of bacteria) techniques are employed to enhance the effectiveness of bioremediation (Trindade et al. 2005).

One type of microbiological treatment commonly used in petroleum wastewater treatment is the aerated lagoon (Stephenson and Blackburn 1998). Basically, wastewater is discharged into a shallow containment pond and aeration is employed to stimulate aerobic microbial transformations of the organic contaminants. The success of aerated lagoons is limited by several factors, including mixing efficiency (also described as bioavailability of substrate), temperature, pH, and the concentrations of dissolved oxygen and nutrients (e.g. N and P) (Metcalf & Eddy, Inc. 2003). Using lagoons also introduces the potential risk of groundwater contamination due to seepage or overflow if containment dykes or liners become compromised.

Engineered wastewater treatment systems that exploit microbiological processes are called biological reactors or bioreactors for short. Suspended growth and attached growth bioreactors are the two main types used in industrial wastewater treatment (Stephenson and Blackburn 1998, Metcalf & Eddy, Inc. 2003). Application of microbiological processes for petroleum wastewater treatment has been documented (Baker and Dold 1992, Tyagi et al. 1993, Sarathy et al. 2002, Lee et al. 2004).

The basic principle of suspended growth bioreactors is to facilitate microbial contact with substrate by maintaining them as a suspension through constant mixing or using hydraulic gradients (Stephenson and Blackburn 1998, Metcalf & Eddy, Inc. 2003). There are three versions of this type of bioreactor: the batch reactor, the continuous-flow reactor, and the activated sludge process.

Batch reactors, as their name suggests, are used to treat batches of wastewater. They are aerated tanks that contain biomass sludge previously acclimated to the wastewater components. Wastewater is added to the batch reactor and is incubated with the microorganisms under conditions of high aeration and mixing. After a suitable time (i.e. such that no further biodegradation can occur), aeration and mixing are discontinued and the suspension is left to settle. The liquid portion is discharged from the reactor and excess sludge is removed, leaving a sufficient quantity in place to treat the next batch of wastewater.

Conversely, continuous-flow reactors, as their name suggests, operate continuously, meaning that wastewater is constantly flowing through them versus being contained for a time and then released. The amount of biomass is kept constant by dilution. Continuous-flow reactors may allow some amount of substrate to make it through without being degraded. Furthermore, biomass is also carried in the effluent and must be removed in a subsequent step prior to discharge.

Activated sludge processes are the most common suspended growth reactors. They consist of a batch-like reactor followed by a separation tank. Biomass (sludge) is recovered from the separation tank and recycled to the reactor in order to maintain a high ratio of microorganisms to substrate. Treatment efficiency is higher under these conditions, but activated sludge processes have the disadvantage of requiring extensive controls and monitoring systems for optimal performance.

Attached growth bioreactors are designed based on the principles of biofilm formation, which involves microbial attachment to a solid medium (Stephenson and Blackburn 1998, Metcalf & Eddy, Inc. 2003). Wastewater is passed over "attached" bacteria and the organic constituents are aerobically biodegraded. Because it is possible to maintain an extremely high density of microorganisms, attached growth systems are more efficient at handling wastewaters with high concentrations of organic contaminants

than suspended growth reactors. They also have the advantage of establishing low specific growth rates, reducing the amount of biomass accumulation that must be disposed. Fixed bed reactors, plug flow reactors, and fluidized bed reactors are all variations of the attached growth process (Stephenson and Blackburn 1998, Metcalf & Eddy, Inc. 2003). Fixed beds are just the basic system described above. Plug flow reactors are similar but allow the wastewater to flow through the media as well as over it. Rocks, plastic sheets, and redwood slats are typical solid supports used in these systems. Examples of plug flow reactors are the trickling filter, biological activated filter, and multistage rotating biological contactor. Fluidized beds use particulate media, such as sand, coal, activated carbon, ion-exchange beads, and metal oxides, which become fluidized or suspended by the directional force of the wastewater. This limited mixing improves the efficiency of microbial contact with substrate, thereby enhancing biodegradation.

The wide variety of wastewater treatment designs involving microbiological processes is indicative of how the original concept has evolved to meet the diverse and distinct needs of many wastewater-producing industries. If microbiological processes were to be applied for treatment of OSPW, a suspended growth bioreactor would likely be most appropriate because the organic contaminants, namely naphthenic acids, are present at relatively low concentrations (MacKinnon and Boerger 1986, Holowenko et al. 2002). The potential for microbial treatment of naphthenic acids in OSPW is discussed in Section 1.8.

1.7.3 Monitoring the effectiveness of wastewater treatment

There are many methods for monitoring the effectiveness of wastewater treatment, including measurement of general parameters such as turbidity or solids content, color, hardness, and pH (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). Wastewater-specific parameters may also be monitored. For example, quantification of chloride, fluoride, nitrogen, sulfate, phosphorus or phosphate, heavy metals, and other trace contaminants may be necessary depending on the type of wastewater. In cases where a wastewater contains chemicals that are considered priority pollutants (i.e. identified as being mutagenic, carcinogenic, teratogenic, or acutely toxic), these

chemicals must be monitored directly to ensure that treatment has been adequate for their reduction or removal. For example, polyaromatic hydrocarbons such as naphthalene, anthracene, pyrene, and benzo(a)pyrene are priority pollutants (USEPA 2006) that may be present in petroleum refinery wastewaters. The oil and grease test is also an important indicator of treatment success for petroleum refinery wastewaters, because it determines the residual petroleum content. Additional parameters for monitoring the effectiveness of wastewater treatment are described below.

1.7.3.1 TOC

Total organic carbon (TOC) is a measurement of the concentration of organic compounds in wastewater (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). It is determined using a TOC analyzer, which utilizes heat and oxygen, UV radiation, or chemical oxidants (in combination or alone) to oxidize the organic carbon to CO_2. CO_2 is then quantified by infrared spectroscopy or other means. TOC measurements may also be divided into particulate TOC and dissolved (soluble) TOC, where the former is defined as the residue from filtration of wastewater through filter paper with a pore size of 0.45 μm (Standard Methods 1999). A major advantage of the TOC test is that it takes only 5 to 10 min to complete.

1.7.3.2 COD and BOD

Organic contaminants are often considered in terms of their chemical oxygen demand (COD) and biochemical oxygen demand (BOD). These parameters are indicative of the potential oxygen demand a wastewater will exert on a receiving body of water. If oxygen demand is not mitigated prior to discharge, the receiving water will undergo a depression in dissolved oxygen concentration, often referred to as an oxygen sag, which can be detrimental to aquatic species that depend on dissolved oxygen for survival.

COD is a measurement that describes the amount of oxygen needed for complete oxidation of all the organic compounds in a given wastewater (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). It is usually determined by reaction with an excess of strong chemical oxidizing agent, such as potassium dichromate in acid solution (Standard Methods 1999). Residual dichromate is then titrated with ferrous ammonium sulfate

(FAS). A blank sample (i.e. no dissolved organics) is also treated with excess dichromate and titrated with FAS. Chemical oxygen demand, in mg O_2/L, is then calculated as shown in Equation 1.1.

$$\text{COD as mg } O_2/L = \frac{(a-b) \cdot M \cdot 8000}{c} \qquad \text{(Equation 1.1)}$$

where a = mL FAS used for blank
b = mL FAS used for sample
c = mL of sample
M = molarity of FAS (mol/L)
8000 = conversion factor to oxygen equivalent (mg/mol)

BOD is a measurement of the amount of oxygen required for "complete" oxidation of the organic compounds via microbiological processes (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). The word "complete" has been noted in quotations because the test does not actually require total mineralization of the organic compounds, which could take a very long time to achieve. Instead, BOD is determined by a 5- or 20-d bioassay, which is considered representative of the majority of the total BOD (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). The test involves seeding a known volume of wastewater with an appropriate microbial consortium and incubating the sealed bottle under aerobic conditions. The concentration of dissolved oxygen is measured before and after incubation, and the difference is the BOD of the wastewater. BOD is always lower than COD due to recalcitrance of some organics and the fact that only the short-time (5-d) BOD is usually measured.

In terms of evaluating wastewater treatment efforts, a reduction in COD or BOD (or, ideally, in both) is evidence for the successful removal of organic contaminants. Furthermore, the ratio of BOD/COD is regarded as the biodegradability of a given wastewater (Sawyer et al. 2003, Metcalf & Eddy, Inc. 2003). Therefore, determining BOD and COD is also useful for predicting the success of bioremediation efforts or the suitability of microbiological processes as a post-physicochemical treatment step.

Basically, the closer the BOD/COD value is to 1, the more effective microbiological treatment will be. As a reference, BOD/COD of domestic wastewater, which is considered treatable by microbiological processes, ranges from 0.3 to 0.8 (Metcalf & Eddy, Inc. 2003).

1.7.3.3 Fingerprinting

Bioremediation can be monitored by following changes in the general parameters of BOD and COD. However, depending on the availability of suitable methods, it is also possible to monitor specific groups of compounds, using changes in their so-called "fingerprint" to evaluate the effectiveness and extent of treatment. Roques et al. (1994) suggested that monitoring the compounds most altered by bioremediation or only those associated with toxicity, reduces the complexity of trying to follow changes to an entire mixture of contaminants. To illustrate this, they applied a method of GC-MS fingerprinting to follow oil degradation.

Naphthenic acids are a primary concern with OSPW and are also a major target of petroleum refinery wastewater treatment because these mixtures are acutely toxic to aquatic organisms (FTFC 1995, CONRAD 1998, Schramm et al. 2000, Headley and McMartin 2004, Clemente and Fedorak 2005). For this reason, naphthenic acids are a good candidate for fingerprinting analyses. A few analytical methods have been developed for this purpose. For example, Holowenko et al. (2002) used GC-MS to characterize differences in the naphthenic acids fingerprint of several OSPW samples and correlated the fingerprint with toxicity. Specifically, they showed that samples with a greater proportion of naphthenic acids assigned as $n \geq 22$ were less toxic.[2] Later, Clemente et al. (2004) used the same GC-MS method to monitor changes in the naphthenic acids fingerprint during biodegradation of commercial naphthenic acids preparations. Wong et al. (1996) also used a fingerprinting technique (fast ion bombardment MS) to monitor adsorption of naphthenic acids onto GAC in order to determine the extent of their removal from a refinery effluent.

[2] As discussed in Chapter 6, it is now known that GC-MS analyses of oil sands naphthenic acids (i.e. extracted from OSPW) do not generate accurate profiles due to the misassignment of derivatized hydroxylated naphthenic acids to high carbon numbers (n-values) and incorrect Z-values. Therefore, these data are no longer valid for drawing conclusions about toxicity in relation to the naphthenic acids fingerprint of a given sample.

1.7.3.4 Toxicity

Where microbiological processes are aimed at reducing or removing toxic compounds, methods to directly evaluate changes in toxicity are also needed. Toxicity is considered in terms of *acute* toxicity and *chronic* toxicity (Metcalf & Eddy, Inc. 2003). Acute toxicity describes a substance or solution that will cause a significant response in the test organism shortly (within 48 to 96 h) after exposure. Chronic toxicity describes a substance or solution that will cause a sublethal response over long-term exposure ($\geq$ 1/10 of the organism's lifespan).

Various standard methods have been developed for assessing acute and chronic toxicity of water and wastewater (Standard Methods 1999, USEPA 2002). One such technique is the Microtox™ bioassay (Microbics Corporation 1991). In this test, acute toxicity is evaluated using the photoluminescent bacterium *Vibrio fischeri* and is expressed as IC_x: the concentration (C) of a compound or mixture that causes x percent inhibition (I) of luminescence. IC_{20} and IC_{50}, denoting 20 and 50% inhibition respectively, are the concentrations most frequently reported. Microtox™ has been used to evaluate toxicity of naphthenic acids (Holowenko et al. 2002, Clemente et al. 2004) and OSPW (MacKinnon and Boerger 1986).

Additional methods for evaluating changes in toxicity involve bioassays with other aquatic organisms, such as fish, zooplankton (eg. *Daphnia magna*), ciliates, and algae. Acute toxicity is the most common effect measured and is usually based on mortality (death) or survival. For example, Wong et al. (1996) used rainbow trout (*Salmo gairdneri*) mortality to evaluate toxicity of a petroleum refinery wastewater before and after treatment using GAC. They observed 95 to 100% survival post-GAC (based on a 96-h static bioassay) compared with 20 to 45% survival after exposure to untreated (pre-GAC) effluent. When fish or other multicellular organisms are used in toxicity testing it is also possible to monitor physiological parameters such as growth or reproductive rate, hematology, or histology, in order to assess chronic toxicity. Studies that have investigated the biological effects (acute and chronic) of naphthenic acids and OSPW are discussed in Section 1.6.

Dorn (1992) published a case study describing biomonitoring techniques for evaluating toxicity of a petroleum refinery effluent known to contain naphthenic acids.

Test organisms for acute toxicity evaluations included opossum shrimp (*Mysidopsis bahia*), green algae (*Thalassiora sipeins*), the marine bacterium *Photobacterium phosphoreum* (now *Vibrio fischeri*; used in the Microtox™ bioassay), and three species of fish: sheepshead minnow (*Caprinodon variegates*), threespine stickleback (*Gasterosteus aculeatus*), and rainbow trout (*S. gairdneri*). Acute toxicity was measured weekly or biweekly over ~ 5 months. Rainbow trout, opossum shrimp, and algae were affected similarly by exposure to the refinery effluent (based on LD_{50} values). Results from bioassays with sheepshead minnow, stickleback, and Microtox™ were also in good agreement with each other but these organisms were much less sensitive than the trout, shrimp, and algae. In fact, sheepshead minnow and stickleback exhibited 100% survival in all but 3 tests, and *V. fischeri* (Microtox™) luminescence was uninhibited in all but 2 tests. Opossum shrimp was consistently the most sensitive test species. Dorn (1992) suggested that opossum shrimp could thus be considered representative of the most sensitive species in the receiving water and recommended that acute toxicity of the effluent be evaluated using opossum shrimp as the sole biomonitoring species.

Chronic exposure tests were also conducted using the water flea *Ceriodaphnia dubia reticula*, fathead minnow (*Pimephales promelas*), and opossum shrimp. In these tests, fathead minnows were the most sensitive test organism. Dorn (1992) recommended that chronic toxicity testing be conducted using a 7-d exposure period with fathead minnows.

The case study by Dorn (1992) presented results that were specific to a particular petroleum refinery effluent and, therefore, the recommendations included in that publication may not be applicable for biomonitoring of other petroleum refinery wastewaters. Nevertheless, the findings of Dorn (1992) demonstrated that response can vary depending on test organism, highlighting the importance of screening to establish the most appropriate test organisms for evaluating toxicity.

1.8 Potential for microbiological treatment of oil sands process water

Clearly the effectiveness of using microbiological processes or bioremediation to eliminate naphthenic acids in OSPW is contingent upon the biodegradability of these compounds. This area of research has not been well developed. In fact, only one study

has actually looked at biodegradation of oil sands naphthenic acids (Herman et al. 1994). However, some information can be gained from literature that has documented biodegradation of compounds structurally similar to oil sands naphthenic acids.

1.8.1 Biodegradation of surrogate naphthenic acids

Surrogate naphthenic acids have been used by researchers to predict the potential biodegradability of naphthenic acids in OSPW. They are commercially available pure compounds with structures corresponding to the chemical formula of aliphatic and alicyclic naphthenic acids ($C_nH_{2n+Z}O_2$) (Brient et al. 1995).

Herman et al. (1993) studied biodegradation of cyclopentane carboxylic acid (CPCA), cyclohexane carboxylic acid (CHCA), 1-methyl-1-cyclohexane carboxylic acid (1MCHC), and 2-methyl-1-cyclohexane carboxylic acid (2MCHC) acid (Figure 1.5) by bacteria in OSPW. The first two compounds were selected because they are known components of petroleum and have structures comparable to simple naphthenic acids. The latter compounds, which have alkyl substitutions on the ring in addition to an alkanoic substitution, were considered representative of slightly more complex naphthenic acids. Known amounts of each test compound were added to separate microcosms of tailings pore water (TPW), which was obtained by centrifuging OSPW at 30 000 X g for 1 h, filtering the supernatant, and recovering the clarified water. Nutrients (N and P) were supplemented by adding a mineral salts medium to the clarified pore water. Viable bacteria from the original OSPW sample were added to the TPW and degradation of test compounds was monitored using GC with a flame ionization detector. CPCA and CHCA were rapidly biodegraded within 10 d whereas 2MCHC was not completely degraded until day 40. No biodegradation of 1MCHC was observed in the 40-d incubation period.

In a second experiment, the researchers tested biodegradation of CPCA and 1MCHC by bacteria in unaltered OSPW (i.e. no nutrients added and with suspended solids present) (Herman et al. 1993). Once again there was a difference in the biodegradability of these two compounds, with CPCA being readily biodegraded and 1MCHC being recalcitrant. However, when N and P were added to the cultures (week 6),

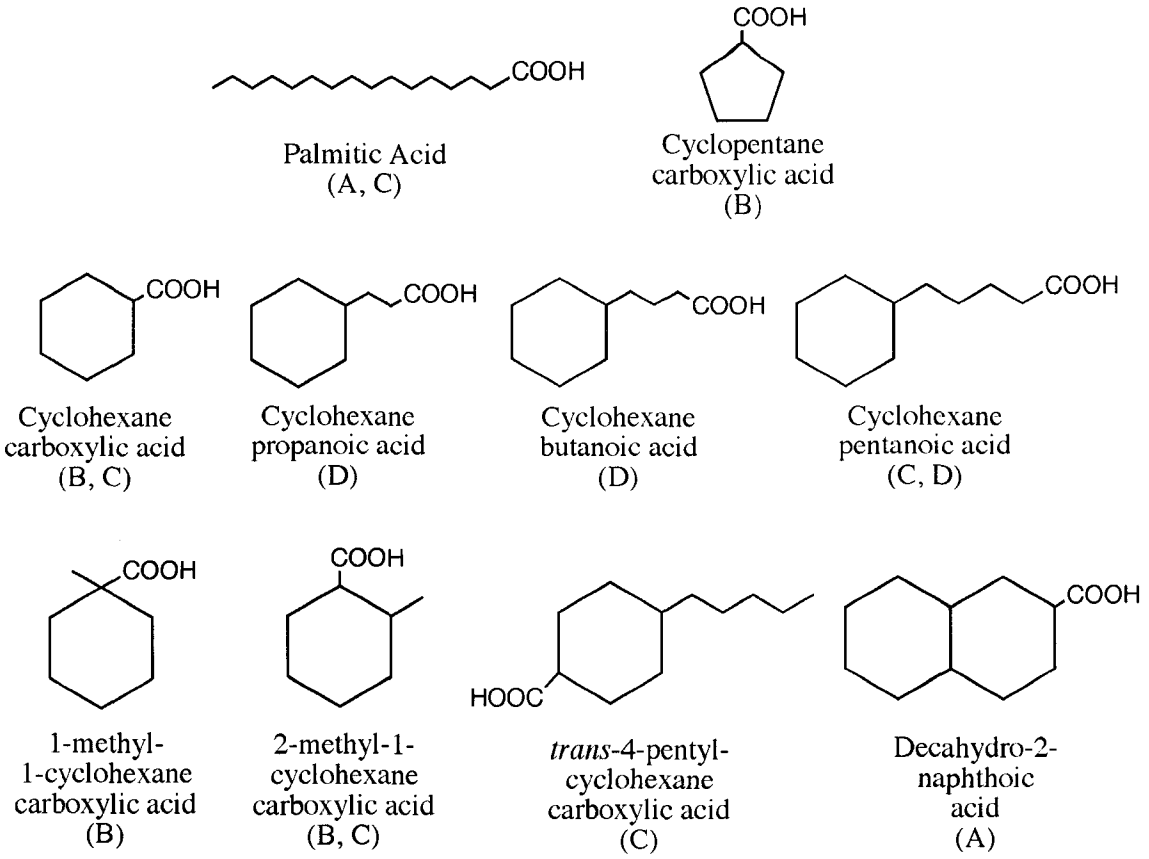

Figure 1.5 Chemical structures of surrogate naphthenic acids used in biodegradation studies by: (A) Lai et al. (1996), (B) Herman et al. (1993), (C) Herman et al. (1994), and, (D) Holowenko et al. (2001).

biodegradation of 1MCHC was stimulated. Complete biodegradation of 1MCHC was achieved by week 9, 3 weeks after the addition of nutrients.

The results of Herman et al. (1993) showed that nutrient availability may be a limiting factor for biodegradation in OSPW. Moreover, they demonstrated that structural variations influence the biodegradability of cycloalkane carboxylic acids, with more complex molecules displaying greater recalcitrance.

This last concept was revisited in a later study by Herman et al. (1994), in which they examined biodegradation of three additional surrogate naphthenic acids, whose structures were either simpler (palmitic acid) or more complex (cyclohexane pentanoic acid and pentylcyclohexane carboxylic acid) than those of the first study (Figure 1.5). They also reexamined biodegradation of CHCA and 1MCHC. Test cultures for this study included oil sands tailings bacteria, tailings bacteria enriched for growth on a commercial

naphthenic acids preparation, and tailings bacteria enriched for growth on an extract of oil sands naphthenic acids. The extent of biodegradation was estimated by using GC to monitor mineralization (production of CO_2) of the surrogate naphthenic acids. As expected, the linear test compound (palmitic acid) was, on average, the most rapidly biodegraded by all three cultures. CHCA was the next best substrate, followed by cyclohexane pentanoic acid, pentylcyclohexane carboxylic acid, and 1MCHC, in that order. This supports their earlier finding that structural complexity affects biodegradability of surrogate naphthenic acids. Specifically, longer alkanoic groups, additional alkyl substitutions on the ring, and quaternary carbons reduce biodegradability. The rate and extent of biodegradation was similar among all three test cultures for most of the surrogate naphthenic acids. However, cultures enriched for growth on the extract of oil sands naphthenic acids were only able to mineralize ~7% of the initial amount of 1MCHC, whereas the other cultures mineralized 37 to 67%. The researchers did not comment on this difference. It is possible that the oil sands extract did not contain 1MCHC or structurally similar compounds and, thus, the enrichment culture had reduced capacity for biodegradation of this surrogate naphthenic acid. Herman et al. (1994) also observed that the culture enriched for growth on commercial naphthenic acids was specialized for degradation of carboxylic acids and was not able to degrade hexadecane: a model alkane also tested in the study. These findings illustrate how essential it is to determine the effectiveness of potential seed cultures before using them in bioremediation efforts, particularly when the target compounds are structurally diverse, as with oil sands naphthenic acids.

Lai et al. (1996) added universally labeled ^{14}C-palmitic acid and decahydro-2-naphthoic acid-8-^{14}C (DHNA) to microcosms of nutrient-amended OSPWs (Syncrude and Suncor) and monitored their mineralization. The former compound is a 16-carbon linear carboxylic acid and the latter a bicyclic monocarboxylic acid (Figure 1.5). The researchers believed that mineralization of these compounds could be taken as an indicator of overall biodegradation of naphthenic acids in the OSPWs. They also looked for a reduction in toxicity using Microtox™ and bioassays with fathead minnows (*Pimephales promelas*). After 4 weeks of incubation, 10 to 15% of the radioactivity from palmitic acid was converted to $^{14}CO_2$, while less than 5% of the DHNA was mineralized

to $^{14}CO_2$ after 8 weeks of incubation. These results demonstrated that an acid with a fused ring is less biodegradable than linear carboxylic acids. Moreover, only partial biodegradation of the two surrogates was observed after several weeks of incubation in favorable nutrient conditions. Acute toxicity of the OSPWs to fathead minnows decreased after 4 weeks of incubation, but there was no change in toxicity according to Microtox™ data. If these observations are in fact representative of the rate and extent of oil sands naphthenic acids biodegradation, the success of microbiological treatment may be quite limited.

Anaerobic metabolism of surrogate naphthenic acids has also been investigated. Holowenko et al. (2001) followed biodegradation of cyclohexane propanoic acid, cyclohexane butanoic acid, and cyclohexane pentanoic acid (Figure 1.5) in methanogenic microcosms. Oil sands mature fine tailings and sewage sludge served as the inocula in this study. Cultures were established in serum bottles containing anaerobic medium with various concentrations of each surrogate naphthenic acids (individually) and methane production was monitored by GC. Incubation times were extremely lengthy, ranging from 220 to 450 d. The sewage sludge cultures were able to partially degrade cyclohexane propanoic acid and cyclohexane pentanoic acid but not cyclohexane butanoic acid. Methane production was greatest in the cultures grown on cyclohexane propanoic acid, exceeding the amount expected if only the side-chain had been metabolized. This result indicated that alicyclic ring cleavage and subsequent degradation is possible under anaerobic conditions. Methanogenesis was observed in tailings-containing cultures only when grown on cyclohexane propanoic acid or cyclohexane butanoic acid. In this case, the extent of degradation could not be directly calculated from methane production because other carbon sources (from the tailings pore water) were present besides the surrogate naphthenic acids. The pattern of methane production showed a diauxic shift, which Holowenko et al. (2001) attributed to the bacteria first using the readily available organic compounds from the pore water and then adapting to growth on the surrogate naphthenic acids. This phenomenon could be a concern for bioremediation of OSPW because current industry practices involve continuous input of fresh tailings to the ponds. Thus, if the tailings contain a preferred carbon source, biodegradation of naphthenic acids might not occur. However, this diauxic shift has only

been observed under anaerobic conditions whereas microbiological treatment will likely be attempted under aerobic conditions.

1.8.2 Biodegradation of commercial naphthenic acids

There are several commercially available naphthenic acids but only two have been used in biodegradation studies. Herman et al. (1994) established microcosms with Kodak naphthenic acids sodium salts (Kodak salts) in mineral salts medium and inoculated them with bacteria from OSPW. These cultures were incubated to enrich for naphthenic acids-degraders and then a portion was transferred to fresh microcosms with more Kodak salts. GC analysis showed that these cultures mineralized 48% of the carbon from the Kodak salts.

Ten years later, Clemente et al. (2004) reported biodegradation of Kodak salts and Merichem Naphthenic Acids by microorganisms indigenous to OSPW storage ponds. Like Herman et al. (1994), these researchers monitored mineralization of the naphthenic acids preparations. However, a recently developed analytical method using HPLC (Yen et al. 2004) also enabled them to directly monitor changes in naphthenic acids concentration. Furthermore, changes in the naphthenic acids profile were visualized using the GC-MS method described by Holowenko et al. (2002). Thus, Clemente et al. (2004) were able to obtain the most detailed information to that date on naphthenic acids biodegradation. Both Kodak salts and Merichem acids were rapidly degraded, stabilizing at less than 10% of their initial concentrations within 10 d. Mineralization results showed that about 60% of the estimated total carbon was released as CO_2. Although the extent of mineralization was less than predicted based on a mass balance of quantification data, some carbon is incorporated into biomass during aerobic heterotrophic degradation of organics and therefore not released as CO_2 (Maier et al. 2000). GC-MS data provided evidence that naphthenic acids with less complex structures (low Z value) and lower molecular weights (carbon number less than 13) are degraded preferentially, as was expected based on the results from studies with surrogate naphthenic acids (Herman et al. 1993). Analysis of culture supernatants using the Microtox™ test revealed a reduction in toxicity corresponding to the decrease in naphthenic acids concentration.

This study by Clemente et al. (2004) clearly showed that naphthenic acids can be

degraded by microorganisms from OSPW storage ponds. However, it should be noted that the commercial preparations are comprised of lower molecular weight (and possibly less complex) naphthenic acids than those from OSPW (Holowenko et al. 2002). Therefore, the results of Clemente et al. (2004) may not be suitable for predicting the biodegradability of oil sands naphthenic acids.

1.8.3 Biodegradation of oil sands naphthenic acids

To date, only one publication has actually documented biodegradation of oil sands naphthenic acids (Herman et al. 1994). Microorganisms indigenous to OSPW storage ponds (tailings ponds) were grown in laboratory cultures where the only carbon source was from an extract of the organic acids fraction of tailings water (i.e. OSPW). This tailings extract (TEX) was believed to consist mainly of naphthenic acids with a few aromatic acids and other contaminants. Herman et al. (1994) monitored mineralization over a 20-d incubation period and reported that, on average, 20% of the carbon in the TEX was released as CO_2. This suggested that naphthenic acids were being degraded in the cultures, but, because the analytical method of Yen et al. (2004) was not available at this time, the researchers were not able to quantify the actual change in naphthenic acids concentration. Clemente and Fedorak (unpublished) endeavored to biodegrade oil sands naphthenic acids in aerobic microcosms. These attempts were unsuccessful for undetermined reasons. Thus, more research is needed in this area before a treatment strategy involving microbiological processes is implemented.

1.8.4 Intrinsic bioremediation of oil sands process waters

Despite the lack of laboratory studies on biodegradation of oil sands naphthenic acids, there is some evidence that bioremediation is naturally occurring in the OSPW ponds at Syncrude and Suncor (MacKinnon and Boerger 1986, Nix and Martin 1992, Holowenko et al. 2002). MacKinnon and Boerger (1986) reported a reduction in the toxicity of OSPW from a Syncrude storage pond after 2 years of undisturbed aging (i.e. a period during which no fresh OSPW was added to the pond). Nix and Martin (1992) observed the same effect in undisturbed OSPW ponds at Suncor. Both research groups commented that naphthenic acids were likely a major contributor to the original toxicity

of the pond water. MacKinnon and Boerger (1986) suggested that the toxicity reduction was therefore indicative of naphthenic acids biodegradation whereas Nix and Martin (1992) believed that the effect was attributable to biodegradation of more than one type of toxic constituent. For example, they noted that phenolic compounds and inorganic constituents, such as ammonia, iron, copper, and zinc, could play a role in the overall toxicity of the waters.

Holowenko et al. (2002) also observed lower toxicity in aged Syncrude and Suncor storage ponds compared to fresh OSPW. In that study, the differences in toxicity were correlated with naphthenic acids concentration: lower concentrations of naphthenic acids were found in ponds that had remained undisturbed for several years compared to fresh OSPW. The naphthenic acids profile, as determined by GC-MS, was also variable depending on the age of the OSPW. Specifically, low molecular weight naphthenic acids (carbon numbers ≤ 21), which represented a large portion of the naphthenic acids in fresh OSPW, were less abundant in aged waters. Based on these findings, it was hypothesized that biodegradation of acutely toxic naphthenic acids had occurred over time, resulting in reduced toxicity of the pond water.

MacKinnon and Boerger (1986) and Nix and Martin (1992) stated that intrinsic bioremediation is typically masked by continuous input of fresh, toxic OSPW. Others have noted that nutrient limitation is a factor in reducing the apparent rate of biodegradation (Nix and Martin 1992, Herman et al. 1993, Lai et al. 1996).

1.8.5 Possible biodegradation pathways

Due to the chemical complexity of naphthenic acids mixtures and the absence of a method for resolving individual naphthenic acids, research to date has not elucidated the biodegradation pathways for naphthenic acids. Thus, the best way to gain some understanding of how naphthenic acids might be biodegraded is to consider the literature on biodegradation of pure carboxylic acids (aliphatic and alicyclic).

By far, the majority of studies have focused on biodegradation of cyclohexanecarboxylic acid (CHCA). Through investigations with many different species of bacteria and, in some cases, mixed cultures, two major pathways have been described (Figure 1.6). One consists of alicyclic ring cleavage followed by typical β-oxidations

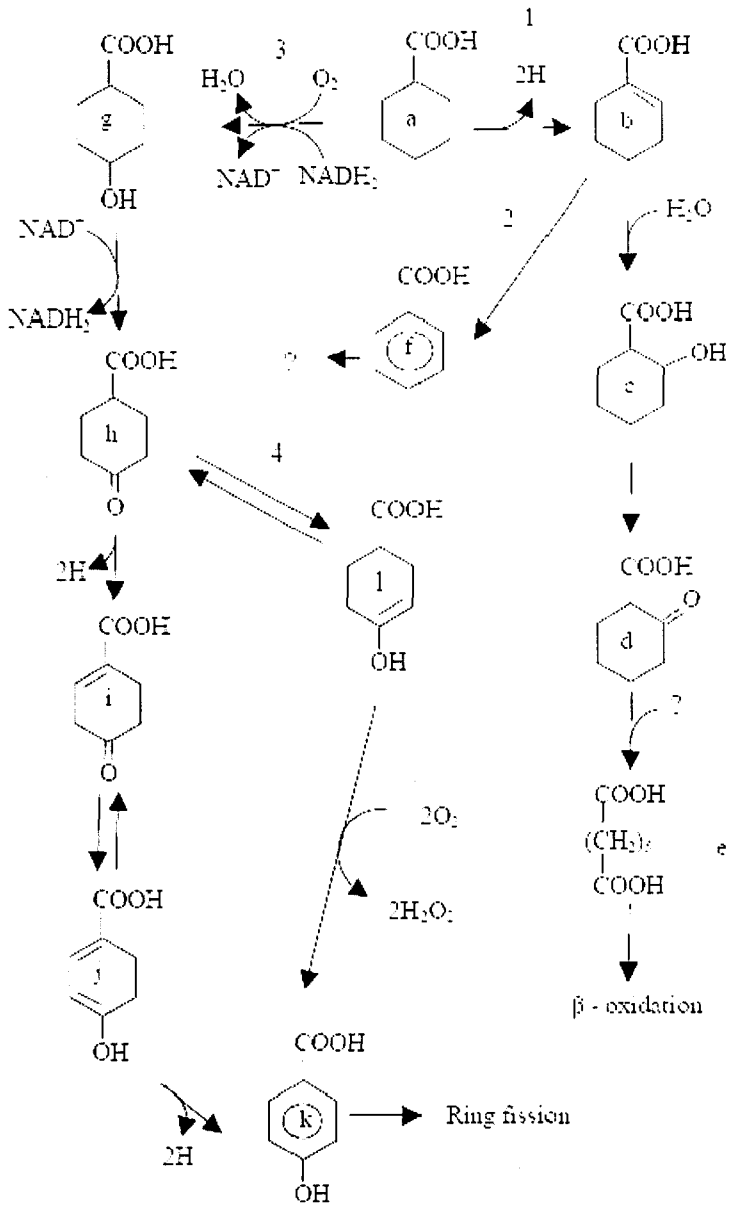

Figure 1.6 Summary of possible cyclohexanecarboxylic acid aerobic biodegradation pathways. Pathways described in the text are indicated by the numbers 1 through 4. (a) cyclohexanecarboxylic acid, (b) 1-cyclohexenecarboxylic acid, (c) *trans*-2-hydroxycyclohexanecarboxylic acid, (d) 2-ketocyclohexanecarboxylic acid, (e) pimelic acid, (f) benzoic acid, (g) *trans*-4-hydroxycyclohexanecarboxylic acid, (h) 4-ketocyclohexanecarboxylic acid, (i) 4-keto-1-cyclohexenecarboxylic acid, (j) 4-hydroxycyclohex-1,3-dienecarboxylic acid, (k) *p*-hydroxybenzoic acid, (l) 4-hydroxycyclohex-1-enecarboxylic acid. Pathways were determined by: (1) Rho and Evans (1975), Blakley (1978), Blakley and Papish (1982) Küver et al. (1995), (2) Dutta and Harayama (2001), (3) Smith and Callely (1975), Taylor and Trudgill (1978), (4) Blakley (1974). Adapted from Clemente (2004).

(Rho and Evans 1975, Blakley 1978, Blakley and Papish 1982, Küver et al. 1995), whereas the other involves aromatization followed by ring fission and subsequent oxidations (Tokuyama and Kaneda 1973, Kaneda 1974, Blakley 1974, Smith and Callely 1975, Taylor and Tudgill 1978, Dutta and Harayama 2001).

Rho and Evans (1975) determined that alicyclic ring cleavage of CHCA could be achieved by *Acinetobacter anitratum* in laboratory cultures. Pimelic acid, a 7-carbon linear dicarboxylic acid (Figure 1.6, compound e), was identified as an intermediate by two techniques. The first technique involved incubating *A. anitratum* with CHCA that had a ^{14}C-labeled carboxyl group. Intermediates were then isolated and separated by thin-layer chromatography. Elution profiles were compared to those of known standards, leading to the identification of pimelic acid as a primary metabolite. This finding was confirmed by the second technique, which involved derivatization of intermediates to their methyl esters and subsequent analysis by MS. Besides pimelic acid, other intermediates identified by MS included 1-cyclohexenecarboxylic acid (Figure 1.6, compound b) and *trans*-2-hydroxycyclohexanecarboxylic acid (Figure 1.6, compound c).

Further support for alicyclic ring fission was established by Blakley (1978), Blakley and Papish (1982), and Küver et al. (1995), who conducted biodegradation studies using *Alcaligenes faecalis*, *Pseudomonas putida*, and *Rhodopseudomonas palustris*, respectively. These studies focused more on characterizing the enzymes involved in CHCA biodegradation, relying mostly on the validity of previous experiments (e.g. Rho and Evans 1975) when selecting metabolites to test. Blakley (1978) and Blakley and Papish (1982) did, however, use MS to detect the same intermediates found by Rho and Evans (1975). They also detected glutaric acid, which provided evidence that ring cleavage is followed by β-oxidation of pimelic acid. In addition, their results, and those of Küver et al. (1995), showed induction of β-oxidation enzymes when the bacteria were grown in cultures containing CHCA. Enzyme analyses also showed the involvement of coenzyme A in metabolism of this compound. Taylor and Trudgill (1978) showed that bacteria from soil utilize this pathway more frequently than the alternative, aromatization pathway: only one of 33 isolates used the aromatization pathway rather than alicyclic ring cleavage.

A biodegradation pathway involving aromatization of CHCA (Figure 1.6) has been shown to occur in cultures of *Corynebacterium cyclohexanicum* (Tokuyama and aneda 1973, Kaneda 1974), *Arthrobacter* sp. (Blakley 1974, Smith and Callely 1975), *Alcaligenes* strain W1 (Taylor and Trudgill 1978), and *Alcanivorax* sp. (Dutta and Harayama 2001). However, the actual mechanism of aromatization was variable depending on the microorganism.

Initially, the only evidence for aromatization was detection of the aromatic intermediate, *p*-hydroxybenzoic acid (Figure 1.6, compound k) in *C. cyclohexanicum* cultures grown on CHCA (Tokuyama and Kaneda 1973). A subsequent study using the same bacterium showed that 4-ketocyclohexanecarboxylic acid (Figure 1.6, compound h) is a precursor in the formation of *p*-hydroxybenzoic acid (Kaneda 1974). Smith and Callely (1975) obtained analogous results when they examined CHCA metabolism in cultures of *Arthrobacter* sp. isolated from marsh mud. The remainder of this aromatization pathway was deduced by Taylor and Trudgill (1978) in their studies using *Alcaligenes* strain W1: the name given to the one bacterial isolate out of 33 that used the aromatization pathway rather than alicyclic ring cleavage. Growth studies were conducted with potential intermediates in mineral salts medium. Enzyme and chemical assays, thin-layer chromatography, and GC, enabled identification of pathway 3 shown in Figure 1.6. Subsequent cleavage of the aromatic ring has been shown to occur by both *ortho*-fission (Blakley 1974) and *meta*-fission (Smith and Callely 1975).

Blakley (1974) and Dutta and Harayama (2001) described two additional pathways leading to aromatization of CHCA. Conversion of 4-ketocyclohexanecarb-oxylic acid (Figure 1.6, compound h) to *p*-hydroxybenzoic acid by cultures of *Arthrobacter* sp. was observed by Blakley (1974) (Figure 1.6, pathway 4). Hydrogen peroxide was generated as a byproduct of this pathway and 4-hydroxycyclohex-1-enecarboxylic acid (Figure 1.6, compound l) was identified as an intermediate. Dutta and Harayama (2001) showed that aromatization could also proceed via pathway 2 from 1-cyclohexenecarboxylic acid (Figure 1.6, compound b): the first intermediate of the alicyclic ring cleavage pathway. This was determined by GC-MS analysis of culture supernatants from *Alcanivorax* sp. grown with CHCA. Furthermore, they demonstrated

that alkyl substitutions on the original cycloalkane ring are degraded by β-oxidation prior to metabolism of the ring structure.

Naphthenic acids with structures comparable to CHCA are likely to be biodegraded in similar ways as those described above. Arguably, this compound is only a good model for simple naphthenic acids. More complex naphthenic acids (i.e. polycyclic, alkyl substituted) would certainly have more complicated pathways and be more difficult to degrade. However, if microbial transformations led to production of CHCA, biodegradation could proceed as above.

1.9 Chemical oxidation of recalcitrant organics

The application of microbiological processes *in situ* or in an engineered setting is typically the most cost-effective method for treating wastewaters contaminated with organic compounds. However, some wastewaters contain organic compounds that are recalcitrant to microbial oxidation or that are toxic or inhibitory to microorganisms. Biodegradation of surrogate and commercial naphthenic acids has been clearly demonstrated (see Section 1.8), but attempts to biodegrade oil sands naphthenic acids have suggested that recalcitrant compounds are likely present in these mixtures (Herman et al. 1994, Clemente and Fedorak, unpublished results).

Chemical oxidation can alter the chemical structure of recalcitrant compounds such that they may become more amenable to microbial degradation. Similarly, chemical oxidation can change toxic or inhibitory compounds so that they are no longer an impediment to microbial degradation (Ledakowicz et al. 2006, Quen and Chidambara Raj 2006). Complete mineralization of organic contaminants is often possible through the application of strong oxidizing agents but can be costly, particularly for wastewaters with high organic content (Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). Coupling chemical and biological processes is more economically favorable than using chemical oxidation alone and, thus, this coupling technique has been studied for application in the treatment of a variety of industrial wastewaters.

1.9.1 Chemical oxidation and advanced oxidation processes

In wastewater treatment, chemical oxidation involves the application of strong oxidizing agents such as potassium permanganate, chlorine, hydrogen peroxide (H_2O_2), Fenton's reagent (H_2O_2 with Fe^{2+}), and ozone with the goal of making recalcitrant organic compounds more biodegradable (Scott and Ollis 1995, Wang et al. 2003, Ikehata and Gamal El-Din 2004). Advanced oxidation processes (AOPs) are a specialized type of chemical oxidation in which the formation of hydroxyl radicals is encouraged through UV irradiation of ozone or H_2O_2, or by combining H_2O_2 and ozone under certain conditions. Ferrous iron (Fe^{2+}) can also be used alone or with UV to generate hydroxyl radicals from ozone or H_2O_2. The efficacy of AOPs is affected not only by the types of organic compounds (e.g. aliphatic, aromatic, carboxylic acids, halogenated organics) but also by their concentration and the composition of the wastewater (Lin et al. 2001, Wang et al. 2003). AOPs are considered preferable to chlorination because there is no risk of producing potentially carcinogenic chlorinated organics.

Oxidation using ozone is often termed ozonation. Depending on the pH and composition of the wastewater being treated, ozonation can proceed via molecular ozone reactions or hydroxyl radical reactions (Scott and Ollis 1995, Wang et al. 2003). Low pH conditions favor molecular ozone reactions, which are referred to as selective oxidation because they only occur at specific sites, namely areas of unsaturation or electron deficiency (Alvares et al. 2001). For this reason, aromatic compounds and unsaturated aliphatics are the most susceptible to oxidation by molecular ozone. At higher pH (>8), ozone decomposes to form hydroxyl radicals (Tomiyasu et al. 1985, Scott and Ollis 1995, Langlais et al. 1991, Wang et al. 2003). Hydroxyl radicals will react with a variety of functional groups, referred to as hydroxyl radical scavengers (Hoigne et al. 1985). These include alkyl groups, tertiary alcohols, carbonate, and bicarbonate. The non-selective nature of hydroxyl radical reactions makes them ideal for oxidation of wastewaters that contain a mixture of organic compounds. AOPs are designed to facilitate the decomposition of ozone to hydroxyl radicals using H_2O_2, UV light, or Fenton's reagent. One disadvantage of using ozone is that it must be generated on-site using electricity, which adds to operating costs.

H_2O_2 is usually applied as an AOP in combination with ozone or in the Fenton process, in which its decomposition into hydroxyl radicals is catalyzed by Fe^{2+} (Harber and Weiss 1934). The Fenton reaction proceeds at a slower rate than other oxidation reactions, making it unsuitable when low residence time is required or when mobile wastewater treatment is needed (Scott and Ollis 1995). However, the photo-Fenton process, which incorporates light to assist in the decomposition of H_2O_2, can circumvent this problem (Wang et al. 2003). One disadvantage of the Fenton process is production of a wet, bulky sludge that requires disposal, adding to the overall operating costs.

1.9.2 Advanced oxidation processes as a pretreatment before microbiological processes

Although AOPs can often achieve complete mineralization of the organic compounds in a wastewater, they can also be used as a pretreatment before microbiological processes (discussed by Langlais et al. 1989, Scott and Ollis 1995, Alvares et al. 2001). This involves arresting oxidation processes when biodegradability of the mixture is optimal. Determining the actual point at which this occurs requires monitoring changes in COD and BOD (defined above) throughout the oxidation treatment, paying particular attention to the ratio of BOD/COD, which is a measure of biodegradability. Optimization studies are conducted to establish the best combination of oxidizing agents and catalysts, as well as reaction conditions like temperature and pH (Langlais et al. 1989, Scott and Ollis 1995, Alvares et al. 2001). In cases where the wastewater has a substantial biodegradable fraction to begin with, biological treatment may precede chemical oxidation followed by another biological treatment step to further reduce the organic content. This type of sequential treatment ensures that AOPs are targeted to recalcitrant organics and not wasted in the oxidation of organics that could be treated much more economically though biodegradation (Scott and Ollis 1995; Quen and Chidambara Raj 2006).

It is critical to ensure that oxidation does not produce toxic or inhibitory compounds that might limit the effectiveness of subsequent microbiological treatment (Manilal et al. 1992, Alvares et al. 2001). Furthermore, the oxidizing agents themselves are generally toxic to bacteria, and, therefore, must be purged from the wastewater before microbiological treatment. AOPs are well-suited for preceding microbiological processes

because hydroxyl radicals are not persistent and therefore will not interfere with biodegradation (Alvares et al. 2001, Langlais et al. 1989). Removal of residual ozone can be achieved by simply sparging the mixture with nitrogen, helium, or air for approximately 15 min (Takahashi 1994, Tzitzi et al. 1994). Excess thiosulfate can also be added to reduce residual ozone (Hijnen et al. 2004, Mazloum et al. 2004, Wang et al. 2004, Wei et al. 2007, Rodriguez-Romo et al. 2007).

Removal of residual H_2O_2 prior to biological treatment is often unnecessary because microorganisms are tolerant to low concentrations of H_2O_2. If removal is required, incubation with a bovine peroxidase will effectively destroy the H_2O_2 (Wang et al. 2003). The enzyme can then be removed by oxidation with potassium dichromate or may be biodegraded during the subsequent microbiological process. Depending on the volume of wastewater to be treated, H_2O_2 oxidation may not be practical due to the potential cost associated with using an enzyme for its removal.

1.9.3 Pre-ozonation for improving biodegradability of industrial wastewaters

Ozonation has been studied as a pretreatment for a variety of industrial wastewaters, including pulp and paper wastewater (Oeller et al. 1997, Helble et al. 1999, Gonzalez et al. 2003, Bijan and Mohseni 2004, 2005, Nakamura et al. 1997, 2004), olive wash waters and olive oil production wastewaters (Rivas et al. 2000, Amat et al. 2003, Bettazzi et al. 2006), petrochemical wastewater (Lin et al. 2001) and oil field drilling wastewater (Wang et al. 2004). In general, these researchers found that ozonation improved biodegradability by removing compounds that are toxic to microorganisms, reducing the molecular weight of organic contaminants, or by changing the form of organic contaminants to be more susceptible to microbial attack.

Pulp and paper process wastewater is typically contaminated with chlorinated organics, phenols, aromatic compounds, and highly recalcitrant lignins. One group of researchers used ozone with UV light to treat a pulp mill wastewater that had previously undergone microbiological treatment but retained high COD and TOC (Oeller et al. 1997). Following oxidation, BOD_5/COD of the wastewater had increased from 0.05 to 0.37, indicating improved biodegradability. Helble et al. (1999) also measured an increase in BOD_5/COD of approximately 0.3 for a paper mill wastewater treated with

ozone. Gonzalez et al. (2003) subjected chlorine bleaching wastewater to ozone treatment and measured an increase in BOD_5/COD from 0.36 to 0.44 after only 5 min. Moreover, they found that the extent of biodegradation (based on TOC reduction) was more than 6-fold greater when ozonation was employed as a pretreatment and that the final effluent after ozonation and biodegradation was no longer mutagenic (based on the Ames test; Ames et al. 1975, Maron and Ames 1983).

Oeller et al. (1997), Helble (1999), Gonzalez et al. (2003), and Wang et al. (2004) did not attempt to characterize the organic compounds in the wastewaters before or after treatment with ozone. Bijan and Mohseni (2004) determined that ozonation had the greatest effect on biodegradability of high molecular weight compounds (>1000 Da) present in pulp and paper wastewater, increasing the BOD_5/COD ratio of these compounds by about 50% after 120 min of treatment (~2400 mg ozone). The BOD_5/COD of low molecular weight compounds (<1000 Da) did not change with ozonation but the proportion of low molecular weight compounds increased as larger compounds were broken down by oxidation. They observed similar trends in a subsequent study where biodegradation experiments were conducted after ozonation of the wastewaters (Bijan and Mohseni 2005). Nakamura et al. (1997, 2004) reported that ozonation improved the biodegradability of pulp mill wastewater by producing smaller organic acids, such as maleic, oxalic, and acetic acid, as well as low molecular weight esters from the large recalcitrant lignins. Overall, these studies showed that ozonation could be applied as a pretreatment to enhance biodegradability of pulp and paper process wastewater.

Rivas et al. (2000) showed improved biodegradability of table olive processing wastewater after ozonation. The improvement was attributed to removal of inhibitory polyphenols and a decrease in pH to around neutral. The researchers measured decreases in COD and increases in BOD during ozonation but they did not characterize the oxidation products beyond showing the disappearance of polyphenols. Amat et al. (2003) used a mixture of four phenolic acids as a surrogate for olive oil wastewater in order to study the effectiveness of ozonation as a pretreatment. They found that biodegradability (based on BOD/COD) of the mixture increased during the first 5 min of ozonation and then decreased slowly over the remainder of the 60-min treatment. A subsequent ozonation experiment was conducted using only one of the phenolic acids (cinnamic

acid) in order to look for oxidation products and attempt to correlate their formation with changes in biodegradability. Using HPLC, the researchers identified benzaldehyde and *p*-hydroxybenzoic acid as biodegradable intermediates formed during the oxidation of cinnamic acid by ozone. Ozonation of actual olive oil processing wastewater improved biodegradability as predicted and, as was observed by Rivas et al. (2000), coincided with the disappearance of polyphenols. Based on this observation, Amat et al. (2003) proposed that ozonation of the wastewater produced readily-biodegradable intermediates such as benzaldehyde and *p*-hydroxybenzoic acid from phenolic compounds and suggested that ozonation would be a useful pretreatment for olive oil process wastewater.

Bettazzi et al. (2006) also studied pretreatment options for olive mill wastewater. They did not report the initial BOD/COD ratio for the raw wastewater but they did measure a BOD/COD of 0.17 for wastewater that was centrifuged to remove suspended solids. The centrifuged wastewater was then treated with lime and subsequently ozonated. BOD/COD of the resulting effluent was 0.27. The researchers used a non-acclimated activated sludge to determine these BOD values (Bettazzi et al. 2006). However, they also determined the BOD of the centrifuged wastewater using fungal biomass present in the raw wastewater and obtained a value more than double that measured using the activated sludge. No explanation was provided for their choice of seed when measuring the BOD of ozonated wastewater. Nevertheless, they did see biodegradability improve after ozonation, further supporting the validity of using ozone as a pretreatment for olive processing wastewaters.

Lin et al. (2001) investigated the potential for using ozone to pretreat petrochemical wastewater contaminated with recalcitrant organics including acrylonitrile butadiene styrene. They found that ozonation increased BOD_5/COD of the wastewater only slightly, from 0.2 to 0.3 after 0.5 h of treatment (~150 mg ozone) and that longer ozonation actually decreased biodegradability.

Wang et al. (2004) studied the application of ozone for pretreatment of oil field drilling wastewater. They showed that ozonation reduced the proportion of compounds with molecular weights > 3000 Da, which comprised 60% of the initial TOC, to less than 8% of the residual TOC. The ozonated mixture was reportedly more biodegradable than the untreated oil field drilling wastewater, based on a higher BOD/TOC value after

ozonation and a marked increase in the TOC reduction achieved by subsequent microbiological treatment using an acclimated activated sludge. Specifically, biodegradation of untreated wastewater achieved only ~10% decrease in TOC after 12 h, whereas biodegradation of wastewater that was ozonated for 5 min (~120 mg ozone, room temperature, pH 10.5) resulted in a 50% decrease in the residual TOC after 12 h of incubation. Wang et al. (2004) also demonstrated that coupling a short ozonation (less ozone consumption) with subsequent microbiological treatment was more efficient than ozonating for a longer period of time. They found that 5 min of ozonation followed by aerobic biodegradation resulted in a 55% decrease in TOC and only 121 mg of ozone was consumed. To achieve the same decrease in TOC using ozone alone, the treatment had to be extended to 30 min and 400 mg of ozone was consumed. The researchers did not specify the duration of the biodegradation treatment they used in the combined ozonation-biodegradation experiment. Another important finding by Wang et al. (2004) was that ozonation for more than 20 min generated a mixture that was less biodegradable than the mixtures generated after 5 or 10 min of ozonation. This observation is similar to those of Lin et al. (2001) and Amat et al. (2003) who reported an initial increase in biodegradability followed by a slow decrease during a 60-min ozonation. Wang et al. (2004) suggested that some of the biodegradable oxidation products that formed during the early stages of ozonation may have been transformed into recalcitrant compounds by further ozonation. This is an essential factor to consider when optimizing treatment protocols.

The studies described previously dealt with ozonation of several industrial wastewaters, each having different recalcitrant compounds that were made more biodegradable by partial oxidation. From these examples alone it is not possible to predict the effectiveness of ozonation or other AOPs for the treatment of potentially recalcitrant oil sands naphthenic acids. However, the fact that ozonation has been successful for pretreatment of such a variety of wastewaters is encouraging in itself.

1.10 Objectives and overview of thesis

The two primary objectives of this thesis were:

1) To study biodegradation of commercial naphthenic acids and naphthenic acids in OSPW using established quantification and characterization methods.

2) To investigate the potential for using ozonation as a pretreatment to improve biodegradability of naphthenic acids in OSPW.

The thesis is organized such that Chapter 2 describes biodegradation experiments in which four commercial naphthenic acids preparations and two OSPWs that contained naphthenic acids were incubated with microorganisms from OSPW storage ponds. Biodegradation of naphthenic acids was monitored by HPLC and GC-MS, and the relative biodegradability of commercial naphthenic acids was compared to that of oil sands naphthenic acids. Chapter 3 describes a study in which the effects of ozonating a sample of OSPW were evaluated based on changes in the naphthenic acids concentration and other characteristics of the water (eg. BOD, COD, TOC, toxicity). Chapter 4 describes several experiments that were conducted to investigate the effects of ozonation on a commercial naphthenic acids preparation (Merichem Refined naphthenic acids). Biodegradability of non-ozonated and ozonated naphthenic acids was compared during subsequent incubation with microorganisms from an OSPW storage pond. Chapter 5 describes experiments that were carried out to demonstrate that thiosulfate, which is added to quench ozone, interferes with naphthenic acids biodegradation and to elucidate the reason for the observed interference. Chapter 6 describes applications of a recently-developed high-resolution MS method for characterization of naphthenic acids during biodegradation and ozonation. Results obtained using the traditional GC-MS method of analysis are compared with those generated via the new method and limitations of the former are discussed. Chapter 7 gives a summary of the major findings reported in this thesis and suggests some possible future directions for the continuation of naphthenic acids research.

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2 NAPHTHENIC ACIDS IN ATHABASCA OIL SANDS TAILINGS WATERS ARE LESS BIODEGRADABLE THAN COMMERCIAL NAPHTHENIC ACIDS*

2.1 Introduction

Naphthenic acids are complex mixtures of predominately alkyl-substituted cycloaliphatic carboxylic acids (containing cyclopentane and cyclohexane rings) and small amounts of acyclic acids (Brient et al. 1995). They are described by the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z is zero or a negative, even integer that specifies the hydrogen deficiency resulting from ring formation. Although simple saturated fatty acids found in biological membranes fit this formula for Z = 0, these acids are very susceptible to biodegradation, so they would not persist as naphthenic acids. Some possible naphthenic acids structures are given in Chapter 1 (Figure 1.3).

Naphthenic acids occur naturally in a variety of petroleums (Brient et al. 1995, Seifert and Teeter 1969, Seifert et al. 1969, Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Jones et al. 2001, Tomczyk et al. 2001, Qi et al. 2004) and are thought to have originated from aerobic microbial degradation of petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Watson et al. 2002). Naphthenic acids are also found in Athabasca oil sands ores (Clemente et al. 2003, Clemente 2004). Commercial naphthenic acids preparations, obtained via extraction of petroleum distillates (Brient et al. 1995, Schramm et al. 2000), are used as textile and wood preservatives, emulsifiers, surfactants, paint driers, and adhesion promoters in the manufacture of tires (Brient et al. 1995).

The complexity of naphthenic acid mixtures provides a major challenge in the development of suitable analytical methods for studying them. Separation and identification of individual compounds have not been achieved, and most studies refer to naphthenic acids as a group. Accepted quantification methods include Fourier transform

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infrared (FT-IR) spectroscopy (Jivraj et al. 1995, Holowenko et al. 2001, Yen et al. 2004) and high-performance liquid chromatography (HPLC) (Clemente et al. 2003, Yen et al. 2004). Characterization of naphthenic acids by mass spectrometry (MS) (Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Hsu et al. 2000, Rudzinski et al. 2002, Lo et al. 2003, Barrow et al. 2004) and gas chromatography-electron impact mass spectrometry (GC-MS) (St. John et al. 1998, Holowenko et al. 2001, 2002) can provide qualitative data useful for comparing naphthenic acids from different sources. The total ion chromatogram (TIC) from GC-MS analysis of a naphthenic acids preparation is an unresolved "hump" (St. John et al. 1998, Clemente et al. 2004). Holowenko et al. (2002) presented GC-MS data by plotting relative ion intensities as a function of n and Z values. In this case, only ions having mass-to-charge ratios consistent with plausible naphthenic acid structures were included. The resulting three-dimensional bar graphs illustrate the distribution of compounds in a particular naphthenic acid mixture.

Syncrude Canada Ltd. and Suncor Energy Inc. (Fort McMurray, Alberta, Canada) employ a caustic hot water extraction method for the separation of bitumen from oil sands ore (Schramm et al. 2000). During this process, the release of naphthenic acids from the bitumen into the aqueous phase is enhanced (Schramm et al. 2000, MacKinnon and Boerger 1986). The resulting oil sands tailings water is transported to on-site ponds where it is retained and a portion is recycled back to the plant (Schramm et al. 2000, MacKinnon 1989). Storage of tailings water is part of the "zero discharge" policy specified in the licenses of operating companies. Currently, there is more than 600×10^6 m^3 of tailings water stored at Syncrude's Mildred Lake site.

Naphthenic acids are acutely toxic to a range of organisms (Headley and McMartin 2004, Clemente and Fedorak 2005). MacKinnon and Boerger (1986) demonstrated that with chemical and microbiological treatment approaches, the toxicity of tailings water could be reduced, presumably by removal or biodegradation of naphthenic acids, although this was not shown directly. Herman et al. (1994) followed biodegradation of naphthenic acids extracted from Mildred Lake Settling Basin (Syncrude) in laboratory cultures and also observed detoxification, as determined by the Microtox method. Clemente et al. (2004) used enrichments of naphthenic acid-degrading microorganisms to biodegrade commercially available naphthenic acids (Kodak Salts and

Merichem). Microtox analyses of culture supernatants revealed a reduction in toxicity after less than 4 weeks of incubation (Clemente et al. 2004).

On the basis of the findings of previous studies, it was hypothesized that naphthenic acids in the oil sands tailings water would be readily biodegraded. However, repeated attempts to extensively biodegrade naphthenic acids from Syncrude, Suncor, and Albian Sands Energy Inc. were unsuccessful using laboratory cultures of tailings water bacteria (Clemente, Scott, Fedorak, unpublished results).

Comparison of three-dimensional plots from GC-MS analyses of some commercial naphthenic acids (Clemente et al. 2004, Clemente and Fedorak 2004) with those of naphthenic acids in oil sands tailings water (Holowenko et al. 2002) clearly shows differences in the relative distributions of high and low molecular mass acids. Commercial naphthenic acids tend to have n from about 7 to 17, whereas naphthenic acids in tailings water have a broader range of n from about 7 to 28.[3] There are also differences in the distribution of Z families within a group of acids sharing the same n. These dissimilarities may account for the differing biodegradation rates and have prompted the current study in which four commercial naphthenic acids preparations were individually added to tailings water from two tailings ponds in order to observe biodegradation patterns resulting from the activities of microbial communities indigenous to these tailings waters. Biodegradation was monitored by measuring the decrease in naphthenic acid concentrations by HPLC and by following changes in the naphthenic acid profile of each mixture using GC-MS.

2.2 Materials and methods

2.2.1 Naphthenic acids

Kodak naphthenic acids ("Kodak acids") (lot 115755A) and Kodak naphthenic acids sodium salt ("Kodak salts") (lot B14C) were purchased from The Eastman Kodak Company (Rochester, NY). The sodium content of the salt preparation was 9 wt %

[3] As discussed in Chapter 6, it is now known that GC-MS analyses of oil sands naphthenic acids (i.e. extracted from OSPW) do not generate accurate profiles due to the misassignment of derivatized hydroxylated naphthenic acids to high carbon numbers (n-values) and incorrect Z-values. The carbon numbers of naphthenic acids in tailings water are actually clustered between 7 and 20 (based on HPLC/QTOF-MS).

(Clemente et al. 2004). Merichem refined naphthenic acids ("Merichem acids") were provided by Merichem Chemicals and Refinery Services LLC (Houston, TX). Fluka naphthenic acids ("Fluka acids") were obtained from Fluka Chemie (Buchs, Switzerland). Total acid number (TAN) for each commercial preparation was determined at the National Centre for Upgrading Technology (Devon, Alberta, Canada) according to American Standard Test Method D664 (American Standard Test Method 2001). The Kodak salts were converted to their acid form prior to submission for TAN analysis. Solubility of the naphthenic acids at different pH was assessed using the Merichem acids preparation (Appendix A).

2.2.2 Tailings waters

Samples of tailings water from active settling basins were provided by Syncrude Canada Ltd. and Suncor Energy Inc. in June 2004. Syncrude tailings water was collected from West In Pit (SCL WIP), whereas the Suncor tailings water was sampled from the Consolidated Tailings pond (Suncor CT).

2.2.3 Incubation methods

Biodegradation experiments were conducted to monitor the loss of naphthenic acids from viable incubations and changes in the naphthenic acids composition during incubation for a total of 40 d (Syncrude tailings water experiment) or 49 d (Suncor tailings water experiment). Microbial communities indigenous to tailings water were the sources of microorganisms used in these experiments.

Individual stock solutions of the four commercial naphthenic acid mixtures were prepared at approximately 1 g/L in dilute NaOH. Solution pH was adjusted to between 10 and 11, to dissolve the naphthenic acids as sodium naphthenates.

All incubations had a final liquid volume of 200 mL in 500-mL Erlenmeyer flasks. Incubations of each combination of the tailings water samples and the four commercial naphthenic acids were prepared in triplicate with 180 mL of well-mixed Syncrude or Suncor tailings water plus 10 mL stock naphthenic acid solution. Each incubation was also supplemented with 10 mL modified Bushnell-Haas medium (Wyndham and Costerton 1981) to ensure nitrogen and phosphorus were not limiting

nutrients. The initial concentrations of N and P were 1 and 0.7 mM, respectively, and the total naphthenic acids concentration ranged from 30 to 100 mg/L.

Four sets of positive control flasks (one for each commercial preparation) containing only commercial naphthenic acids were prepared in triplicate by adding 20 mL stock naphthenic acid solution to 170 mL sterile MilliQ water. An inoculum for each of these flasks was prepared by centrifuging 200 mL tailings water at 12 000*g* for 15 min, discarding the supernatant, and resuspending the resulting pellet in 10 mL modified Bushnell-Haas medium. The entire 10-mL suspension was then transferred to a positive control flask, providing nitrogen and phosphorus as well as viable microorganisms in approximately the same proportion as incubations set up directly in tailings water. Negative controls contained 20 mL filter-sterilized (using Millex-GS, 0.22 μm, Millipore, Bedford, MA) stock naphthenic acids solution and 180 mL sterile MilliQ water. Filter-sterilized tailings water could not be used for this purpose because they contain naphthenic acids.

Viable and sterile controls, with either Syncrude or Suncor tailings water as the only source of naphthenic acids, were also prepared in triplicate. In this case, 190 mL tailings water and 10 mL medium were added to 500-mL Erlenmeyer flasks. Tailings water used for the sterile controls was heat-killed by autoclaving at 121°C, 100 kPa, twice for 20 min, with 24 h between treatments.

Incubations were carried out under aerobic conditions at room temperature (approximately 20°C) on a shaker at 200 rpm. Samples were taken from the incubations and stored at -20 or 4 °C prior to analysis by HPLC or GC-MS.

2.2.4 *Analysis of incubation supernatants*

Naphthenic acids quantification was carried out using the derivatization protocol and HPLC method described earlier (Yen et al. 2004). Individual calibration curves were prepared with each commercial naphthenic acids preparation, and the specific slopes and intercepts from each calibration curve were used to calculate the naphthenic acids concentration in samples containing the corresponding commercial naphthenic acids. Changes in naphthenic acids concentration were plotted against incubation time and the initial biodegradation rates were determined from the slope over the first 10 d of

incubation. Statistical comparisons of the slopes were then done as described by Kleinbaum and Kupper (1998). Final naphthenic acids concentrations (i.e. on the last day of incubation) in the sterile and viable incubations that contained only tailings water were compared by t-test.

GC-MS analyses were conducted to obtain qualitative data showing the naphthenic acids profile in incubation supernatants. Prior to analysis by GC-MS, naphthenic acids were extracted from incubation supernatants and derivatized using previously published methods (Clemente et al. 2004). A GC-MS protocol (Holowenko et al. 2002) was used to generate TICs and average mass spectra of the unresolved "humps" of naphthenic acids.

2.3 Results

2.3.1 Incubations with Kodak Acids and Syncrude tailings water

Figure 2.1 summarizes the naphthenic acids concentrations in various incubations with Kodak acids and Syncrude tailings water. These concentrations were determined by HPLC using Kodak acids to prepare the calibration curves. The Kodak acids were readily biodegraded by bacteria in the Syncrude tailings water. For example, when Kodak acids were the only source of naphthenic acids in the incubations, the concentration was rapidly depleted between days 4 and 7 (Figure 2.1). Sterile controls containing only Kodak acids showed no change in naphthenic acids concentration after 40 d (Figure 2.1).

In contrast, the naphthenic acids that occurred naturally in the Syncrude tailings water proved to be more recalcitrant than the commercial naphthenic acids. The concentrations of these native naphthenic acids in the viable incubations remained nearly equivalent to those in the sterile control throughout the incubation period (Figure 2.1). At the end of the 40-d incubation time, naphthenic acids concentrations in the viable incubations with Syncrude tailings water were about 20% less than in the sterile control with Syncrude tailings water and this difference was significant ($P<0.05$). In another set of incubations, Kodak acids were added to the Syncrude tailings water to give a total initial naphthenic acids concentration of 94 mg/L. Within 7 d of incubation, the naphthenic acids concentration was depleted to 64 mg/L: the approximate concentration

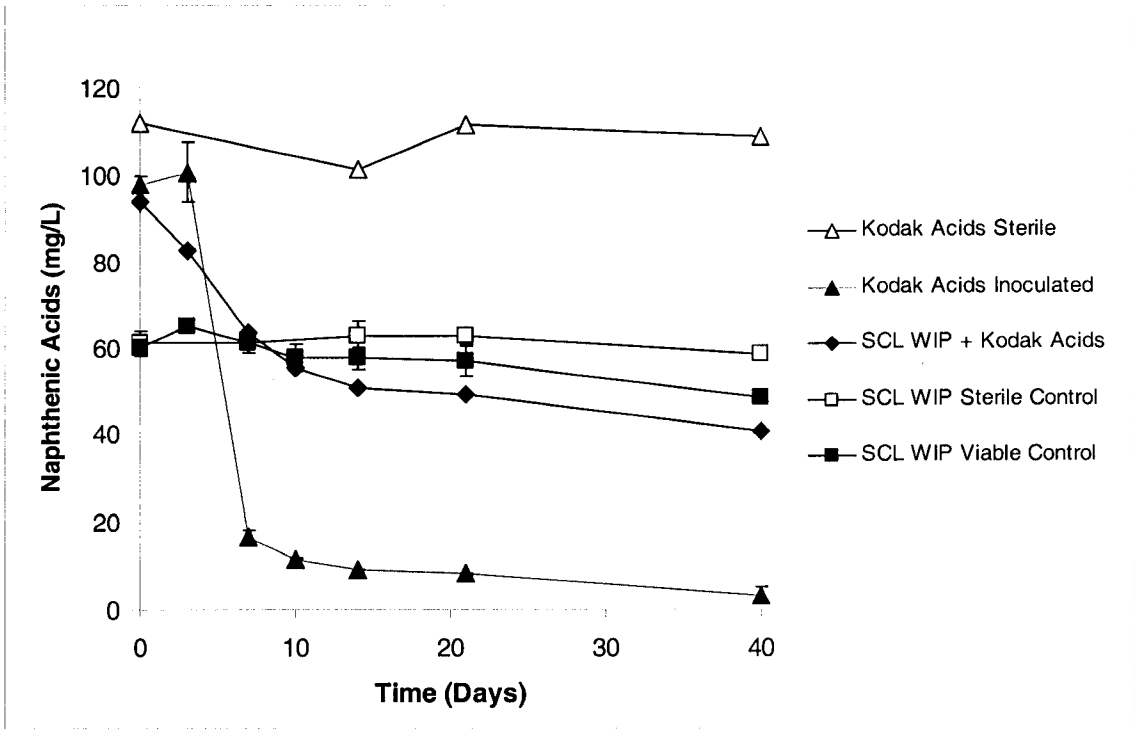

Figure 2.1 Aerobic biodegradation of Kodak acids and Syncrude naphthenic acids (SCL WIP) in laboratory incubations of tailings water bacteria. Naphthenic acid concentrations in Syncrude incubations and controls were determined from a Kodak acids calibration curve. Error bars (often smaller than the symbols) represent one standard deviation from the average of triplicate incubations. Minimum detection limit of the HPLC method is ~5mg/L (Yen et al. 2004).

contributed by the native naphthenic acids in the Syncrude tailings water (Figure 2.1). By the end of the 40-d incubation period, the naphthenic acids concentration in these incubations was about 40 mg/L or 30% lower ($P<0.05$) than in the sterile control with Syncrude tailings water alone. This final concentration was about 15% lower ($P<0.05$) than the final concentration the the viable incubations that only contained Syncrude tailings water. The results in Figure 2.1 suggest that the naphthenic acids in the Syncrude tailings water were more resistant to biodegradation than commercial naphthenic acids.

From the data in Figure 2.1, it appeared that, in the incubations with Kodak acids added to Syncrude tailings water, most of the commercial naphthenic acids were degraded leaving mainly those that originated in the tailings water. To test this hypothesis, extracts of the Kodak acids, Syncrude tailings water, and an incubation

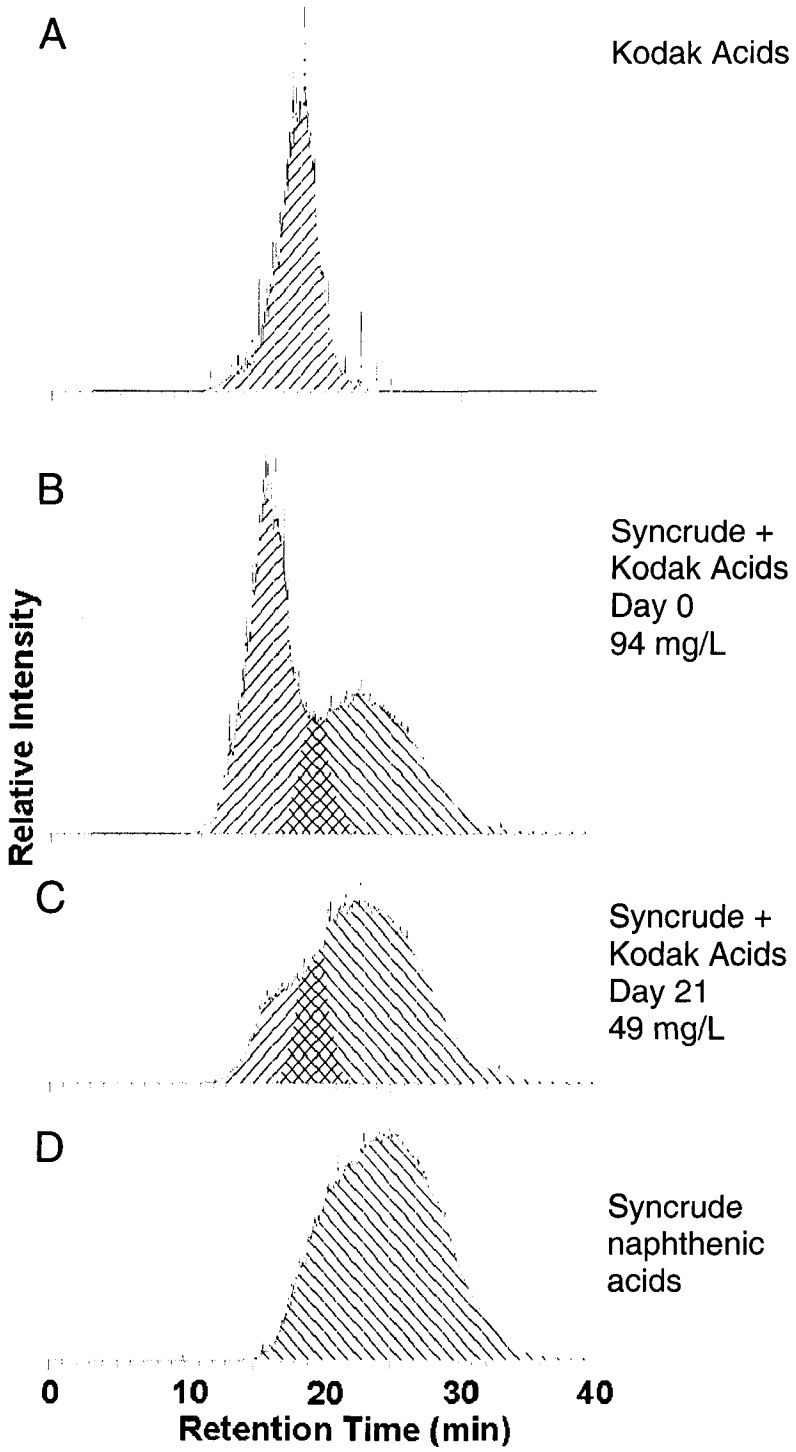

Figure 2.2 TICs for (A) Kodak acids, (B) Kodak acids plus Syncrude naphthenic acids on day 0, (C) this mixture on day 21, and (D) Syncrude naphthenic acids. Crosshatching illustrates possible overlapping areas of the two naphthenic acids "humps". Average naphthenic acid concentration in incubation supernatants is given for both time-points.

containing Syncrude tailings water amended with Kodak acids were derivatized and analyzed by GC-MS. The "humps" that were observed in TICs of these samples were quite revealing and are shown in Figure 2.2. The TICs for Kodak acids (Figure 2.2A) and Syncrude naphthenic acids (Figure 2.2D) were clearly different. The shape of their respective "humps" and the retention times over which they occurred were visibly distinct. The Kodak acids sample eluted from the GC column with a shorter retention time than the naphthenic acids extracted from Syncrude tailings water. Using this same GC-MS method, Clemente and Fedorak (2004) demonstrated that, in general, lower molecular mass naphthenic acids eluted earlier than higher molecular mass naphthenic acids. Thus, the Kodak acids mixture has a high proportion of low molecular mass naphthenic acids. The Kodak acids hump was also narrower, suggesting that the composition of naphthenic acids in this mixture is less diverse than the naphthenic acids from the Syncrude tailings water, which gave a wider hump.

The two middle panels of Figure 2.2 show the TICs of extracts from incubations that contained Syncrude tailings water supplemented with Kodak acids. Figure 2.2B is the analysis of the extract taken just after the incubation was started. It showed the presence of two overlapping "humps", corresponding to Kodak acids and naphthenic acids from the Syncrude tailings water. By day 21, the Kodak acids hump had almost completely disappeared (Figure 2.2C). This was attributed to biodegradation because a decrease in the naphthenic acids concentration also occurred between these two times (Figure 2.1). The hump that remained on day 21 (Figure 2.2C) more closely resembled the hump from naphthenic acids in the Syncrude tailings water (Figure 2.2D) than the hump from the Kodak acids (Figure 2.2A). These results, like those from the HPLC analyses (Figure 2.1), are consistent with the preferential biodegradation of the commercial naphthenic acids.

Data used to generate three-dimensional plots, as in Holowenko et al. (2002), (Figure 2.3) indicated a shift in the naphthenic acids composition during incubation. For example, at time zero in incubations with a mixture of Syncrude naphthenic acids and Kodak acids, 65% of the ions corresponded to naphthenic acids with $n \leq 17$. On day 7, the proportion of ions in this n range was 51%, and on day 21 the proportion had

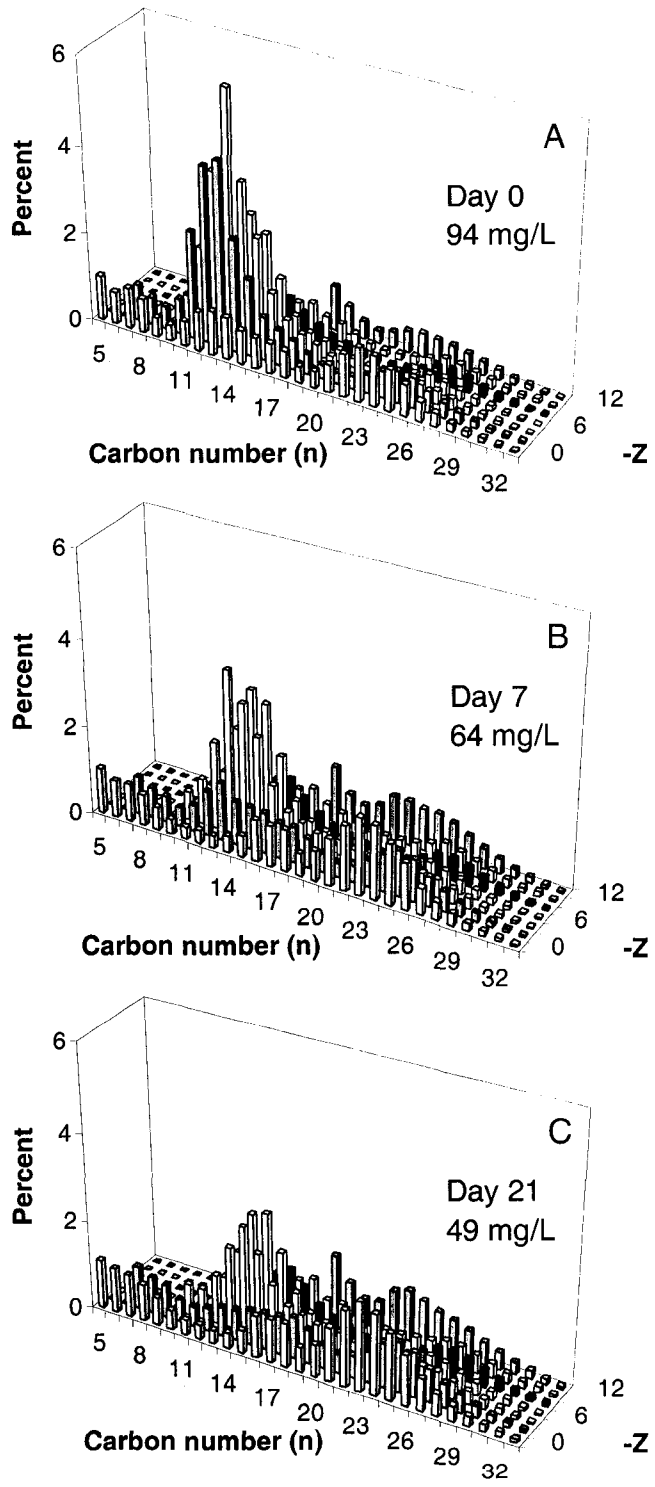

Figure 2.3 Changes in the distribution of residual acids recovered from incubations grown on Syncrude naphthenic acids plus Kodak acids. Results are from GC-MS analyses of samples taken after (A) 0, (B) 7, and (C) 21 d of incubation. The sum of all the bars in each panel is 100%. Average naphthenic acid is concentration is given for each time.

decreased to 44%. These results corroborate the TICs in Figure 2.2B and Figure 2.2C showing the preferential removal of lower molecular mass naphthenic acids.

2.3.2 Incubations with Kodak Acids and Suncor tailings water

Microorganisms from the Suncor tailings water were also able to degrade the Kodak acids samples. Changes in the naphthenic acids concentrations (determined by HPLC with Kodak acids used for the calibration curves) are shown in Figure 2.4. With only the Kodak acids present, the naphthenic acids concentration decreased quickly from 81 to 11 mg/L during the first 10 d, and then remained essentially constant over the rest of the 49-d incubation.

Biodegradation of naphthenic acids in the incubations of Suncor tailings water supplemented with Kodak acids exhibited a trend similar to that observed in the experiment with Syncrude tailings water supplemented with Kodak acids (Figure 2.1). With the supplemented Suncor tailings water, the initial naphthenic acids concentration (68 mg/L) decreased rapidly during the first 14 d of incubation when it reached the concentrations (37 mg/L) measured in the sterile controsl and viable incubations that contained only the Suncor tailings water. By day 49, the concentration of residual naphthenic acids in the Kodak acid-supplemented Suncor tailings water incubations had dropped to about 26 mg/L, which was equivalent to the concentration in the viable incubations that contained only Suncor tailings water ($P=0.915$) and 25% less ($P<0.05$) than the concentration in the sterile control that contained only Suncor naphthenic acids (Figure 2.4).

Initially, there was little change in the concentration of naphthenic acids in the incubations that contained only the Suncor naphthenic acids (Figure 2.4). However, after 49 d, the concentration in these incubations had decreased to approximately 25% less than that in the sterile control, which was similar to the extent of biodegradation in the incubations with Suncor tailings water amended with Kodak acids. Overall, the results from the HPLC analyses suggested that the naphthenic acids in the Suncor tailings water were less susceptible to biodegradation than the Kodak acids.

Figure 2.5 compares the TICs from GC-MS analyses of incubation supernatants. The "humps" shown in Figure 2.5A and D are from the Kodak acids and the naphthenic

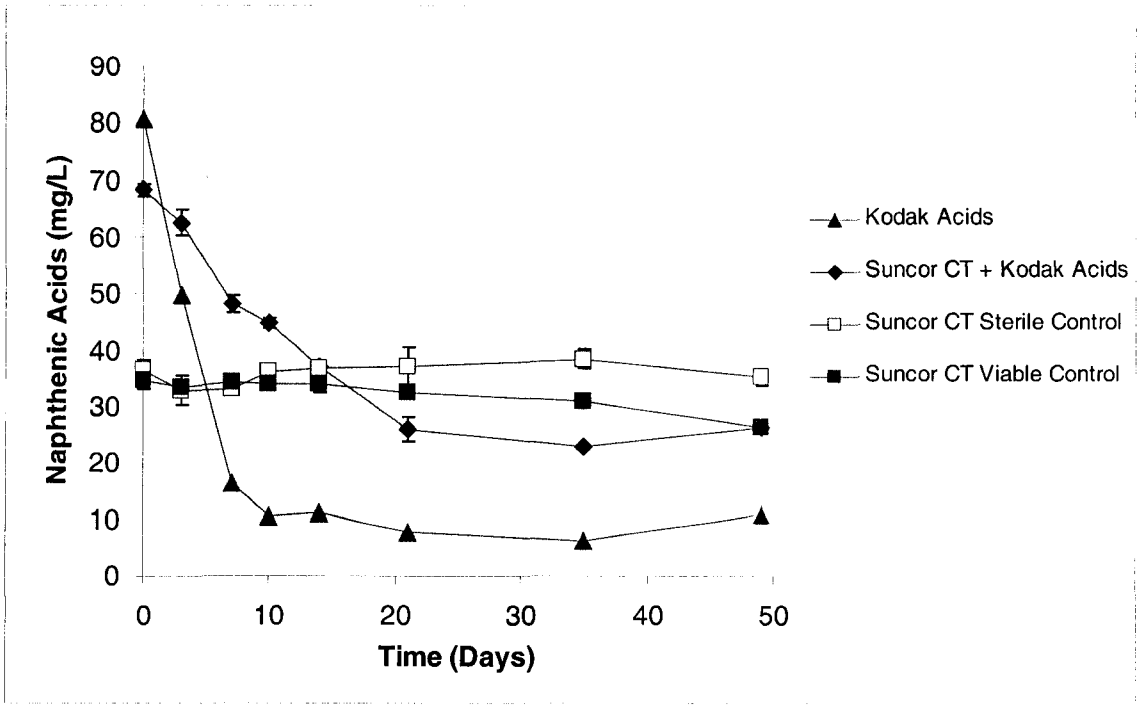

Figure 2.4 Aerobic biodegradation of Kodak acids and Suncor naphthenic acids in laboratory incubations of tailings water bacteria. Naphthenic acid concentrations in Suncor incubations and controls were determined from a Kodak acids calibration curve. Error bars (often smaller than the symbols) represent one standard deviation from the average of triplicate incubations. Minimum detection limit of the HPLC method is ~5mg/L (Yen et al. 2004).

acids in the Suncor tailings water, respectively. The day 0 sample from the incubation containing Suncor tailings water amended with Kodak acids (Figure 2.5B) shows a combined hump composed of the naphthenic acids shown in Figure 2.5A and D. After 49 d of incubation, the naphthenic acids concentration had decreased to about one-third of the original concentration, and the composition had changed, as evident by the TIC (Figure 2.5C). The hump with the shorter retention time (corresponding to the Kodak acids) disappeared, and the residual hump had a different shape than the original Suncor naphthenic acids hump (Figure 2.5D), particularly in the material that eluted with retention times between 10 and 25 min. These losses of the early eluting naphthenic acids are consistent with the preferential biodegradation of the lower molecular mass naphthenic acids.

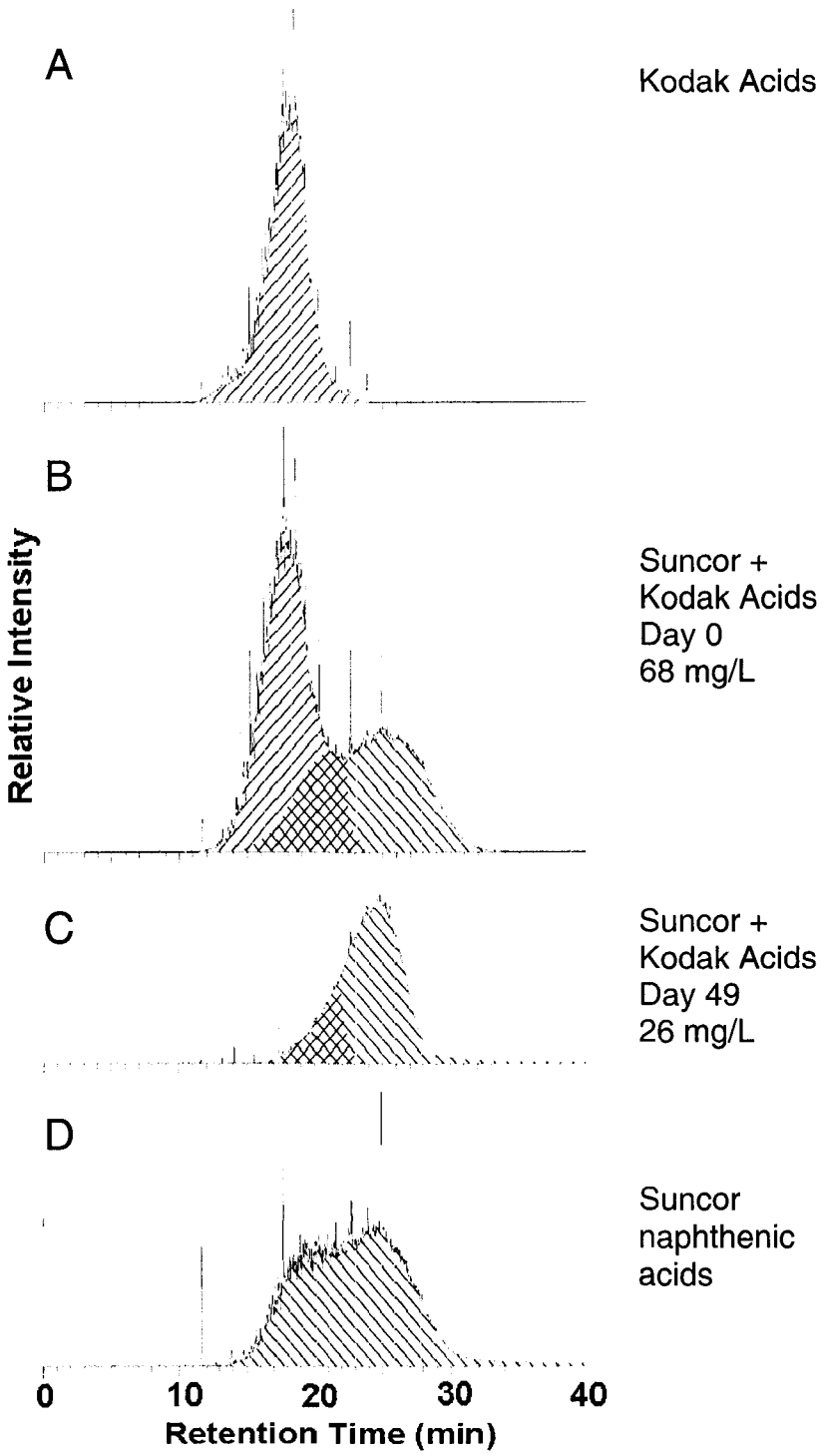

Figure 2.5 TICs for (A) Kodak acids, (B) Kodak acids plus Suncor naphthenic acids on day 0, (C) this mixture on day 49, and (D) and Suncor naphthenic acids. Crosshatching illustrates possible overlapping areas of the two naphthenic acid humps. Average naphthenic acid concentration in incubation supernatants is given for both time-points.

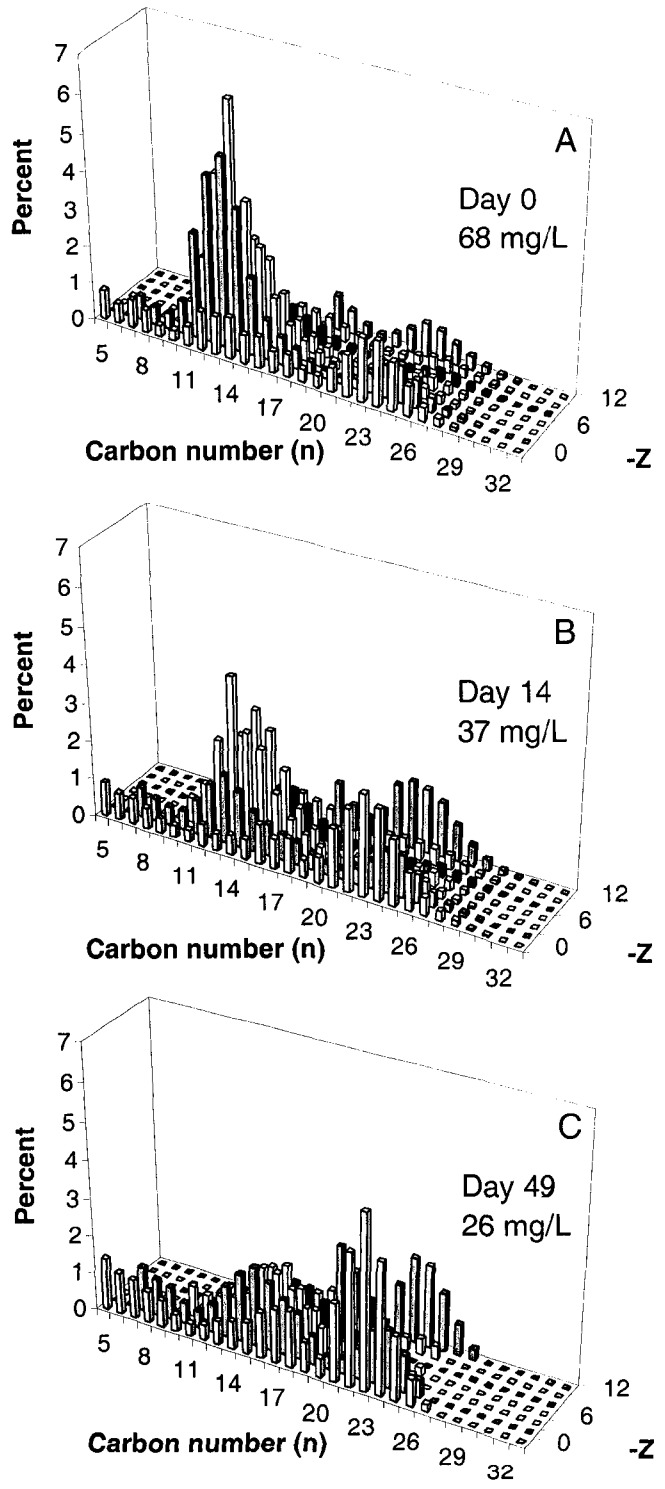

Figure 2.6 Changes in the distribution of residual acids recovered from incubations grown on Suncor naphthenic acids plus Kodak acids. Results are from GC-MS analyses of samples taken after (A) 0, (B) 14, and (C) 49 d of incubation. The sum of all the bars in each panel is 100%. Average naphthenic acid is concentration is given for each time.

Analyses of the data from three-dimensional plots (Figure 2.6) illustrate the preferential removal of the lower molecular mass naphthenic acids over the 49-d incubation time. For example, at time zero, 68% of the ions corresponded to naphthenic acids with $n \leq 17$. On day 14, 53% of the ions corresponded to naphthenic acids with $n \leq 17$, and on day 49, 47% of the ions corresponded to naphthenic acids with $n \leq 17$.

2.3.3 Results from incubations with other commercial naphthenic acids

During the early stages of these experiments, the Kodak acids were used to prepare the calibration curves for all of the incubations, regardless of which commercial naphthenic acids preparation was added to the tailings water. However, this gave unreliable results. For example, when an incubation of Syncrude tailings water was amended with 50 mg/L Fluka acids, the results from the calibration curve prepared with Kodak acids showed the naphthenic acids concentration was only 8 mg/L above that in the tailings water. This discrepancy was rectified by preparing individual calibrations curves with each of the four commercial naphthenic acids preparations and using the appropriate calibration curve for analyses of incubations amended with the corresponding commercial naphthenic acids. Table 2.1 summarizes typical parameters from four calibration curves.

The Kodak acids gave a calibration curve with the highest slope, which was nearly double the slope of the Fluka preparation (Table 2.1). It was presumed that the slopes would vary with the TAN of the naphthenic acids preparations because the derivatizing reagent reacts with the carboxylic acid moiety. However, there is no correlation between TAN values and the slopes of the calibration curves (Table 2.1). The reason for the different slopes remains unknown. The consequence of using the four different calibration curves was that concentrations of naphthenic acids measured in the tailings water varied depending upon which commercial naphthenic acids preparation was used for the calibration curve (Table 2.1). For example, the apparent concentrations of naphthenic acids in the Syncrude tailings water ranged from 60 to 97 mg/L.

Despite the difficulties determining the true concentrations of naphthenic acids in these incubations, the HPLC method was useful for following changes in naphthenic acids concentrations over time to determine whether the commercial preparations were

Table 2.1 Total acid number (TAN) values, parameters from typical calibration curves of four different naphthenic acid standards, and naphthenic acid concentrations in tailings water determined with the different naphthenic acid standards

Naphthenic acids standard	TAN (mg KOH/g)	Slope[a] (mAU/ mg/L)	Y-intercept (mAU)	R^2	Initial naphthenic acids concentrations[b] (mg/L)	
					Syncrude tailings water	Suncor tailings water
Kodak acids	264	12.7	212	0.9997	60 ± 1	35 ± 2
Merichem acids	268	10.4	243	0.9982	73 ± 2	42 ± 2
Kodak salts	195	9.1	234	0.9993	69 ± 1	41 ± 2
Fluka acids	235	6.7	225	0.9959	97 ± 4	61 ± 3

[a] mAU = milli-Absorbance Units, mg/L refers to the concentration of naphthenic acids.
[b] Average of triplicates, ± one standard deviation.

more susceptible to biodegradation than naphthenic acids in the oil sands tailings water. Incubations containing each of the commercial naphthenic acids alone and in combination with the naphthenic acids in the two tailings water samples were established and monitored for naphthenic acids concentrations. Results from these eight incubations are summarized in Table 2.2. Plotting the naphthenic acids concentrations over time resulted in graphs that had the same general shape as Figure 2.1. For example, Figure 2.1 shows that after 10 d of incubation there was 88% removal of the Kodak acids when they were the only naphthenic acids in the incubations. This figure also shows that the concentration of naphthenic acids in incubations with Syncrude tailings water amended with Kodak acids reached the concentration in the Syncrude tailings water sterile control after 7 d of incubation. The values "88%" and "7 d" are the entries in the first line of Table 2.2.

Each of the commercial naphthenic acids preparations were biodegraded by microorganisms from the two tailings water samples. After 10 d of incubation, over 80% of the Kodak acids, Merichem acids, and Fluka acids was degraded when they were the

only source of naphthenic acids in the incubations (Table 2.2). The Kodak salts were not biodegraded to the same extent as the other commercial preparations within 10 d of incubation; only 69% of the Kodak salts were degraded by microorganisms from the Syncrude tailings water and 77% were degraded when the source of microorganisms was Suncor tailings water. Similarly, the Kodak-salts amended incubations required 21 d of incubation before the naphthenic acids concentrations decreased to those in the sterile controls with tailings water. Incubation times of ≤ 14 d were required for the same decrease in incubations that were amended with the other commercial naphthenic acids (Table 2.2). On the basis of the results from HPLC analyses, all four commercial naphthenic acids preparations were more readily biodegraded than the naphthenic acids in the two tailings water samples.

Table 2.2 Summary of the biodegradation studies with two tailings water samples and four commercial naphthenic acids preparations.

		Commercial naphthenic acids alone	**Commercial naphthenic acids added to tailings water**
Tailings water	**Commercial naphthenic acids**	**Percent removal after 10 d**	**Time to reach conc. equivalent to tailings water sterile control (d)**
Syncrude	Kodak acids	88	7
	Merichem acids	90	7
	Fluka acids	81	14
	Kodak salts	69	21
Suncor	Kodak acids	87	14
	Merichem acids	93	7
	Fluka acids	87	10
	Kodak salts	77	21

Statistical comparison of biodegradation rates over the first 10 d of incubation showed that Merichem and Fluka naphthenic acids were degraded more rapidly ($P<0.05$) than the Kodak salts when microorganisms from Syncrude tailings water were used as an inoculum. However, the rate of biodegradation for Kodak acids and Kodak salts was not

significantly different when incubated with this inoculum. There were also no significant differences among the biodegradation rates for the four commercial naphthenic acids preparations when Suncor tailings water was the source of microorganisms.

Samples from each of the incubations amended with commercial naphthenic acids were extracted and analyzed by GC-MS. The time zero samples all yielded TICs that were similar to those of Figure 2.2B and Figure 2.5B. That is, two "humps" were apparent: one that eluted early, composed of the commercial naphthenic acids, and one that eluted late, corresponding to the naphthenic acids in the tailings water. Extended incubations resulted in the loss of the early hump, as illustrated by Figure 2.2C and Figure 2.5C. All of these results indicated that the naphthenic acids in the tailings water samples were more persistent than those in the commercial preparations.

2.4 Discussion

The various concentrations of naphthenic acids in a given tailings water sample determined using different commercial naphthenic acids for calibration curves (Table 2.1) demonstrate the difficulties associated with the analysis of naphthenic acids. During the development of the HPLC method, Clemente et al. (2003) did not observe differences in slopes of calibration curves prepared with Kodak acids and Merichem acids as standards. Yen et al. (2004) improved this HPLC method, but they did not determine the slopes of calibration curves with various commercial naphthenic acids preparations. The results in Table 2.1 are the first evaluation showing how the measured concentrations of naphthenic acids in tailings water samples determined using the HPLC method can be affected by the commercial naphthenic acids preparation chosen for the calibration curve. The reason for this difference is yet to be determined, but the slopes of the calibration curves are not related to the TAN values (Table 2.1) of the naphthenic acids preparations. The oil sands industry standard method for measuring naphthenic acids concentration in water samples uses a FT-IR spectroscopy method (Jivraj et al. 1995) with Kodak acids as the calibration standard. Using the same calibration standard, Yen et al. (2004) showed that the HPLC method was in good agreement with the FT-IR method.

GC analyses of complex mixtures often produce "humps" in the chromatograms, commonly known as unresolved complex mixtures (Blumer et al. 1970, Ahmed et al.

1998, Reddy et al. 2002, Frysinger et al. 2003). Little detailed information can be obtained from these "humps", but they can be quite different with respect to their shape and retention times. For example, Frysinger et al. (2003) presented two GC chromatograms of organic materials extracted from different marine sediments. The "humps" in both chromatograms were distinctly different from each other. Likewise, the TICs of naphthenic acids presented in Figures 2.2 and 2.5 show distinctly different shapes and retention times. These TICs were used to glean information about the naphthenic acids extracted from our various laboratory incubations.

On the basis of GC-MS analyses of Syncrude tailings water samples that had aged for various lengths of time in pits that receive no fresh input of tailings water, Holowenko et al. (2002) hypothesized that naphthenic acids with $n \leq 21$ are more susceptible to biodegradation than those with $n \geq 22$. Figures 2.2 and 2.5 illustrate that the naphthenic acids with the shorter retention times and lower molecular masses are biodegraded more rapidly than those with the longer retention times.

Using three-dimensional plots to summarize the data from the GC-MS can be a convenient method to observe differences between naphthenic acids preparations (Holowenko et al. 2002). For example, the naphthenic acids in oil sands tailings water are typically composed of a wide range of molecular mass, with n = 5 to 28 (Holowenko et al. 2002) or even 40 (Lo et al. 2003).[4] In contrast, commercial preparations are often composed of mainly low molecular mass naphthenic acids, with $n \approx 10$ to 14 (Clemente et al. 2003, 2004). This narrow range of molecular mass in the commercial naphthenic acids depends on the boiling range of the petroleum fractions from which the naphthenic acids are recovered. Kodak salts are somewhat different than the other commercial naphthenic acids because the majority (80%) of naphthenic acids in these salts fall in the n = 14 to 21 range. On the basis of the molecular mass distributions from GC-MS analyses, the Kodak salts more closely resemble the naphthenic acids in Syncrude tailings water (Clemente et al. 2003) and Suncor naphthenic acids (data collected during this study) than do any of the other three commercial preparations.

The data in Table 2.2 show that, among the commercial preparations, Kodak salts were most resistant to biodegradation by microorganisms in the tailings water from

[4] See footnote 3, page 72.

Syncrude and Suncor. This is consistent with the fact that the Kodak salts contain higher molecular mass naphthenic acids than the other three commercial preparations. In addition, the Kodak salts contain a high proportion of multi-ring acids, similar to the naphthenic acids in the tailings waters. The proportion of ions that correspond to 3-, 4-, and 5-ring acids (Z = -6, -8, and -10, respectively) comprises 23% of the ions detected in the GC-MS analysis of these salts, and the proportions of 3- to 5-ring acids in the Syncrude and Suncor naphthenic acids are 37% and 35%, respectively. By comparison, the proportions of ions that correspond to 3- to 5-ring acids in the Fluka, Merichem, and Kodak acids are only 16%, 8%, and 9% of the total ions, respectively. These comparisons suggest that the structures of the naphthenic acids in the Kodak salts are more complex than those in the other three commercial preparations.

In contrast to the data presented in Table 2.2 for the Kodak salts, Clemente et al. (2004) showed that the Kodak salts were nearly completely removed from laboratory incubations within only 10 d. However, Clemente et al. (2004) used an enrichment culture that had been maintained for several months by repeated transfer to fresh medium with Kodak salts, increasing the biodegradative capability of the culture. No enrichment procedure was used in the current study. Instead, the commercial preparations were inoculated on day 0 with a suspension of microorganisms taken directly from the tailings water samples.

In one study, carboxylic acid fractions were separated from 33 crude oils, including some crudes that were biodegraded and some that were not biodegraded (Meredith et al. 2000). In general, the greater the degree of biodegradation, the higher the concentration of carboxylic acids in the crude oils. Bitumen in the Athabasca oil sands deposit is generally believed to be the residue of conventional crude that has undergone extensive biodegradation (Hunt 1979). Thus, the presence of naphthenic acids in the oil sands is expected. Recently, Clemente (2004) reported that the average naphthenic acids concentration in seven oil sands ore samples from Syncrude was 200 mg/kg of ore. Presumably, as naphthenic acids in the oil sands "incubated" over geological time, those acids that were most susceptible to biodegradation would have been degraded, leaving mainly the recalcitrant naphthenic acids in the ore. These would be released during the

alkaline, hot water extraction process used to recover the bitumen (Schramm et al. 2000), and the recalcitrant naphthenic acids would remain in the tailings water.

In laboratory studies, Watson et al. (2002) subjected a weathered, light Arabian crude oil to microbial degradation under aerobic conditions for up to 80 d. They observed that, after extensive biodegradation of the crude oil, there was an increase in the concentrations of branched and cyclic carboxylic acids with $n > 20$. These eluted from the GC as a hump, and they were considered to be naphthenic acids. These acids resisted further biodegradation for the duration of the experiment. The observed persistence of the high molecular mass naphthenic acids in our incubations is consistent with the findings of Watson et al. (2002).

Several biodegradation studies (Herman et al. 1994, Lai et al. 1996, Clemente et al. 2004) have used commercial naphthenic acids preparations as surrogates for naphthenic acids in tailings water. At the time that Herman et al. (1994) did their biodegradation studies, the GC-MS method used in the current study was not available for monitoring changes caused by microbial metabolism. Microbial activity was monitored by measuring microbial respiration and the release of CO_2 from the naphthenic acids (or other organic compounds) in their cultures (Herman et al. 1994). In retrospect, the results reported by Herman et al. (1994) showed the same trends as those of the current study. For example, microbial cultures oxidized 48% of the carbon from the Kodak salts to CO_2, whereas they oxidized only 20% of the carbon in a naphthenic acids extract from tailings water to CO_2 (Herman et al. 1994). These results demonstrated that the commercial naphthenic acids are more susceptible to biodegradation than the naphthenic acids in tailings water, as shown in Figure 2.1 and Figure 2.4.

Because it is now known that commercial naphthenic acids generally have lower molecular masses than naphthenic acids in tailings water,[5] it is not surprising that the latter are more resistant to biodegradation. Our investigation has shown that commercial naphthenic acids, with predominantly low molecular mass acids, are not appropriate surrogates for predicting the biodegradability of naphthenic acids in the tailings water

[5] See footnote 3, page 72. Based on the information mentioned in footnote 3 and data generated using a novel HPLC/QTOF-MS method (see Appendix C), differences in the biodegradability of commercially-available and oil sands naphthenic acids are now being attributed to the chemical structures of oil sands naphthenic acids being less susceptible to microbial metabolism due extensive alkylation and branching. See Section 6.3.2 for more details.

because the commercial naphthenic acids are much more readily biodegraded than the naphthenic acids in the tailings water. Thus, subsequent studies should use naphthenic acids from oil sands sources to accurately assess their biodegradability. Because of the demonstrated recalcitrance of the high molecular mass naphthenic acids in tailings water, oil sands companies are exploring new methods to remove the toxicity of these compounds.

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3 PARTIAL OXIDATION OF NAPHTHENIC ACIDS IN AN OIL SANDS PROCESS WATER

3.1 Introduction

Naphthenic acids are a mixture of naturally-occurring, aliphatic or alicyclic carboxylic acids that are described by the general formula $C_nH_{2n+Z}O_2$, where n represents the number of carbons in the molecule and Z specifies hydrogen deficiency in the case of cyclic naphthenic acids (Brient et al. 1995). Some possible naphthenic acid structures are shown in Chapter 1 (Figure 1.3). Naphthenic acids are thought to have originated from aerobic microbial degradation of petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Watson et al. 2002) and are found in petroleum reservoirs ubiquitously (Brient et al. 1995, Seifert and Teeter 1969, Seifert et al. 1969, Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Jones et al. 2001, Tomczyk et al. 2001, Qi et al. 2004).

Naphthenic acids are also present in Athabasca oil sands ores (Clemente et al. 2003, Clemente 2004), which contain a viscous, tar-like form of petroleum known as bitumen. Bitumen recovery involves extraction of the oil sands ore with caustic hot water and during this process naphthenic acids become dissolved in the aqueous phase (MacKinnon and Boerger 1986, Schramm et al. 2000). The resulting oil sands process water (OSPW) is retained on-site in large ponds (MacKinnon 1989) in accordance with the "zero discharge" policy specified in the licenses of operating companies. Storage of OSPW is also necessary because naphthenic acids are acutely toxic to a range of organisms (Headley and McMartin 2004, Clemente and Fedorak 2005). To date, more than 600 x 10^6 m^3 of OSPW are stored at Syncrude's Mildred Lake site.

The recalcitrance of oil sands naphthenic acids discussed in Chapter 2 has since been confirmed by Del Rio et al. (2006), who observed limited biodegradation of the naphthenic acids using an inoculum of sediment microorganisms (from an OSPW storage pond) that were enriched for growth on commercial naphthenic acids. The biorefractory nature of oil sands naphthenic acids was an unanticipated challenge in the remediation of OSPWs generated by the oil sands companies. Biodegradation is generally the most cost-

effective way to mitigate toxicity and other undesirable characteristics, such as biochemical oxygen demand (BOD), associated with wastewaters contaminated with organic compounds. However, in cases where the organic compounds are recalcitrant, pretreatment of the wastewater with a strong oxidizing agent can improve biodegradability through partial oxidation of the organic compounds. When the oxidation treatment involves the use of ozone or H_2O_2, sometimes in combination with UV light, to generate hydroxyl radicals it is termed an advanced oxidation process (AOP) (Scott and Ollis 1995, Wang et al. 2003, Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). Although AOPs can often completely mineralize the organic compounds in a given wastewater, they are not typically applied for this purpose due to the high cost of the required chemicals (in the case of H_2O_2) or energy (in the case of UV and ozone generation). It is more economical to combine an AOP with microbiological treatment, usually in that order. However, if the raw wastewater has biodegradable compounds present in high proportion relative to the recalcitrant compounds, a biodegradation step may precede the AOP to ensure efficient use of the more expensive chemical treatment by removing the biodegradable compounds from the mixture (Scott and Ollis 1995; Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). The recalcitrant compounds may then be completely oxidized using an AOP or partially oxidized and subjected to another round of biodegradation, provided the oxidation step improved their biodegradability.

AOPs, and particularly ozonation, have been successfully applied for improving the biodegradability of various industrial wastewaters. For example, several research groups have looked at using ozonation to pretreat wastewaters from pulp and paper processing because these wastewaters contain lignins and other poorly-biodegradable organics (Oeller et al. 1997, Helble et al. 1999, Gonzalez et al. 2003, Nakamura et al. 1997, 2004, Bijan and Mohseni 2004, 2005). In all cases, ozonation increased the ratio of BOD to chemical oxygen demand (COD) of the wastewaters, indicating improved biodegradability. One group of researchers showed that ozonation had the greatest effect on compounds of molecular weight >1000 Da, which were thought to include the majority of the original recalcitrant fraction (Bijan and Mohseni 2004, 2005). These high molecular weight compounds were likely broken down into smaller, readily-

biodegradable compounds, including organic acids such as maleic, oxalic, and acetic acid, which were detected by Nakamura et al. (1997, 2004) in their ozonation experiments with pulp mill wastewater.

Olive and olive oil processing wastewaters are also good candidates for pre-ozonation due to the presence of polyphenols, which are inhibitory to microbial oxidation but susceptible to ozone oxidation. Several studies have measured improved biodegradability of these wastewaters with limited ozonation (Rivas et al. 2000, Amat et al. 2003, Bettazzi et al. 2006) but there is also evidence that extending ozone treatment past a certain point can actually be detrimental to biodegradability (Amat et al. 2003). Improved biodegradability was found to coincide with decreased concentrations of polyphenols, as predicted, and Amat et al. (2003) identified benzaldehyde and *p*-hydroxybenzoic acid as possible oxidation products using cinnamic acid as model phenolic compound. Both products are more susceptible to aerobic microbial degradation than cinnamic acid.

Oil field drilling wastewater, which is characterized by recalcitrant high molecular weight (> 3000 Da) compounds, also showed improved biodegradability following ozonation (Wang et al. 2004). The improvement coincided with a shift in the proportion of the high molecular weight compounds from 60% of the initial total organic carbon (TOC) to only 8% of the residual TOC.

In Chapter 2, differences in the biodegradability of naphthenic acids in OSPWs and commercially-available naphthenic acids were attributed in part to the oil sands naphthenic acids having a greater proportion of high molecular weight ($n \geq 22$) compounds.[6] Holowenko et al. (2002) also reported that aged OSPW ponds (i.e. those that did not receive fresh OSPW for 7 or more years) had retained high molecular weight naphthenic acids whereas naphthenic acids with $n < 22$ were present in lower proportion than in fresh OSPW. Holowenko et al. (2002) proposed that oil sands naphthenic acids with $n < 22$ were more susceptible to biodegradation.[6] Based on the trends observed in

[6] As discussed in Chapter 6, it is now known that GC-MS analyses of oil sands naphthenic acids (i.e. extracted from OSPW) do not generate accurate profiles due to the misassignment of derivatized hydroxylated naphthenic acids to high carbon numbers (n-values) and incorrect Z-values. Based on this new information and data generated using a novel HPLC/QTOF-MS method (see Appendix C), differences in the biodegradability of commercially-available and oil sands naphthenic acids are now being attributed to the chemical structures of oil sands naphthenic acids being less susceptible to microbial metabolism due extensive alkylation and branching. See Section 6.3.2 for more details.

the ozonation studies described above, partial oxidation by ozone may be an effective method for breaking down the high molecular weight oil sands naphthenic acids into smaller, more readily-biodegradable compounds.

To investigate the potential for using ozone to pretreat OSPW, ozonation was conducted in a SEAIR diffusion system. This system employs a proprietary technology that enhances the mass transfer of gases into fluids in order to generate what the company terms a "supersaturate solution" (www.seair.ca). Samples taken before, during, and after treatment were analyzed by FT-IR spectroscopy, HPLC, and GC-MS in order to monitor changes in the naphthenic acids concentration and profile. The parameters of BOD, COD, and TOC were also measured.

3.2 Materials and methods

3.2.1 Sampling and preparation of OSPW

OSPW was sampled from the clarified zone of the Recycle Water Pond at Syncrude Canada Ltd., Fort McMurray, AB. The water was filtered using Zenon's ZeeWeed ZL-10 system with a standard membrane that excludes all particles nominally larger than 0.04 microns (www.zenon.com). The sediment-free OSPW had a pH of ~8 and was stored at 4°C.

3.2.2 Ozone treatments using a SEAIR diffusion system

Approximately 100 L of the filtered water was put into a SEAIR diffusion system (model no. SA28) equipped with an ozone generator (SEAIR Diffusion Systems Inc., Edmonton, AB). Air was passed through an oxygen concentrator and the captured oxygen was then passed through the ozone generator to produce ozone. Ozone was fed into the diffusion system, producing micro-bubbles (5 μm) of ozone that were fed into the reactor. The dissolved ozone concentration in the reactor was approximately 35 mg O_3/L. Ozonation was conducted at room temperature (~20°C) for 130 min and samples were taken after 0, 11, 50, and 130 min. The 11- and 50-min samples were sparged with nitrogen immediately after sampling to purge residual ozone. All samples were stored at 4°C. These treatments were conducted by Warren Zubot (Syncrude Canada Ltd.).

3.2.3 *Analysis of water samples*

The naphthenic acids concentration in samples was quantified by HPLC according to the method of Yen et al. (2004) except that the baseline hold was turned off and integration was started at 3.1 min instead of 2.9 min. Naphthenic acids were extracted from samples using the method of Clemente et al. (2004) and GC-MS analyses were conducted as described by Holowenko et al. (2002). Two-sided t-test comparisons of GC-MS data were performed using the method of Clemente et al. (2003). BOD, COD, TOC, and routine water chemistry (inorganic) analyses were carried out by EnviroTest Laboratories (now ALS Environmental), Edmonton, AB, according to American Public Health Association Standard Methods (Standard Methods 1999) for the analysis of water and wastewater. BOD was determined based on a 5-d incubation period. FT-IR spectroscopy was done by EnviroTest Laboratories, Winnipeg, MB, using the method of Jivraj et al. (1995).

Microtox™ toxicity assays were conducted using the Basic protocol to measure changes in toxicity with ozonation (Microbics Corporation 1991). A Microbics model M500 (Strategic Diagnostics Inc., Newark, DE, USA) was used to measure the luminescence of reconstituted *Vibrio fischeri* before and 15 min after exposure to each sample. The percent v/v of sample that caused 20 and 50% decreases in luminescence was determined and reported as the IC_{20} and IC_{50} values respectively. Phenol toxicity was tested as a positive control prior to the analysis of samples and the measured IC_{50} was within the recommended range of 13 to 26 mg/L (Microbics Corporation 1991).

3.3 Results and discussion

Treatment with ozone in the SEAIR reactor for just over 130 min resulted in a greater than 80% decrease in the apparent naphthenic acids concentration, according to both the HPLC and FT-IR quantification methods (Figure 3.1). To the author's knowledge this is the first experimental evidence that ozone can react with naphthenic acids. Samples taken during ozonation were less colored than the non-ozonated water. Aqueous solutions of oil sands naphthenic acids are typically a transparent pale yellow or beige color and, depending on pH and the concentration of naphthenic acids, may also

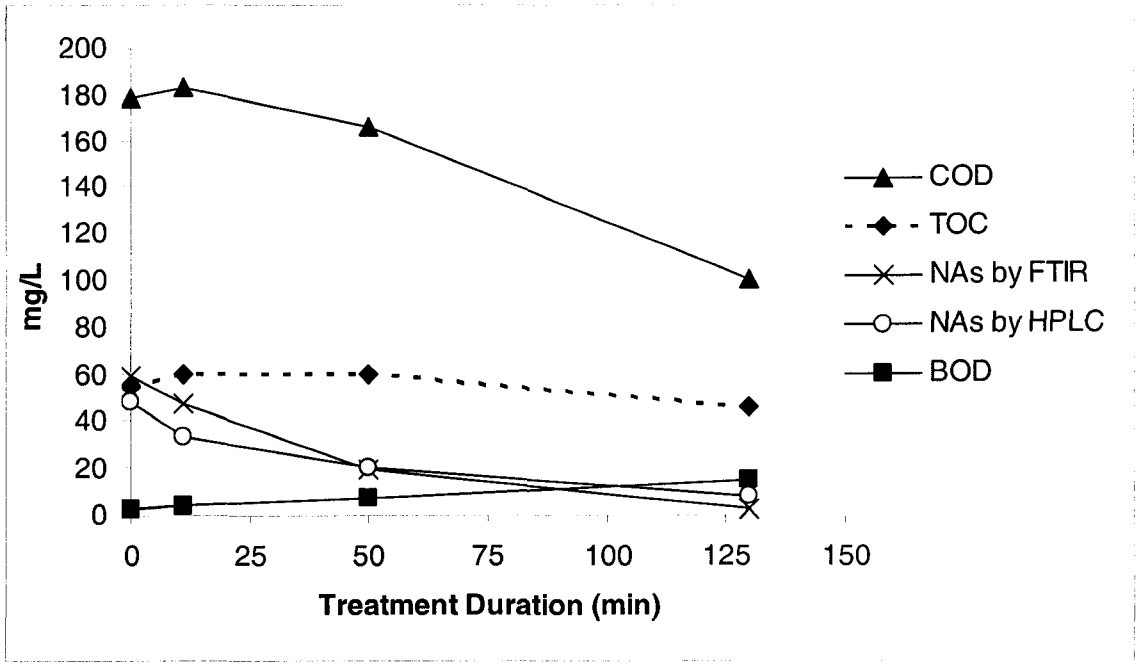

Figure 3.1 The effect of ozonation in a SEAIR diffusion system on TOC, BOD, COD, and apparent naphthenic acids (NAs) concentration in a sample of pre-filtered Syncrude OSPW. Except for HPLC data, all results provided by EnviroTest Laboratories (now ALS Environmental). Sample analyses were performed in singlet.

appear somewhat cloudy. The disappearance of color observed in this study reflects the change in the apparent naphthenic acids concentration due to oxidation through the ozonation process.

COD nearly halved over the treatment duration whereas BOD increased from 2 to 15 mg/L (Figure 3.1). BOD/COD increased from 0.01 to 0.15. This indicated that treatment with ozone met the objective of the experiment: to improve biodegradability of the mixture. It is possible that manipulating certain conditions, such as pH and temperature, could achieve an even greater increase in BOD/COD. The increase measured here (0.14) was within the range reported in the literature; Oeller et al. (1997) and Helble et al. (1999) calculated that BOD/COD increased by about 0.3 after ozonation of pulp and paper wastewaters, whereas Gonzalez et al. (2003) and Lin et al. (2001) measured increases of less than 0.1 following ozonation of bleaching wastewater and petrochemical wastewater, respectively. However, it is difficult to make comparisons between these studies and the current study because the target compounds were different

in each case. Furthermore, the current study was only intended to provide a preliminary assessment of ozonation as treatment option for OSPW and, therefore, reaction conditions were not necessarily optimal. It is perhaps more useful to consider what constitutes a "good" BOD/COD value. Municipal wastewaters, which are considered treatable by microbiological processes, typically have BOD/COD values between 0.3 and 0.8 (Metcalf & Eddy, Inc. 2003). Ozonation of the OSPW for 130 min did not increase BOD/COD of the water to within this range but it is possible that better results could be obtained with optimization.

TOC remained fairly constant during ozonation, decreasing by less than 15%, from 54 to 46 mg/L, after 130 min (Figure 3.1). Figure 3.1 clearly shows that the residual TOC was comprised of compounds other than naphthenic acids because the naphthenic acids concentration was <10 mg/L whereas TOC was 46 mg/L. This suggested that the naphthenic acids were not being completely mineralized but were instead being converted to other carbon forms. Other studies have also reported little change in TOC with ozonation. For example, ozonation of pulp and paper process waters resulted in overall TOC decreases of less than 20% for the specific ozone dosages and contact times tested (Gonzalez et al. 2003, Bijan and Mohseni 2004, 2005). On the other hand, Nakamura et al. (2004) reported that the TOC of a solution containing a model lignin decreased by approximately 50% during the first 30 min of ozonation and then remained fairly constant for the next 30 min. Wang et al. (2004) observed a steady decrease in TOC with ozonation to less than 50% of the initial TOC. Had ozonation been continued, it is possible that TOC would have decreased further. Differences in the reaction conditions, contact time, ozone dosage, and wastewater composition are likely responsible for the variable effect of ozonation on TOC observed in these studies.

The concentrations of trace metals including sodium, calcium, potassium, and magnesium did not change with ozonation (data not shown). Similarily, the concentrations of chloride, sulfate, carbonate, and bicarbonate remained constant during ozonation (data not shown). These results indicated that ozonation had no effect on the major inorganic constituents of the OSPW.

Changes in the toxicity of the OSPW during ozonation were evaluated using the Microtox™ method. IC_{50} values were all >100%, and therefore could not be used to

Table 3.1 Microtox™ toxicity data for samples taken during ozonation of sediment-free Syncrude OSPW in a SEAIR reactor.

Treatment duration (min)	IC_{20} (%v/v)[a]
0	23
11	31
50	>100
130	>100

[a] The amount of sample (volume percent) required to reduce bioluminescence of the test bacterium by 20% (IC_{20}), based on a 15-min bioassay.

monitor the change in toxicity. However, the IC_{20} values changed with ozonation, and these served as a measure of toxicity removal. A slight increase in IC_{20}, corresponding to reduced toxicity, was observed after only 11 min of treatment (Table 3.1) and, after 50 min, the water was completely detoxified. Although these results indicated that ozonation alone could mitigate toxicity of the OSPW, such a treatment process might not be economically feasible for handling the huge volumes of process water produced by the oil sands companies, due to the high cost of generating ozone. Interestingly, the water was non-toxic after 50 min of ozonation despite a residual naphthenic acids concentration of about 20 mg/L (based on both HPLC and FT-IR quantification). This showed that complete degradation of naphthenic acids was not necessary for producing a non-toxic effluent. Holowenko et al. (2002) showed that the toxicity of oil sands naphthenic acids was related not only to concentration but was also dependent on the size of the naphthenic acids. Specifically, OSPWs that contained a greater proportion of naphthenic acids with $n \geq 22$ were less toxic than those characterized by lower molecular weight naphthenic acids.[7]

Figure 3.2 shows three-dimensional plots of the naphthenic acids present before and at two times during ozonation. These plots were generated from GC-MS data using the method of Holowenko et al. (2002). The 11-min sample is not shown because the

[7] See footnote 6, page 99. Based on this new information, these data are no longer valid for for drawing conclusions about toxicity in relation to the naphthenic acids fingerprint of a given sample.

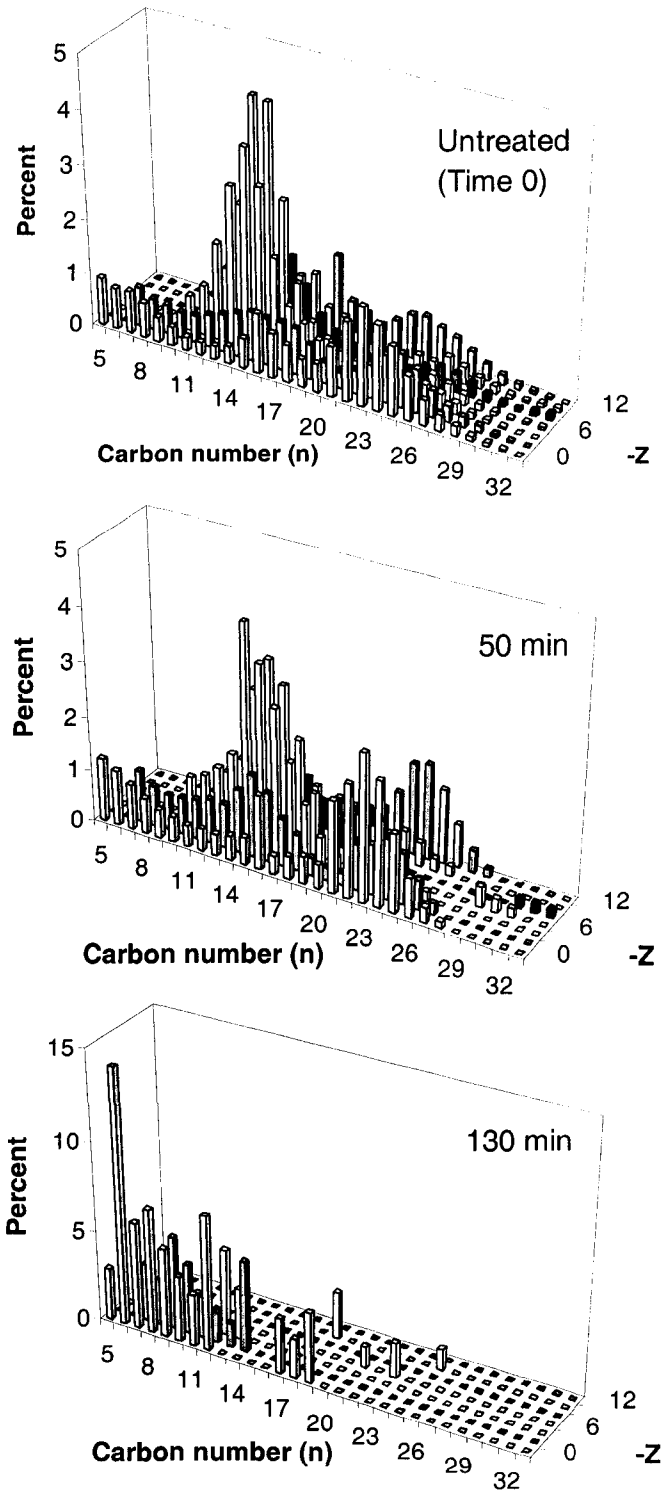

Figure 3.2 Three-dimensional plots showing changes in the distribution of naphthenic acids in Syncrude OSPW with ozone treatment in a SEAIR reactor. Note the change in y-axis scale in the third panel. The sum of the bars in each panel is 100%.

naphthenic acids profile was almost identical to that of the untreated water. The naphthenic acids profile after 50 min of ozonation was also very similar to the profile of the untreated water, but some changes were observed, particularly in the higher carbon-number ($n \geq 20$) range with $Z = -4$, -6 and -8. After 130 min of ozonation, the naphthenic acids profile had a very different distribution than that of the untreated water (Figure 3.2).

The residual ions were clustered between $n = (5 \text{ to } 15)$ with $Z \geq -4$. This shift in the distribution of naphthenic acids towards a greater proportion of low molecular weight compounds is comparable to trends observed during ozonation of pulp and paper process wastewaters (Bijan and Mohseni 2004, 2005), as well as oil field drilling wastewater (Wang et al. 2004).

To simplify comparison of the GC-MS data, three carbon-number "groups" were defined and the proportion of ions falling into each group was determined for the untreated water and the 50- and 130-min samples (Table 3.2). It was expected that the 50-min sample would contain a larger proportion of ions assigned to the $n = (22 \text{ to } 33)$ group because this sample was non-toxic (Table 3.1) and a previous investigation of the toxicity of naturally-aged OSPW from various ponds and wetlands correlated reduced toxicity with abundance of high molecular weight ions (Holowenko et al. 2002).[7] However, the proportion of ions assigned to the $n = (22 \text{ to } 33)$ group was not significantly different ($P=0.89$) from the untreated water, based on two-sided t-test comparison (Table 3.2). In fact, there were no significant changes in any of the three carbon-number groups after 50 min of ozonation. Therefore, the improvement in toxicity could not be correlated with a specific shift in the naphthenic acids distribution, with respect to carbon number. This suggests that the mechanism of detoxification by ozonation is different from the mechanism of "natural aging" in OSPW.

Two-sided t-test comparison revealed that all three carbon-number groups were significantly different ($P<0.05$) from the untreated water after 130 min of ozonation (Table 3.2). Specifically, the group with $n = (5 \text{ to } 13)$ increased in proportion by more than 4-fold from 19 to 79%, whereas the group with $n = (22 \text{ to } 33)$ decreased by 14-fold from 28 to 3%. The proportion of ions in the $n = (14 \text{ to } 21)$ group did not change as much, decreasing from 53% before treatment to 18% after treatment. As mentioned above, Holowenko et al. (2002) observed that toxicity was inversely related to the

Table 3.2 Proportion of ions in each carbon-number group before ozonation and after 50 and 130 min of ozonation in the SEAIR diffusion system.

	Proportion of Ions (Percent)		
Carbon-number (n) group	Non-ozonated (Time 0)	Ozonated (50 min)	Ozonated (130 min)
5 to 13	19	19	79*
14 to 21	53	54	18*
22 to 33	28	27	3*

* denotes significant difference from Time 0 ($P<0.05$).

proportion of naphthenic acids assigned to $n \geq 22$.[7] In the present study, toxicity of the OSPW did not increase between 50 and 130 min of ozonation despite the disappearance of high molecular weight naphthenic acids (Tables 3.1 and 3.2). The reason for this discrepancy was not known.

The untreated (Time 0) and the 50- and 130-min samples were also compared based on the proportion of ions assigned to each Z-family (Table 3.3). There were no significant differences observed after 50 min of ozonation, meaning that the change in toxicity shown in (Table 3.1) could not be correlated with a change in the naphthenic acids distribution according to Z-family. The Z = 0, -6, -8, and -12 families were all significantly different ($P<0.05$) after 130 min of ozonation (Table 3.3). The greatest change was observed for the Z = 0 family, which increased in proportion from 19% before treatment to 58% after treatment. This increase combined with the decreases in other Z-families may be evidence of ozone reacting with the ring structures of cyclic naphthenic acids to produce acyclic naphthenic acids.

A major ion with $m/z = 281$, corresponding to n = 14, Z = -4, was detected in the 130-min sample. This ion was observed previously by Clemente and Fedorak (2004) and was identified as bleed from the GC column. Therefore, this ion was excluded from the three-dimensional plot for the 130-min sample. Presumably the 130-min sample contained a mixture of parent compounds and partially-oxidized intermediates, but this could not be directly confirmed because the current GC-MS method of analysis

Table 3.3 Proportion of ions in each Z-family before ozonation and after 50 and 130 min of ozonation in the SEAIR diffusion system.

	Proportion of Ions (Percent)		
Z-family	Untreated (Time 0)	Ozonated (50 min)	Ozonated (130 min)
0	19	22	58*
-2	15	17	25
-4	21	20	10
-6	21	18	3*
-8	9	7	0*
-10	7	6	4
-12	8	10	0*

* denotes significant difference from Time 0 ($P<0.05$).

(Holowenko et al. 2002) does not permit elucidation of more detailed structural information than what has been described here. Nevertheless, comparison of isomer classes, carbon-number groups, and Z-families did indicate that ozonation in the SEAIR system changed the distribution of naphthenic acids. Moreover, ozonation generated a mixture that, according to the final BOD/COD value, was more biodegradable than the original mixture of naphthenic acids in the OSPW. Future studies will look at optimizing the ozonation procedure.

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4 OZONATION AND BIODEGRADATION OF MERICHEM REFINED NAPHTHENIC ACIDS

4.1 Introduction

The Athabasca oil sands operations in northern Alberta generate vast quantities of process water during recovery and upgrading of bitumen. A major concern associated with this water is the presence of naphthenic acids, which are acutely toxic to a variety of organisms (Headley and McMartin 2004, Clemente and Fedorak 2005). Naphthenic acids are naturally-occurring components of oil sands ore (Clemente et al. 2003, Clemente 2004) that become concentrated in oil sands process water (OSPW) during the caustic hot water extraction process employed for bitumen recovery (Schramm et al. 2000, MacKinnon and Boerger 1986). Petroleum reservoirs throughout the world also contain naphthenic acids (Brient et al. 1995, Seifert and Teeter 1969, Seifert et al. 1969, Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Jones et al. 2001, Tomczyk et al. 2001, Qi et al. 2004), and the predominant theory is that naphthenic acids are the remnants of partially biodegraded petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Watson et al. 2002).

Naphthenic acids are highly-branched, aliphatic or alicyclic carboxylic acids that fit the general formula $C_nH_{2n+Z}O_2$ (Brient et al. 1995). The number of carbons is indicated by n and the number of hydrogens is related to n but must be corrected by Z to account for ring structures. Specifically, Z is zero for acyclic naphthenic acids or a negative, even integer in the case of naphthenic acids that contain one or more rings. Some examples of possible naphthenic acids are shown in Chapter 1 (Figure 1.3).

OSPW is currently stored on-site in massive ponds (MacKinnon 1989) pending the development of suitable treatment methods that will ensure the ecological impact of discharge is negligible. Mitigating the toxicity contributed by naphthenic acids in the water will be critical to achieving this end. Biodegradation was tested in laboratory incubations with microorganisms indigenous to the storage ponds, but oil sands naphthenic acids were poorly biodegraded (Chapter 2).

Chemical oxidation can improve biodegradability of recalcitrant organics by breaking them into smaller compounds that are more susceptible to microbial attack (Scott and Ollis 1995, Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). Chemical oxidation using ozone, commonly termed ozonation, has been tested for pretreatment of poorly-biodegradable organic compounds in various industrial wastewaters including pulp mill wastewater (Oeller et al. 1997, Helble et al. 1999, Gonzalez et al. 2003, Nakamura et al. 1997, 2004, Bijan and Mohseni 2003, 2005), olive processing wastewater (Rivas et al. 2000, Amat et al. 2003, Bettazzi et al. 2006), and oil field drilling wastewater (Wang et al. 2004). Biodegradability of these wastewaters generally improved with ozonation, but two studies showed that continuing ozonation after a certain time actually had an overall negative impact on biodegradability, possibly due to the conversion of readily-biodegradable oxidized intermediates to recalcitrant products (Amat et al. 2003, Wang et al. 2004). Bijan and Mohseni (2003, 2005) and Wang et al. (2004) demonstrated that improved biodegradability coincided with shifts in molecular weight distribution towards smaller compounds. Other studies detected small organic acids (Nakamura et al. 1997, 2004, Amat et al. 2003) and aldehydes (Amat et al. 2003) as readily-biodegradable products of oxidation.

In a recent study, a pre-filtered sample of OSPW was subjected to ozonation in a SEAIR diffusion system (see Chapter 3). Partial degradation of the naphthenic acids was observed within 11 min of ozonation and complete degradation was achieved within 130 min. Residual total organic carbon (TOC) was more biodegradable than the non-ozonated control. The proprietary nature of the SEAIR design technology precluded the ability to control and monitor ozone dosage or other reaction parameters. Therefore, in order to study ozonation of naphthenic acids in greater detail and with more control over experimental conditions, a bench-scale ozone reactor set-up was developed for the present study.

Aqueous solutions of Merichem refined naphthenic acids, a commercially-available preparation, were used as a surrogate for OSPW in four ozonation experiments. A surrogate naphthenic acids mixture enabled starting at a higher initial naphthenic acids concentration than that of the OSPW (< 100 mg/L). Commercial naphthenic acids can be biodegraded by microorganisms from OSPW storage ponds (Clemente et al. 2004,

Chapter 2). It was hypothesized that ozonation would increase the biodegradation rate by generating partially-oxidized, more readily-biodegradable products. Established GC-MS and HPLC methods were used to monitor the effect of ozonation on naphthenic acids composition and concentration, respectively. The Microtox™ method was used to measure changes in toxicity with ozonation. Biodegradation of naphthenic acids was monitored by HPLC and by measuring mineralization.

4.2 Materials and methods

4.2.1 Ozonation of Merichem naphthenic acids

Merichem refined naphthenic acids were provided by Merichem Chemicals and Refinery Services LLC (Houston, TX).

Ozonations were conducted by David Wang and Rodney K. Guest (Civil and Environmental Engineering, University of Alberta). Three reaction types were tested in individual experiments: continuous ozone bubbling, semi-batch reaction with ozone bubbling halted after a set time, and batch reactions using ozone solutions prepared at limiting concentrations (relative to the naphthenic acids concentration).

4.2.1.1 Continuous ozonation

A single trial was conducted in which ozone was bubbled continuously through a Merichem naphthenic acids solution. Briefly, a 1 g/L stock solution of Merichem naphthenic acids was prepared in ozone-demand free (ODF) 0.1 M NaOH. Solution pH was adjusted to between 10 and 11 to dissolve the naphthenic acids as sodium naphthenates. The stock solution was diluted with ODF phosphate buffer (final concentration 30 mM, pH 8) to give a target final naphthenic acids concentration of 100 mg/L. The diluted naphthenic acids solution was then transferred to an ODF glass reactor and ozone was bubbled through the solution for 2 h using an ozone diffuser (8.2% feed gas concentration; flow rate 1.5 L/min). Ozone was generated using a Welsbach Laboratory Ozonator, Model T-816.

Reaction temperature was 19°C. Samples were removed from the reactor at various times during the ozonation and excess sodium thiosulfate was immediately added

to quench residual ozone. The ozone concentration in the reactor was monitored by UV absorption. This experiment was performed by D. Wang. Samples were stored at 4°C prior to HPLC analysis.

4.2.1.2 Semi-batch ozonations

Two semi-batch reactions were conducted in which ozone was bubbled through phosphate-buffered solutions of Merichem naphthenic acids (prepared as described above) for 16 and 37 min, respectively. These two reaction times were predicted to yield 25 and 50% decreases in the apparent naphthenic acids concentration based on the results obtained with continuous ozonation. Reaction conditions were the same as those used in the continuous ozonation experiment. After the desired reaction time elapsed, bubbling was stopped and excess thiosulfate was added to the reaction mixture to quench residual ozone. The ozone concentration in the reactor was monitored by UV absorption. A non-ozonated control was also prepared by adding excess thiosulfate to a solution of Merichem naphthenic acids in phosphate buffer. This experiment was performed by D. Wang. Samples were stored at 4°C prior to HPLC and GC-MS analyses.

4.2.1.3 Batch ozonations

Batch ozonation was tested in two separate experiments. One-liter solutions of ozone were prepared in ODF phosphate buffer (30 mM, pH 8) at approximately 4°C in an ODF glass reactor with a magnetic stir-bar. A lower temperature was used to increase ozone solubility. Ozone doses tested in the first experiment were 20 and 28 mg/L. Ozone doses tested in the second experiment were 20 and 30 mg/L.

A Merichem naphthenic acids stock solution (1 g/L) was prepared as described above and 10 mL was transferred to the reactor with the ozone solution. The two solutions were mixed for 2 to 5 min. Ozone concentration in the reactor was not monitored. Non-ozonated controls were prepared by adding 10 mL Merichem naphthenic acids stock solution to 1 L ODF phosphate buffer (30 mM, pH 8) and mixing for 2 to 5 min. These experiments were performed by R.K. Guest. Samples were stored at 4°C prior to GC-MS (first experiment only) and Microtox™ analyses.

4.2.2 Shake-flask biodegradation study

A shake-flask biodegradation study was conducted to monitor the loss of naphthenic acids and changes in the naphthenic acids composition during the incubation of ozonated and non-ozonated Merichem naphthenic acids solutions with microorganisms indigenous to a storage pond containing OSPW. Samples generated in the first batch ozonation experiment (see Section 4.2.1.3) were used in this study.

All incubations had a final liquid volume of 200 mL in 500-mL Erlenmeyer flasks. Flasks were prepared in duplicate and contained 190 mL of filter-sterilized (Stericup™, 0.22 μm, Millipore, Bedford, MA) ozonated sample or non-ozonated control. An inoculum was prepared by centrifuging 200 mL of OSPW (West In-Pit, Syncrude Canada Ltd.) at 12 000 *g* for 15 min, discarding the supernatant, and resuspending the resulting pellet in 10 mL modified Bushnell-Haas medium (Wyndham and Costerton 1981). The 10-mL suspensions from several identical centrifugations were combined and then 10-mL portions were transferred to each flask, providing nitrogen and phosphorus as well as viable microorganisms. Addition of Bushnell-Haas medium ensured N and P were not limiting nutrients. The initial concentrations of N and P were 1 and 0.7 mM, respectively, and the total naphthenic acids concentration ranged from 60 to 85 mg/L. Sterile controls were prepared in singlet with 190 mL filter-sterilized ozonated sample or non-ozonated control and 10 mL modified Bushnell-Haas medium.

Incubations and sterile controls were maintained under aerobic conditions at room temperature (approximately 20°C) on a rotary shaker shaking at 200 rpm. Samples were taken on day 0, 2, 3, 4, 5, 7, 9, 11, and 14. At every sampling time, 3 mL was removed from each incubation and sterile control. The pH of each sample was adjusted to ~11 using 2 M NaOH to ensure maximum solubility of the naphthenic acids (Appendix A) and then samples were centrifuged for 10 min at maximum speed in a clinical centrifuge. A 200-μL portion of sample supernatant was transferred to a 1.5-mL glass vial, which was capped and stored at 4°C prior to HPLC analysis. Changes in naphthenic acids concentration were plotted against incubation time and the initial biodegradation rates were determined from the slope over the first 4 d of incubation. Statistical comparisons of the slopes were then done as described by Kleinbaum and Kupper (1998).

4.2.3 Microcosm biodegradation studies

A second biodegradation study was conducted in sealed microcosms so that mineralization (CO_2 release) could be monitored and compared with measured changes in the naphthenic acids concentration. Samples generated in the second batch ozonation experiment (see Secton 4.2.1.3) were used in this study.

The methods used for this experiment were similar to those used by Clemente et al. (2004). Incubations were prepared in 125-mL serum bottles with a final liquid volume of 40 mL. Each serum bottle was sealed with a rubber stopper to trap any microbially-produced CO_2. There was enough oxygen in the headspace gas at the time of inoculation to allow complete mineralization of the organic carbon (i.e. naphthenic acids or partially-oxidized intermediates).

Each incubation contained 38 mL of filter-sterilized (Stericup™, 0.22 μm) ozonated or non-ozonated Merichem naphthenic acids solution. An inoculum was prepared in the same way described in Section 4.2.2 and a 2-mL portion was transferred to each serum bottle to provide nutrients (N and P) and viable microorganisms. Sterile controls contained 38 mL of filter-sterilized ozonated or non-ozonated Merichem naphthenic acids solution and were supplemented with 2 mL sterile modified Bushnell-Haas medium. A sufficient number of serum bottles were prepared for each condition so that viable incubations could be sacrificially sampled in duplicate on every sampling day and sterile controls could be sacrificially sampled in singlet four times during the 2-week incubation period.

Incubations were maintained under aerobic conditions at room temperature (approximately 20°C) on a rotary shaker shaking at 200 rpm. Sacrificial sampling of the viable incubations was carried out on day 0, 1, 2, 3, 4, 5, 7, 10 and 14. Sterile controls were sampled on day 0, 5, 10, and 14. Mineralization was monitored as described in Section 4.2.5. Samples were also taken and prepared for HPLC analysis in the same manner described in Section 4.2.2. Biodegradation rates over the first 4 d of incubation were compared using the method described by Kleinbaum and Kupper (1998) for the statistical comparison of slopes.

4.2.4 *HPLC quantification and GC-MS characterization of naphthenic acids*

Naphthenic acids were quantified by HPLC analysis using the method of Yen et al. (2004) with a few modifications. The pH of all samples was adjusted to ~11 with 2 M NaOH prior to derivatization. This ensured maximum solubility of the naphthenic acids and that samples were at the same initial pH as standards. Calibration standards for the analysis of samples from the continuous and semi-batch ozonations were prepared as outlined by Yen et al. (2004). Calibration standards for the analysis of samples from the batch ozonations and biodegradation studies were prepared according to the procedure given in Appendix B. Briefly, the dilution protocol was changed from that of Yen et al. (2004) so that all standards had final NaOH concentrations of 8 mM. Standards were also made up with the same final concentration of phosphate as samples (30 mM) to ensure that the effects of buffering were the same for standards and samples. Two changes were made to the HPLC method: the baseline hold was turned off and integration was started at 3.1 min instead of 2.9 min. A peak of variable size appeared early on in the naphthenic acids elution profile, contributing inconsistent and substantial error to the total area determined for the naphthenic acids "hump." Chromatograms were manually reintegrated to exclude the aberrant peak.

Prior to GC-MS analysis, naphthenic acids were extracted using the method of Clemente et al. (2004) and derivatized according to the method of St. John et al. (1998) using *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide containing 1% *tert*-butyldimethylsilylchloride (MTBSTFA) (Sigma, St. Louis, MO). GC-MS analyses were conducted as described by Holowenko et al. (2002) except that the instrument was set to scan from *m/z* 150 to 550. Two-sided t-test comparisons of GC-MS data were performed using the method of Clemente et al. (2003).

4.2.5 *Monitoring mineralization*

Headspace CO_2 and dissolved inorganic carbon were quantified using the GC method of Bressler et al. (1999). Viable incubations and sterile controls were sampled as described by Clemente et al. (2004). Briefly, a sterile syringe and needle were used to withdraw 4 mL of headspace and 2 mL of liquid (5% of the volume of each phase) from

the sealed serum bottles. Each sample was then injected into a sealed 35-mL serum bottle containing 2 mL 2 M H_2SO_4 and allowed to equilibrate for at least 24 h. As per the method of Bressler et al. (1999), 0.5-mL of the headspace gas was analyzed by GC using a thermal conductivity detector to determine the amount of CO_2.

Stock solutions were prepared in sealed 158-mL serum bottles using known amounts of $NaHCO_3$ in borax buffer (pH 8). The ratio of headspace to liquid volume was the same as that of the viable incubations and sterile controls. Each stock solution was sampled and acidified in the same manner described above to generate calibration standards that were also analyzed by GC. The amount of CO_2 produced after 14 d of incubation was compared by t-test.

4.2.6 Microtox™ assays

Microtox™ toxicity assays were used to measure changes in toxicity with ozonation. Samples from the batch ozonation experiments (Section 4.2.1.3) were analyzed by the Basic protocol (Microbics Corporation 1991). A Microbics model M500 was used to measure the luminescence of reconstituted *Vibrio fischeri* before and 15 min after exposure to non-ozonated and ozonated Merichem naphthenic acids solutions. The percent v/v of sample that caused a 50% decrease in luminescence was determined and reported as the IC_{50} value. Phenol toxicity was tested as a positive control prior to the analysis of samples and the measured IC_{50} was within the recommended range of 13 to 26 mg/L (Microbics Corporation 1991).

4.3 Results and discussion

4.3.1 Continuous ozonation

A previous study showed that ozone could react with naphthenic acids in a sample of OSPW (Chapter 3). In the present study, continuous ozonation of Merichem naphthenic acids was conducted as a single trial to establish whether ozone could react with commercial naphthenic acids. Figure 4.1A shows that there was an immediate decrease in the ozone concentration, followed by a slow (>80 min) recovery to just less than the initial concentration (~14 mg/L). The depletion of ozone was attributed to

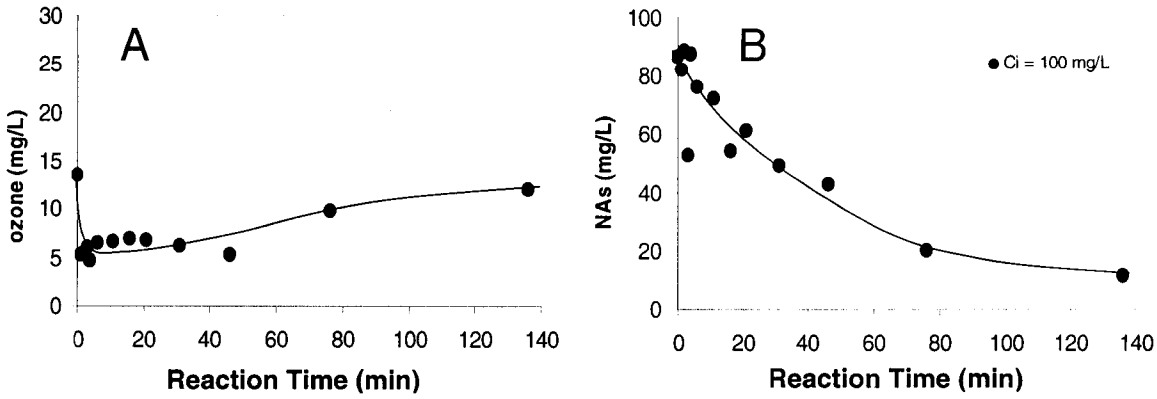

Figure 4.1 Ozone (A) and Merichem naphthenic acids (NAs) concentration (B) with continuous bubbling of ozone. The target initial naphthenic acids concentration (Ci) was 100 mg/L. This trial was performed in singlet.

reaction with naphthenic acids in the solution, which also decreased in concentration during the experiment (Figure 4.1B). Reaction efficiency could not be evaluated because the theoretical stoichiometry of the reaction between ozone and Merichem naphthenic acids is not known and nearly impossible to calculate due to the complexity of the naphthenic acids mixture and the decomposition of ozone to hydroxyl radicals, which have very different oxidizing potential and reaction mechanism than molecular ozone (Scott and Ollis 1995, Wang et al. 2003, Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). However, the results shown in Figure 4.1 clearly demonstrated that ozone reacted with the Merichem naphthenic acids.

4.3.2 Semi-batch ozonation

Samples taken during the continuous ozonation experiment (Figure 4.1B) were too small in volume to be used in a biodegradation study and the final reaction mixture (post-130 min) had a naphthenic acids concentration that was too low (< 20 mg/L) for the purpose of monitoring changes during a subsequent biodegradation study. Therefore, two semi-batch reactions were conducted in which phosphate-buffered solutions of Merichem naphthenic acids were bubbled with ozone for 16 or 37 min. These two treatment times were predicted to yield 25 and 50% decreases in the apparent naphthenic acids concentration based on the results shown in Figure 4.1B. A non-ozonated control was also prepared.

The naphthenic acids concentration in the non-ozonated control was 70 mg/L, whereas the 16-, and 37-min semi-batch samples had final naphthenic acids concentrations of 37 and 22 mg/L, respectively (determined by HPLC). These values were approximately 50 and 70% lower than the naphthenic acids concentration in the non-ozonated control; much greater decreases than were expected based on the continuous ozonation experiment (Figure 4.1B). However, the continuous ozonation experiment was only conducted in singlet and variability of this degree may be expected because of inherent difficulty replicating mass transfer and diffusion of ozone, as well as decomposition of ozone to hydroxyl radicals.

Portions of the non-ozonated control and ozonated samples were extracted, derivatized, and analyzed by GC-MS. Major changes in the naphthenic acids distribution, with respect to both carbon number (n) and Z, were apparent after both treatment times (Figure 4.2). Of particular interest was the appearance of ions in the high carbon-number ($n \geq 15$) range, with Z-values between -12 and -4. The shift towards higher molecular weight compounds with ozonation was not expected based on literature results that showed the opposite trend (Wang et al. 2004, Bijan and Mohseni 2004, 2005). This phenomenon is discussed more below.

Tables 4.1 and 4.2 highlight the observations shown in Figure 4.2. The proportion of ions assigned to n = (22 to 33) increased more than 4-fold after 16 min of ozonation to comprise slightly more than one quarter of the mixture (Table 4.1). This change was statistically significant ($P<0.05$), as was a decrease in the group with n = (5 to 13) from 59% to 36%. With 37 min of ozonation, a more than 6-fold increase in the n = (22 to 33) group was measured, meaning this group comprised more than a third of the mixture, whereas the proportion ions with n = (5 to 13) decreased to less than 20%. The n = (14 to 22) group did not change much with ozonation, increasing by only 2% and 7% with 16 and 37 min of ozonation, respectively.

In the non-ozonated control, 91% of ions were assigned to Z-values between -4 and 0 (Table 4.2). Following ozonation for 16 min this proportion had decreased to 62%, and with 37 min of treatment only 41% of the ions fell into this range. Another notable change occurred in the Z = -10 family, which increased in proportion from 1 to 22% with 37 min of ozonation. An ion with n = 16, Z = -10 was the major contributor to the

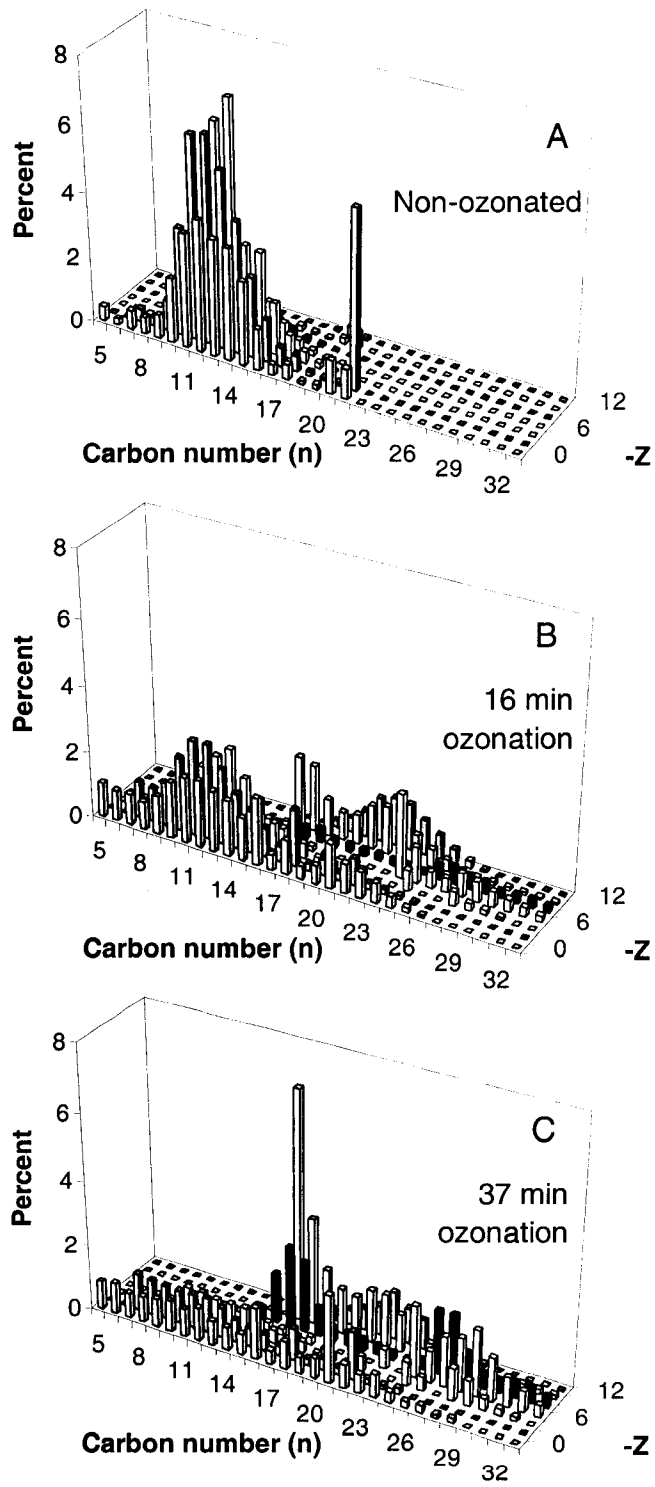

Figure 4.2 Three-dimensional plots showing the distribution of naphthenic acids in a non-ozonated solution of Merichem naphthenic acids (A) and after treatment with ozone for 16 (B) and 37 min (C). The sum of all bars in each panel is 100%.

Table 4.1 Proportion of ions in each carbon-number group before and after ozonation of Merichem naphthenic acids.

	Proportion of Ions (Percent)		
Carbon-number (n) group	Non-ozonated (Time 0)	Ozonated (16 min)	Ozonated (37 min)
5 to 13	59	36*	19*
14 to 21	35	37	42
22 to 33	6	27*	39*

* denotes significant difference from Time 0 ($P<0.05$).

Table 4.2 Proportion of ions in each Z-family before and after ozonation of Merichem naphthenic acids.

	Proportion of Ions (Percent)		
Z-family	Non-ozonated (Time 0)	Ozonated (16 min)	Ozonated (37 min)
0	26	25	18
-2	38	22	13*
-4	27	15	10
-6	6	11	14*
-8	2	6*	16*
-10	1	13*	22*
-12	0	8*	7*

* denotes significant difference from Time 0 ($P<0.05$).

observed increase (Figure 4.2C). Statistically significant ($P<0.05$) increases occurred within 16 min of ozonation in the Z = -8, -10, and -12 families and in the Z = -6, -8, -10, and -12 families with 37 min of ozonation (Table 4.2). The observed decrease from 38 to 13% in the Z = -2 family following 37 min of ozonation was also significant ($P<0.05$).

It was hypothesized that ozonation of naphthenic acids would generate smaller intermediates and catalyze ring cleavage. GC-MS results obtained in this study showed virtually the opposite trend (Figure 4.2). Initially this was explained as a shift in the *relative* proportions of acids with ozonation. That is, these high molecular weight, polycyclic acids may have been present in the original sample but were overshadowed by the greater abundance of low molecular weight aliphatic or mono-cyclic acids. Assuming 1), that ozone was most effective at oxidizing the low molecular-weight acids and, 2), that the majority were mineralized to CO_2, the resulting mixture would be comprised of primarily high molecular-weight acids, hence, the apparent increase in their proportion. However, other studies have demonstrated that ozonation is actually less effective for oxidizing low molecular weight compounds compared with high molecular weight compounds (Wang et al. 2004, Bijan and Mohseni 2004, 2005).

A separate study was conducted to determine the origins and identity of the high molecular weight, $Z \leq -4$ ions that appeared after ozonation. Details of this study are given in Chapter 6 (Section 6.3.3). Basically, it was proposed that ozonation resulted in hydroxylation of naphthenic acids, contributing one or more active hydrogens in addition to the active hydrogen of the carboxylic acid moiety. Active hydrogens are the site of derivatization with MTBSTFA (St. John et al. 1998). Therefore, naphthenic acids with more than one active hydrogen would have more than one derivatizing group added during the derivatization reaction, yielding products with much higher molecular weights. These products are mistakenly assigned as naphthenic acids of high molecular weight (see Chapter 6).

4.3.3 Batch ozonation and biodegradation of Merichem naphthenic acids

Two separate experiments were conducted with batch ozonation of Merichem naphthenic acids followed by biodegradation of the ozonated samples and a non-ozonated control. In the first ozonation experiment, ozone doses of 20 and 28 mg/L were tested.

Portions of the ozonated samples and a non-ozonated control were extracted, derivatized, and analyzed by GC-MS. Three-dimensional plots generated from the GC-MS data illustrate the naphthenic acids profile in each extract (Figure 4.3). The appearance of high molecular-weight ions with Z = -10 to -12 was noted in both ozonated samples (Figure 4.3). This phenomenon has been characteristic of all ozonated naphthenic acids samples analyzed by GC-MS. However, the shift in naphthenic acids distribution observed here was less pronounced than the shifts observed previously with the semi-batch ozonations (Figure 4.2). This may be due to differences in the type of ozonation technique employed (bubbling ozone versus batch reaction with an ozone solution of finite concentration).

Ozonated samples and a non-ozonated control were incubated in aerobic shake-flasks with microorganisms from a Syncrude OSPW pond. Biodegradation of naphthenic acids was observed in all samples and the initial rate of biodegradation appeared to be faster in the ozonated samples than in the non-ozonated control (Figure 4.4). Biodegradation rates over the first 4 d of incubation were calculated to be -4.1 mg/L/d for the incubations with non-ozonated naphthenic acids, -7.0 mg/L/d for the incubations with ozonated (20 mg/L ozone dose) naphthenic acids, and -7.6 mg/L/d for the incubations with ozonated (28 mg/L ozone dose) naphthenic acids. The faster rates observed for incubations with ozonated naphthenic acids suggested that ozonation had improved biodegradability of the Merichem naphthenic acids. However, statistical analysis showed that the rates were not significantly different.

The extent of biodegradation measured for the non-ozonated control was less than expected based on previous studies that showed complete biodegradation of Merichem naphthenic acids within about 10 d (Clemente et al. 2004, Chapter 2). The only difference between the incubations from the present study and those of previous studies was the presence of phosphate buffer (~30 mM) in the former. Phosphate is an essential nutrient for microorganisms and addition of phosphate to OSPW has been shown in laboratory studies to increase the rate of naphthenic acids biodegradation (Lai et al. 1996). No definitive explanation could be found for why the extent of biodegradation observed in this study was lower than expected. However, it was considered possible that HPLC quantification of naphthenic acids in phosphate-buffered samples might be inaccurate despite modifications intended to circumvent such problems (Appendix B).

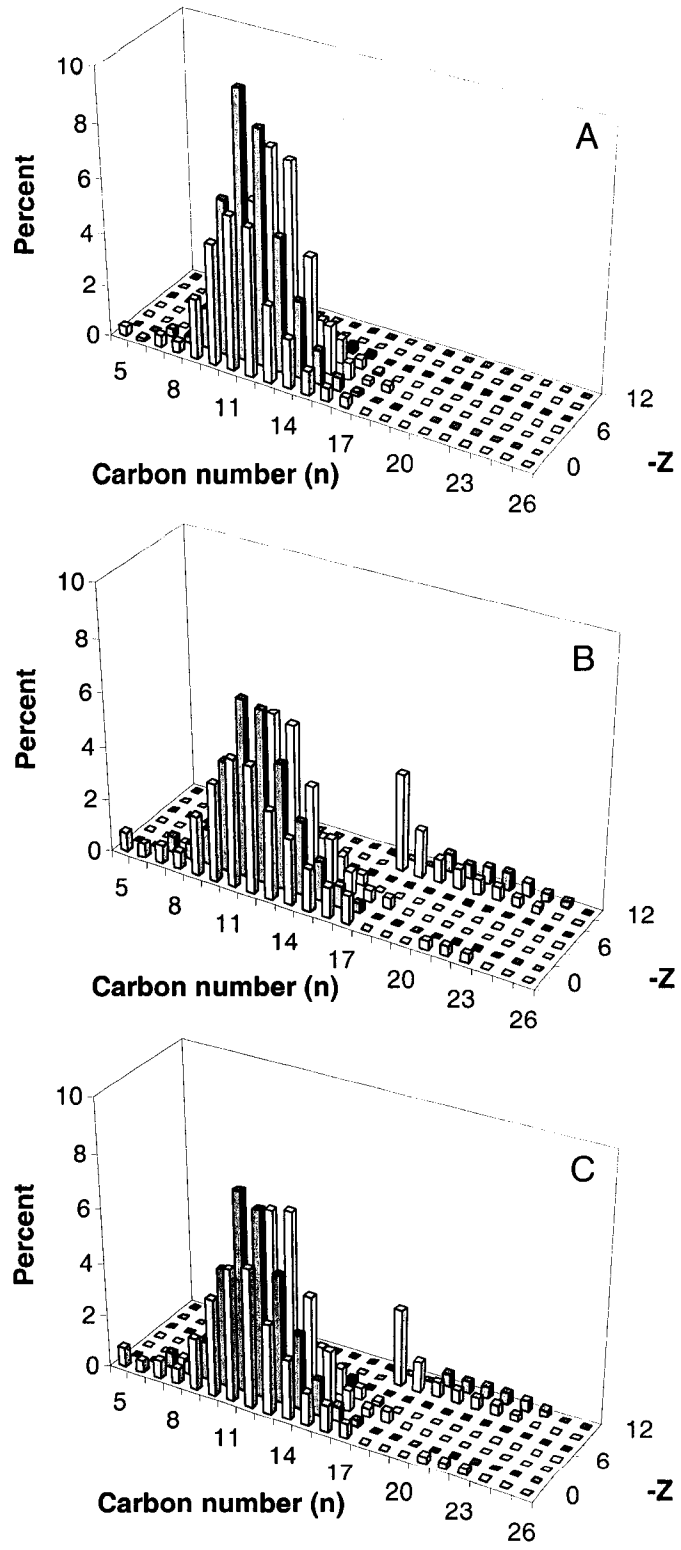

Figure 4.3 Three-dimensional plots showing the distribution of naphthenic acids in a non-ozonated solution of Merichem naphthenic acids (A) and after mixing Merichem naphthenic acids with 20 mg ozone/L (B) and 28 mg ozone/L (C) for a contact time of 2 min. The sum of all bars in each panel is 100%.

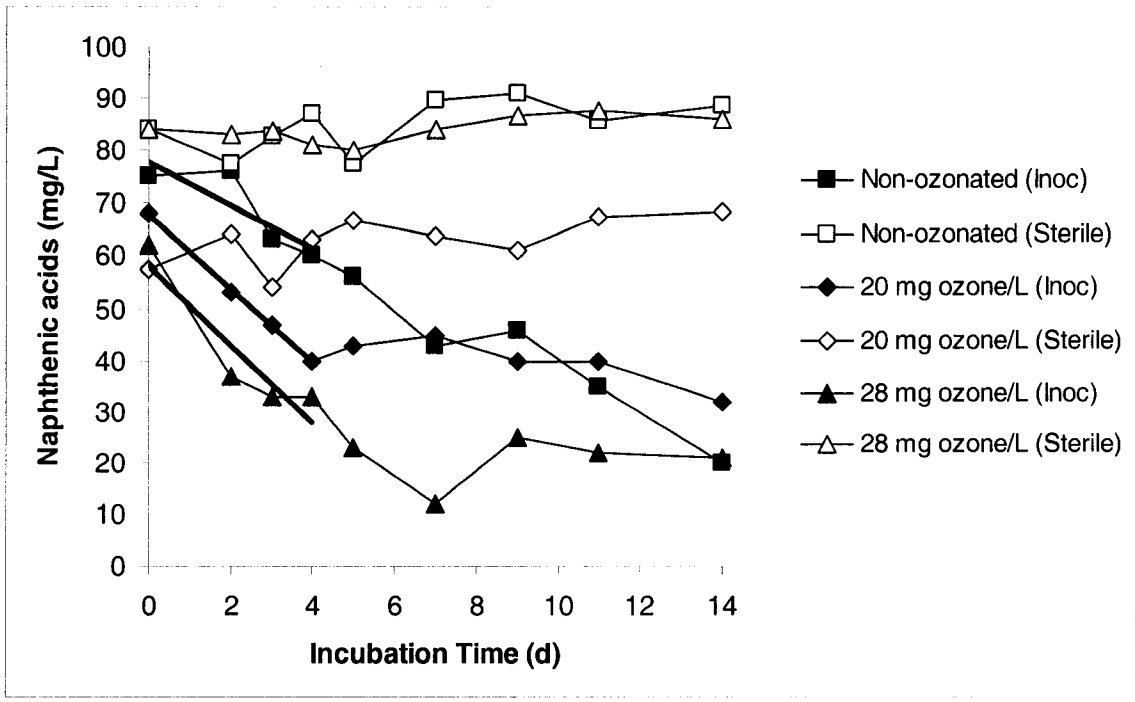

Figure 4.4 Naphthenic acids concentration in sterile controls and inoculated incubations (Inoc) of a non-ozonated Merichem naphthenic acids solution and two ozonated Merichem naphthenic acids solutions. Data points for the inoculated incubations represent the average from duplicate incubations. Error bars are not shown. Sterile controls were prepared in singlet. For inoculated incubations, the initial rate of biodegradation (i.e. over the first 4 d of incubation) is indicated by trendlines.

It was decided that another biodegradation study should be conducted using sealed aerobic microcosms for the incubations in order to concurrently monitor mineralization. Mineralization data could then be compared with HPLC results to confirm biodegradation trends. This required preparation of a new set of ozonated Merichem naphthenic acids solutions.

A second batch ozonation experiment was conducted using ozone doses of 20 and 30 mg/L. Microcosm incubations were set up to evaluate differences in the biodegradability of ozonated Merichem naphthenic acids and a non-ozonated control. Results are shown in Figure 4.5. Biodegradation followed similar patterns to those seen in the previous study with the shake-flask incubations (Figure 4.4), but differences in the initial rate of biodegradation were not as apparent as those observed previously (Figure 4.5). Biodegradation rates over the first 4 d of incubation were calculated to be

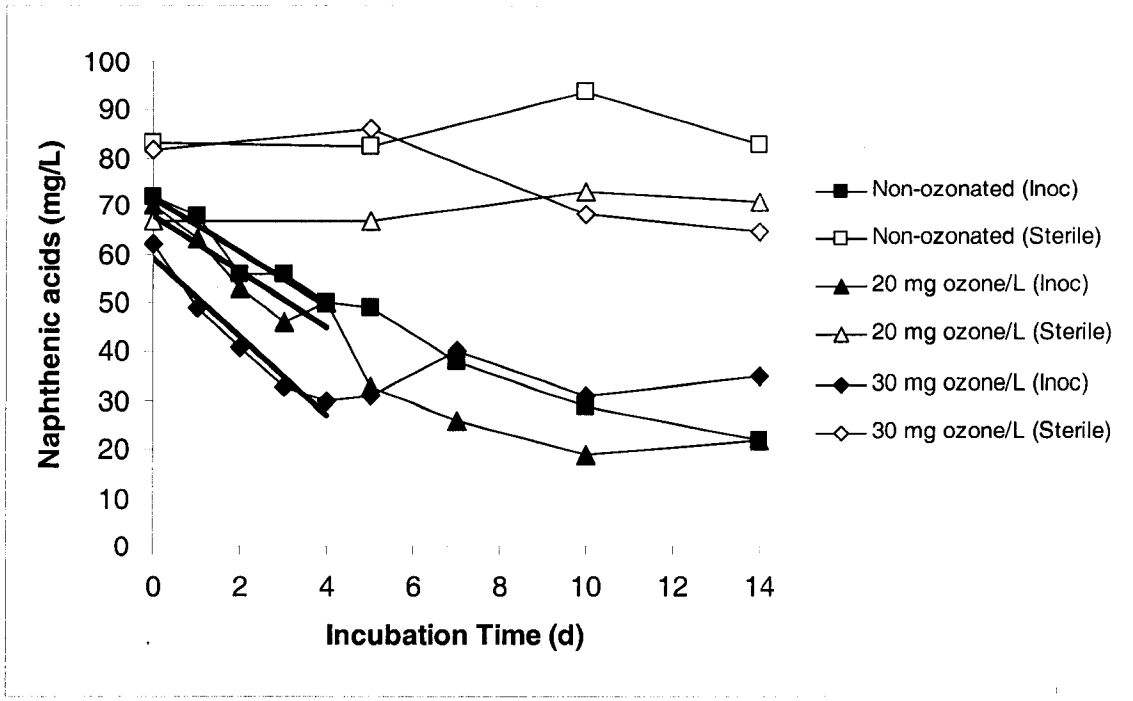

Figure 4.5 Naphthenic acids concentration in sterile controls and inoculated incubations (Inoc) of a non-ozonated Merichem naphthenic acids solution and two ozonated Merichem naphthenic acids solutions. Data points for the inoculated incubations represent the average from duplicate incubations. Error bars are not shown. Sterile controls were prepared in singlet. For inoculated incubations, the initial rate of biodegradation (i.e. over the first 4 d of incubation) is indicated by trendlines.

-5.6 mg/L/d for the incubations with non-ozonated naphthenic acids, -5.7 mg/L/d for the incubations with ozonated (20 mg/L ozone dose) naphthenic acids, and -8.0 mg/L/d for the incubations with ozonated (30 mg/L ozone dose) naphthenic acids. Statistical analyses showed that the biodegradation rates for incubations with ozonated naphthenic acids were not significantly different from that of the non-ozonated control.

The extent of biodegradation in the microcosm incubations with non-ozonated Merichem naphthenic acids was less than expected based on previous studies (Clemente et al. 2004, Chapter 2) but comparable to results obtained with the shake-flask incubations (Figure 4.4). As mentioned above, the reason why naphthenic acids were biodegraded less extensively in the present study could not be determined.

Mineralization was also monitored in this biodegradation study. CO_2 production in sterile controls was minimal compared to production in the inoculated incubations

(Figure 4.6). Because of the variability in the results, it was difficult to compare the rate of CO_2 production in the inoculated incubations. Therefore, the average total amounts of CO_2 generated after 14 d were compared using a t-test. The mean CO_2 produced in the incubations with naphthenic acids treated with 20 mg ozone/L appeared to be greater than the amount measured in incubations with non-ozonated naphthenic acids, but the difference was not significant (P=0.24). In the incubations with naphthenic acids treated with 30 mg ozone/L, one replicate produced 1.98 mg CO_2-C and the other replicate produced 1.82 mg CO_2-C. Mean CO_2 production (1.90 mg CO_2-C) was more than double the mean amount measured in incubations with non-ozonated naphthenic acids. This difference was significant (P<0.01). There was poor agreement between the day 14 duplicates for the incubations with ozonated (20 mg ozone/L) naphthenic acids. Specifically, one replicate produced only 0.72 mg CO_2-C, whereas the other replicate

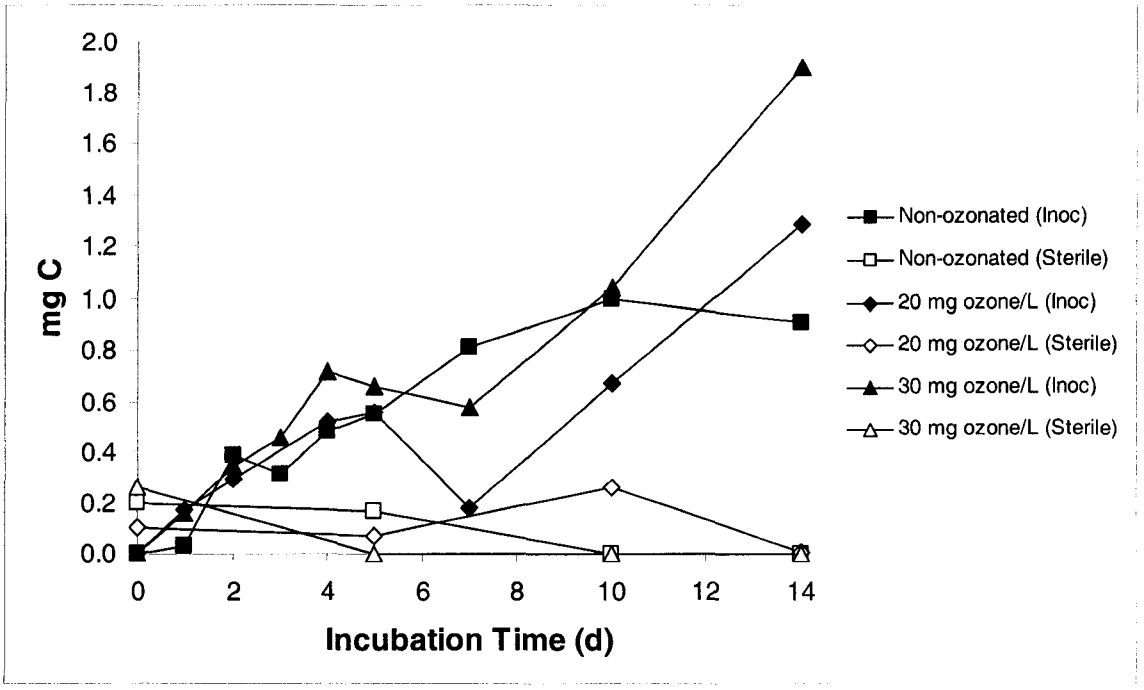

Figure 4.6 CO_2 production in sterile controls and inoculated (Inoc) incubations of non-ozonated and ozonated Merichem naphthenic acids solutions. Data points for inoculated incubations represent the average of duplicates. Error bars are not shown. Sterile controls were prepared in singlet.

produced 1.84 mg CO_2-C, which was comparable to the amount produced in incubations with naphthenic acids treated with 30 mg ozone/L. It was believed that the mineralization rate in the first replicate may have been slower and that CO_2 production would have reached an amount equivalent to that measured for the second replicate if incubation had been continued.

Mineralization results obtained for the incubations with naphthenic acids treated with 30 mg ozone/L indicated that ozonation significantly improved the extent of biodegradation (Figure 4.6), a finding that was not apparent from the naphthenic acids quantification data (Figure 4.5). One explanation for this discrepancy is that monitoring changes in naphthenic acids concentration does not account for biodegradation of potential non-naphthenic acid oxidation products, whereas mineralization data would include CO_2 produced from the biodegradation of all organic compounds in the samples. In other words, monitoring mineralization is a more comprehensive way to follow biodegradation than monitoring one specific group of compounds in a mixture.

To estimate theoretical CO_2 production in incubations, the general chemical formula for naphthenic acids, $C_nH_{2n+Z}O_2$, was considered. For n values between 5 and 20, which is the typical range found in Merichem naphthenic acids mixtures (eg. Figure 4.3), the naphthenic acids are about 70% carbon. Based on this, the estimated theoretical CO_2 production was calculated for the non-ozonated control and ozonated samples (Table 4.3). In incubations with non-ozonated Merichem naphthenic acids, the actual amount of CO_2 produced was 63% of the estimated theoretical amount (Table 4.3). This result is in agreement with the findings of Clemente et al. (2004), who showed that about 60% of the organic carbon in a naphthenic acids preparation was released as CO_2 during aerobic biodegradation (Clemente et al. 2004). In incubations with ozonated Merichem naphthenic acids, the actual amount of CO_2 produced was much greater than the estimated theoretical amount (Table 4.3). No feasible explanation could be found to validate this result.

Most ozonation studies reported in the literature assessed changes in biodegradability by measuring the ratio of biochemical oxygen demand to chemical oxygen demand, BOD/COD, before and after ozonation. However, some conducted actual biodegradation studies on ozonated and non-ozonated samples and monitored the

Table 4.3 Total decreases in the amount of naphthenic acids after incubation for 14 d, estimated theoretical CO_2 production, and actual CO_2 production after 14 d.

Treatment	Decrease in naphthenic acids[a] mg	Estimated theoretical CO_2 production mg C	Actual CO_2 production mg C	Percent of estimated theoretical CO_2 production
Non-ozonated	2.0	1.46	0.91	63
20 mg ozone/L	1.9	1.39	1.28	92
30 mg ozone/L	1.1	0.80	1.90	240

[a] Calculated by subtracting the final naphthenic acids concentration from the initial naphthenic acids concentration (Figure 4.5) and multiplying the result by the incubation volume (0.04 L).

extent of TOC removal, which is comparable to monitoring mineralization. For example, Gonzalez et al. (2003) reported up to 5-fold more TOC removal during biodegradation of ozonated chlorine bleaching wastewater compared with non-ozonated wastewater. Nakamura et al. (2004) reported up to a 10-fold improvement in the extent of biodegradation of sodium lignosulfonate after ozonation. Wang et al. (2004) measured up to a 60% increase in TOC biodegradation with ozonation of oil field drilling wastewater and two studies showed ~50% more TOC removal when pulp mill effluent was ozonated (Bijan and Mohseni 2004, 2005). Mineralization results obtained in the current study were in agreement with these studies, showing up to 2-fold more CO_2 production in incubations with ozonated naphthenic acids than in incubations with non-ozonated naphthenic acids after 14 d of incubation (Figure 4.6).

4.3.4 Microtox™ assays

Samples from the batch ozonation experiments were assayed for toxicity using the Microtox™ method (Table 4.4). Non-ozonated Merichem naphthenic acids solutions were acutely toxic, as expected from previous experiments (Clemente et al. 2004). Average IC_{50} for ozonated Merichem naphthenic acids solutions was more than 3-fold

Table 4.4 Toxicity of non-ozonated and ozonated Merichem naphthenic acids solutions determined by the Microtox™ method.

	IC_{50} (%v/v)
Non-ozonated	10.3 ± 0.6[a]
Ozonated	32.9 ± 3.2[b]

[a] reported value is the average of two non-ozonated samples plus or minus one standard deviation;
[b] reported value is the average of four batch-ozonated samples (ozone dose 20 to 30 mg/L) plus or minus one standard deviation.

higher than the average IC_{50} of the non-ozonated control (Table 4.4). This indicated that the ozone doses tested were effective for reducing toxicity but were not able to generate a completely non-toxic effluent.

4.3.5 Summary

This study showed that ozonation caused statistically significant changes in the distribution of Merichem naphthenic acids, with respect to carbon number (n) and Z-family. Ozonation also reduced toxicity of the naphthenic acids solutions and enhanced their biodegradation by microorganisms indigenous to OSPW storage ponds. Specifically, the extent of biodegradation was greater with ozonated samples, although biodegradation rates were not significantly different than those measured for non-ozonated controls. Overall, this study generated some promising results using a surrogate OSPW to investigate the potential for using ozonation to improve biodegradability of oil sands naphthenic acids. Future studies should test ozonation and biodegradation using actual OSPW.

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5 THIOSULFATE INTERFERES WITH BIODEGRADATION OF NAPHTHENIC ACIDS

5.1 Introduction

Biodegradation is an effective and economical way to treat wastewaters that contain organic compounds. However, some organic compounds are recalcitrant or inhibitory to biodegradation. Chemical oxidation may be employed as a pretreatment in these circumstances to render the organic compounds more susceptible to microbiological oxidation (Scott and Ollis 1995, Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). Ozonation, or chemical oxidation using ozone, has been studied for pretreatment of various industrial wastewaters and has generally been successful for improving biodegradability (Oeller et al. 1997, Helble et al. 1999, Rivas et al. 2000, Amat et al. 2003, Gonzalez et al. 2003, Nakamura et al. 1997, 2004, Bijan and Mohseni 2003, 2005, Wang et al. 2004, Bettazzi et al. 2006).

Process waters produced by the oil sands operations of northern Alberta contain a group of acutely toxic organic compounds known as naphthenic acids (MacKinnon and Boerger 1986, Schramm et al. 2000, Headley and McMartin 2004, Clemente and Fedorak 2005). Naphthenic acids are alkylated aliphatic or alicyclic carboxylic acids that have structures consistent with the chemical formula $C_nH_{2n+Z}O_2$, where n specifies the number of carbons and Z corrects the number of hydrogens to account for ring formation (Brient et al. 1995). Some examples of naphthenic acids are shown in Figure 1.3 (see Chapter 1).

Laboratory biodegradation studies demonstrated that the naphthenic acids present in oil sands process water are recalcitrant (Chapter 2), but a separate study reported that ozonation improved biodegradability (based on the ratio of biochemical oxygen demand to chemical oxygen demand; BOD/COD) (Chapter 3). To further investigate the potential for using ozonation to improve biodegradability of naphthenic acids, a surrogate oil sands process water was prepared using Merichem naphthenic acids; a commercially-available preparation. Solutions of Merichem naphthenic acids were ozonated in batch reactions and the effect on biodegradability was evaluated in a subsequent biodegradation study (Chapter 4). Ozonation did not cause a statistically significant change in the initial rate of

biodegradation, but the highest ozone dose tested (30 mg ozone/L) significantly increased the overall extent of mineralization compared to a non-ozonated control ($P<0.01$).

Prior to the batch ozonations, a preliminary ozonation study was conducted in which solutions of Merichem naphthenic acids were bubbled continuously with ozone for set periods of time (see Sections 4.2.1.2 and 4.3.2.). Immediately after the desired reaction time had elapsed, sodium thiosulfate was added to the solutions to quench residual ozone. The ozonated samples were then used in a biodegradation study along with a non-ozonated control. Naphthenic acids concentrations in inoculated incubations showed almost no change over a 20-d incubation period and were nearly equivalent to the concentration in sterile controls on day 20. This was initially attributed to an inoculum viability problem because previous studies had shown complete biodegradation of untreated commercial naphthenic acids in approximately 10 d (Clemente et al. 2004, Chapter 2). However, an unrelated experiment set up at the same time with the identical inoculum showed biodegradation of Merichem naphthenic acids (non-ozonated) within the expected 10 d of incubation.

Samples used in the unsuccessful biodegradation study differed from those used in the unrelated experiment in that the former contained phosphate buffer and sodium thiosulfate, whereas the latter did not. Neither of these components are typically associated with microbial toxicity. Phosphate is an inorganic nutrient required for carbon metabolism by microorganisms and is therefore unlikely to have a negative effect on biodegradation. In fact, supplementing phosphate has been shown to stimulate biodegradation of naphthenic acids in laboratory cultures (Lai et al. 1996). Thiosulfate addition is a common method for quenching residual ozone and there are examples in the literature of its use when ozonation has been applied in the food sanitization industry (Wei et al. 2007, Rodriguez-Romo et al. 2007) and in wastewater treatment for the removal of pathogenic microorganisms (Hijnen et al. 2004, Mazloum et al. 2004, Wang et al. 2004). The effect of thiosulfate on biodegradation has not been well-studied but one publication reported that thiosulfate interfered indirectly with biodegradation of organic compounds (Kreye et al. 1973). Specifically, total carbon removal decreased from 86 to 0% when wastewater with an influent thiosulfate concentration of ~10 mM was subjected to aerobic biodegradation for 7 d. Decreased biodegradation was attributed to

biooxidation of thiosulfate to sulfuric acid (Equation 5.1) causing an unfavorable drop in pH from 8.3 to 2.7. Contrary to the findings of Kreye et al. (1973), another study showed that the presence of up to 5 mM thiosulfate did not encumber aerobic biodegradation of a synthetic wastewater comprised of glucose, acetate, and citrate (Schreiber and Pavlostathis 1998).

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \qquad \text{(Equation 5.1)}$$

The incubations with ozonated Merichem naphthenic acids were inoculated with a mixed community of aquatic microorganisms that could have included species such as *Thiobacillus*, which are capable of thiosulfate oxidation (Starkey 1934, Vishniac and Santer 1957, Unz and Lundgren 1961, Pepper and Miller 1978). Therefore, it is possible that thiosulfate oxidation occurred in these incubations.

In the present study, biodegradation of Merichem naphthenic acids was monitored in the presence and absence of thiosulfate to determine whether the lack of biodegradation observed with samples from the ozonation study was related to thiosulfate. The final concentration of thiosulfate in the ozonated samples (Chapter 4) was not known; the procedure only specified addition of one drop of a concentrated stock solution. It was estimated that addition of one drop would give an approximate initial thiosulfate concentration of 0.62 mM in the reaction mixture. In the ozonated samples, some thiosulfate would have been oxidized by residual ozone, resulting in a final concentration less than 0.62 mM. For the purpose of the current study, thiosulfate-amendment was tested at 0.62 mM. Biodegradation was monitored by following changes in the naphthenic acids concentration by HPLC.

A second biodegradation experiment was conducted to determine whether thiosulfate oxidation was occurring in the incubations. One set of incubations was amended with 0.62 mM thiosulfate and another set was amended with 4.4 mM to test whether any inhibitory effect would be amplified in the presence of more thiosulfate. Changes in the naphthenic acids concentration were monitored along with pH, thiosulfate depletion, and sulfate formation.

5.2 Materials and methods

5.2.1 Naphthenic acids and sodium thiosulfate

Merichem refined naphthenic acids were provided by Merichem Chemicals and Refinery Services LLC (Houston, TX). A 1 g/L stock solution of Merichem naphthenic acids was prepared in 0.1 M NaOH and the pH was adjusted to ~11 using 2 M NaOH. The stock solution was filter sterilized using Millex-GS 0.22 μm filters (Millipore, Bedford, MA) and stored at 4°C.

Sodium thiosulfate pentahydrate (Lot. 722466) was purchased from Fisher Scientific (Fair Lawn, NJ). A 6.2 mM stock solution of sodium thiosulfate was prepared in double-distilled water. The stock solution was filter sterilized using Millex-GS 0.22 μm filters and stored at room temperature (~20°C).

5.2.2 Incubation methods

A preliminary biodegradation study was conducted to monitor changes in naphthenic acids concentration in the presence or absence of thiosulfate. All incubations had a final liquid volume of 200 mL in 500-mL Erlenmeyer flasks. Flasks without thiosulfate were prepared in triplicate with 170 mL of sterile double-distilled water (autoclaved at 121°C, 100 kPa, for 20 min) plus 20 mL of the Merichem naphthenic acids stock solution. Flasks with thiosulfate were also prepared in triplicate and contained 150 mL sterile double-distilled water plus 20 mL Merichem naphthenic acids stock solution plus 20 mL of sodium thiosulfate stock solution.

An inoculum was prepared by centrifuging 200 mL of oil sands process water (West In-Pit, Syncrude Canada Ltd.) at 12 000*g* for 15 min, discarding the supernatant, and resuspending the resulting pellet in 10 mL modified Bushnell-Haas medium (MMBH) (Wyndham and Costerton 1981). The 10-mL suspensions from several identical centrifugations were combined and then 10-mL portions were transferred to each flask, providing N and P as well as viable microorganisms. Addition of MMBH ensured N and P were not limiting nutrients. The initial concentrations of N and P were 1 and 0.7 mM, respectively, and the total naphthenic acids concentration was ~100 mg/L. The concentration of thiosulfate in the thiosulfate-amended incubations was 0.62 mM.

Incubations were maintained under aerobic conditions at room temperature on a rotary shaker shaking at 200 rpm. Samples were taken on day 0, 2, 6, and 12. At every sampling time, 3 mL was aseptically removed from each incubation and sterile control. The pH of each sample was adjusted to ~11 using 2 M NaOH to ensure maximum solubility of the naphthenic acids and then samples were centrifuged for 10 min at maximum speed in a clinical centrifuge. A 200-μL portion of sample supernatant was transferred to a 1.5-mL glass vial, which was capped and stored at 4°C prior to HPLC analysis. Changes in naphthenic acids concentration were plotted against the incubation time and biodegradation rates were determined from the slope over the entire incubation period. Statistical comparisons of the slopes were then done as described by Kleinbaum and Kupper (1998).

A second biodegradation study was conducted to look for evidence of thiosulfate oxidation. Flasks with and without thiosulfate were prepared and inoculated as described above. A second set of thiosulfate-amended incubations were prepared with 140 mL thiosulfate stock solution to give a higher initial concentration of thiosulfate (4.4 mM). Sterile controls for each thiosulfate condition were prepared in singlet in the same manner as inoculated incubations but were supplemented with 10 mL sterile MMBH instead of the suspension of microorganisms in MMBH.

Incubations and sterile controls were maintained under aerobic conditions at room temperature (approximately 20°C) on a rotary shaker shaking at 200 rpm. Samples were taken on day 0, 2, 4, 7, 9, 11, 14, 18, 25, and 35. At every sampling time, 10 mL was aseptically removed from each incubation and sterile control. The pH of each sample was measured using colorpHast® pH indicator strips (EMD Chemicals Inc., Gibbstown, NJ) and then pH was adjusted to ~11 using 2 M NaOH. Samples were then centrifuged for 10 min at maximum speed in a clinical centrifuge. A 200-μL portion of sample supernatant was transferred to a 1.5-mL glass vial, which was capped and stored at 4°C prior to HPLC analysis. The remaining volume of each sample (~9.8 mL) was stored at 4°C prior to quantification of thiosulfate and sulfate.

5.2.3 Analysis of incubation supernatants

Naphthenic acids were quantified by HPLC analysis using the method of Yen et al. (2004) with a few modifications. Before derivatization, the pH of all samples was adjusted to ~11 with 2 M NaOH to ensure maximum solubility of the naphthenic acids and that samples were at the same initial pH as standards. Calibration standards were prepared according to the procedure given in Appendix B. Briefly, the dilution protocol was changed from that of Yen et al. (2004) so that all standards had final NaOH concentrations of 8 mM. Two changes were made to the HPLC method: the baseline hold was turned off and integration was started at 3.1 min instead of 2.9 min.

Thiosulfate was quantified using a colorimetric assay developed by Nor and Tabatabai (1975). Total reaction volume was scaled down approximately 5-fold from 25 to 4.8 mL to reduce the volume of sample (or standard) required to between 0 and 4 mL. The volume of each reagent was reduced to 1/5 the original volume used by Nor and Tabatabai (1975). Samples taken on days 0, 2, 9, and 35 were analyzed. Measured thiosulfate concentrations were corrected by subtracting the values obtained for incubations with no thiosulfate.

Sulfate analyses were done by the Natural Resources Analytical Laboratory at the University of Alberta. Samples were filter-sterilized using Millex-GS 0.22 μm filters and then analyzed for sulfate by ion chromatography using a Dionex DX-600 (Dionex Corp., Sunnyvale, CA) according to the method of Tabatabai and Basta (1991). Samples taken on days 0, 2, 7, and 25 were analyzed. Measured sulfate concentrations were corrected by subtracting the values obtained for incubations with no thiosulfate.

5.3 Results and discussion

Biodegradation of Merichem naphthenic acids was monitored in incubations amended with 0.62 mM thiosulfate and in control incubations with no thiosulfate (Figure 5.1). The rate of biodegradation in thiosulfate-amended incubations was -3.6 mg/L/d, which was significantly ($P<0.01$) slower than the biodegradation rate in control incubations (-8.1 mg/L/d). Moreover, naphthenic acids in the control incubations were completely degraded within 12 d, whereas only about 50% were degraded in the

thiosulfate-amended incubations (Figure 5.1). These results corroborate the lack of biodegradation observed in incubations with samples from the ozonation study (discussed in Section 5.1) and clearly demonstrate that biodegradation of naphthenic acids is inhibited in the presence of thiosulfate.

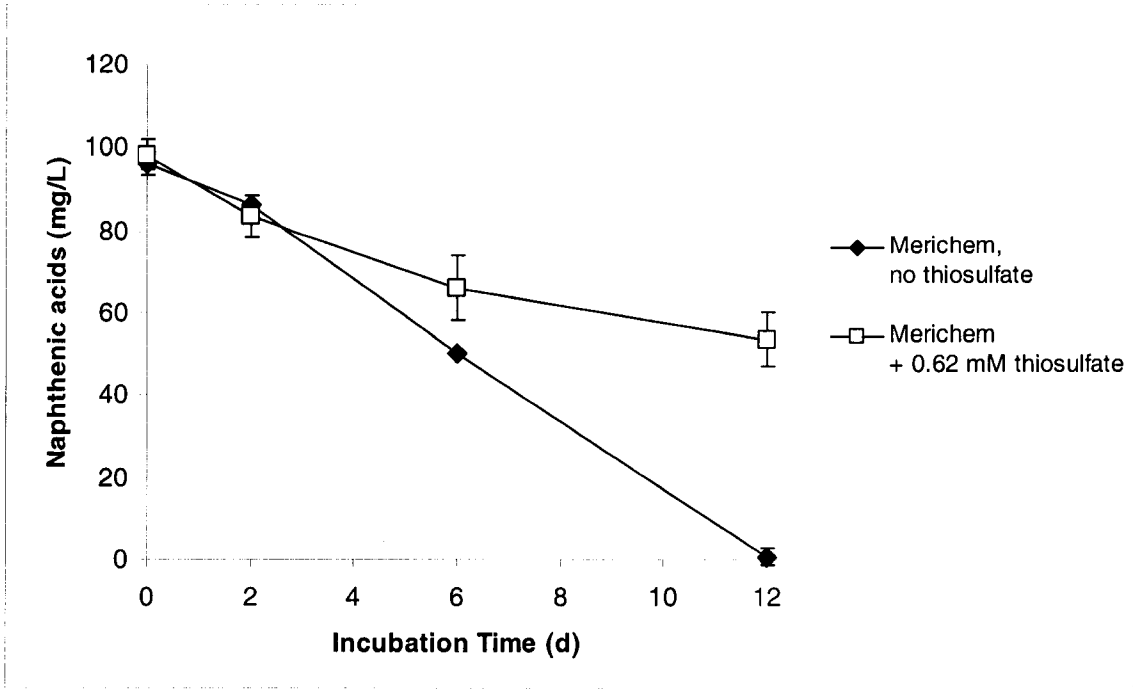

Figure 5.1 Biodegradation of Merichem naphthenic acids in incubations with no thiosulfate and with 0.62 mM thiosulfate. Data-points represent the average of triplicate incubations. Error bars represent one standard deviation and are sometimes smaller than the data points themselves.

The actual mechanism of thiosulfate inhibition could not be determined from the previous biodegradation experiment. However, microbial oxidation of thiosulfate to sulfate (Equation 5.1) has been shown to generate low pH conditions that are detrimental to biodegradation of organic compounds (Kreye et al. 1973). Thiosulfate oxidation was monitored in a second biodegradation study by measuring changes in the concentration of thiosulfate and sulfate. The pH of incubations was also monitored.

Naphthenic acids concentrations in the sterile controls for all conditions did not change much over the 35-d incubation period (Figure 5.2A). Complete biodegradation of naphthenic acids in the inoculated incubations with no thiosulfate was achieved within

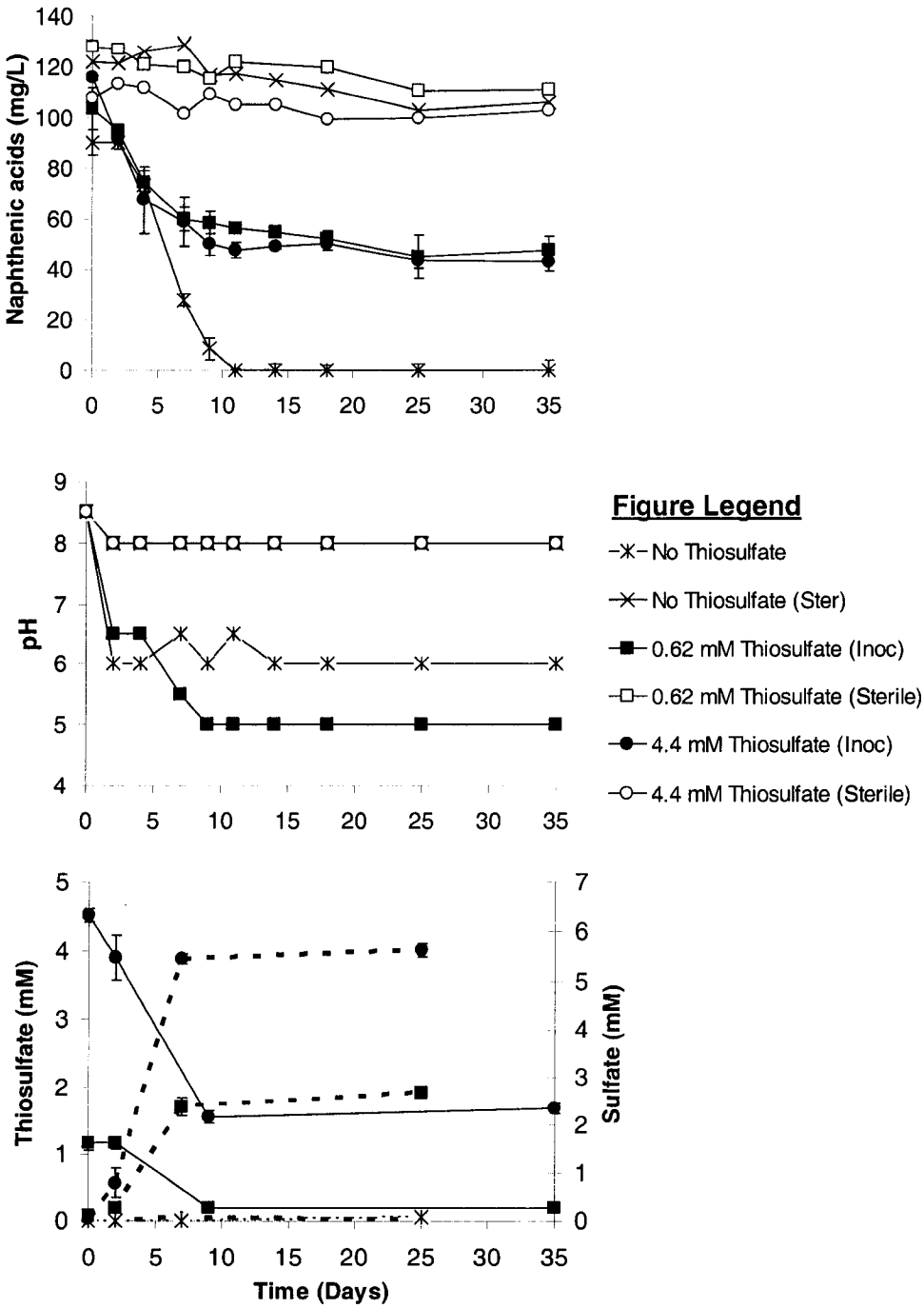

Figure 5.2 Naphthenic acids concentration (A), pH (B), and thiosulfate (—) and sulfate (- - -) concentrations (C) in sterile controls and inoculated incubations (Inoc). Data points represent the average of triplicates except for sterile controls, which were done in singlet. Error bars represent one standard deviation and are sometimes smaller than the data points. In panel B, pH for the incubations with 4.4 mM thiosulfate was identical to pH of the incubations with 0.62 mM thiosulfate. The pH of sterile controls was the same for all conditions.

11 d of incubation (Figure 5.2A). This is comparable to the biodegradation rate observed in the previous study (Figure 5.1). The biodegradation rate in the inoculated, thiosulfate-amended incubations deviated from that of the no thiosulfate incubations after 4 d of incubation (Figure 5.2A). Between days 4 and 9, naphthenic acids concentrations in the thiosulfate-amended incubations decreased by ~15 mg/L, whereas the incubations with no thiosulfate showed a decrease of more than 60 mg/L. After 9 d there was very little change in the naphthenic acids concentration in the incubations with thiosulfate and the total decrease in naphthenic acids over the 35-d incubation period was ~ 60%. These results confirmed the findings of the previous biodegradation study, showing conclusively that thiosulfate inhibits the biodegradation of naphthenic acids. The trends shown in Figure 5.2A are in agreement with those of Kreye et al. (1973), who showed that biodegradation of organic compounds decreased steadily over a 7-d period and then stopped completely after 8 d when a wastewater containing thiosulfate was treated aerobically in an activated sludge reactor.

Interestingly, the biodegradation patterns for the low-thiosulfate (0.62 mM) and high-thiosulfate (4.4 mM) conditions were very similar (Figure 5.2A). It is possible that the two thiosulfate concentrations tested were not separated by a sufficient magnitude to cause observable differences in their respective inhibition of naphthenic acids biodegradation.

Initial pH of all incubations and sterile controls was 8.5 (Figure 5.2B). Within 2 d of incubation, the pH of sterile controls decreased to 8.0 and was constant for the duration of the experiment. The pH of incubations with no thiosulfate decreased to 6.0 after 2 d and remained at this value for the rest of the incubation, except for slight increases to 6.5 measured on days 7 and 11. The pH of thiosulfate-amended incubations (both 0.62 and 4.4 mM) also decreased over the first 2 d of incubation from 8.5 to 6.5. Between days 4 and 9, the pH decreased to 5 and remained at this value for the duration of the incubation. This pattern mirrors the pattern of naphthenic acids biodegradation observed for the thiosulfate-amended incubations (Figure 5.2A), suggesting a correlation. Surprisingly, there was only a difference of one pH unit between incubations with no thiosulfate and the thiosulfate-amended incubations. Despite this fairly small discrepancy, it is possible that pH 6 is the lower limit of the optimal pH range for

naphthenic acids biodegradation. It is also possible that heterotrophs in the original inoculum were outcompeted by thiosulfate autotrophs, as was observed by Kreye et al. (1973). The pH decrease observed for incubations with no thiosulfate may have been caused by the formation of weakly acidic organic acids produced during the biodegradation of naphthenic acids

Figure 5.2C shows that thiosulfate concentrations decreased in the thiosulfate-amended incubations during the first 9 d of incubation, after which the concentration did not change. A corresponding increase in sulfate concentrations was observed over the first 7 d of incubation, followed by a leveling-off period for the remainder of the incubation. Based on Equation 5.1, two moles of sulfate are expected from every mole of thiosulfate oxidized. Considering first the low-thiosulfate (0.62 mM) incubations, the initial concentration of thiosulfate was measured as 1.2 mM, which is almost twice the targeted initial concentration. The reason for this discrepancy was not known, but the measured concentration was well above the detection limit (20 μM) of the thiosulfate assay (Nor and Tabatabai 1975). Thiosulfate in these incubations was almost fully depleted after 9 d, decreasing by a total of 1.0 mM (Figure 5.2C). Over the same time-period, sulfate concentrations increased by 2.69 ± 0.01 mM, which was a greater increase than predicted based on Equation 5.1. The initial concentration of thiosulfate measured in the high-thiosulfate incubations was 4.5 mM, which is very close to the targeted initial concentration of 4.4 mM. The overall decrease in thiosulfate concentration over the incubation period was 2.8 mM. This change was expected to yield a 5.6 mM increase in sulfate and the measured final concentration of sulfate in these incubations was 5.63 ± 0.13 mM. These data conclusively showed that thiosulfate oxidation occurred in the thiosulfate-amended incubations, resulting in the accumulation of sulfate.

The results of this study demonstrated that biodegradation of naphthenic acids is inhibited in the presence of thiosulfate (used to quench ozone). This effect was attributed to the development of unfavorably low pH conditions as thiosulfate was oxidized to sulfuric acid and sulfate (Equation 5.1) by microorganisms in the incubations. In most cases, a phosphate or bicarbonate buffering system could be employed to mitigate pH changes resulting from thiosulfate oxidation. However, the ozonated samples described in the introduction to this chapter (Section 5.1) were not readily biodegraded despite being

prepared in a phosphate-buffered system. Furthermore, bicarbonate interferes with the method used for monitoring mineralization (Bressler et al. 1999), precluding the use of bicarbonate buffer. Formate was considered as an alternative ozone-quenching agent but was also found to interfere with the method for monitoring mineralization (data not shown). Attempts to purge residual ozone by sparging samples with air or nitrogen resulted in excessive foaming due to the surfactant properties of naphthenic acids. It was subsequently decided that all future ozonations should be conducted using a limiting concentration of ozone to eliminate the need for an ozone-quenching agent or removal protocol.

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6 APPLICATION OF HPLC/QTOF-MS FOR STUDYING BIODEGRADATION AND OZONATION OF NAPHTHENIC ACIDS

6.1 Introduction

Naphthenic acids are complex mixtures of saturated acyclic and cyclic carboxylic acids (Brient et al. 1995). The chemical structures of naphthenic acids fit the general formula $C_nH_{2n+Z}O_2$; n specifies the number of carbons, whereas Z accounts for hydrogen deficiency resulting from ring formation. Z is zero for acyclic naphthenic acids, but is a negative, even integer in the case of cyclic naphthenic acids. Figure 1.3 (see Chapter 1) shows some examples of possible naphthenic acids with Z = 0, -2, -4, and -6. Acyclic naphthenic acids exhibit a high degree of alkylation, differentiating them from fatty acids, which also fit the general formula given above for Z = 0 (Rudzinski et al. 2002).

Naphthenic acids are natural constituents of petroleum (Brient et al. 1995, Seifert and Teeter 1969, Seifert et al. 1969, Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Jones et al. 2001, Tomczyk et al. 2001, Qi et al. 2004) and are also found in the bitumen component of oil sands deposits in northern Alberta, Canada (Clemente et al. 2003, Clemente 2004). These compounds are a concern for petroleum refineries because of their propensity to corrode steel (Slavcheva et al. 1999). They also have surfactant properties, which are thought to be responsible for the observed toxicity of naphthenic acids to a variety of organisms (Headley and McMartin 2004, Clemente and Fedorak 2005).

A hot water extraction method used for the recovery of bitumen from oil sand results in naphthenic acids partitioning into the aqueous phase and becoming constituents of the process water (Schramm et al. 2000, MacKinnon and Boerger 1986, MacKinnon 1989). Oil sands process waters (OSPWs) are held on-site in large storage ponds pending treatment to mitigate toxicity, which is primarily attributed to the dissolved oil sands naphthenic acids. Holowenko et al. (2002) reported that naphthenic acids concentrations in these ponds ranged from 20 to 120 mg/L.

The complexity and structural diversity of naphthenic acids mixtures has precluded development of analytical methods capable of isolating individual naphthenic acids. For this reason, all analytical procedures currently applied in naphthenic acids research and monitoring are based on the analysis of these compounds as a group (reviewed by Clemente and Fedorak 2005; described in Section 1.4).

One of the first methods reported for quantification of naphthenic acids involved extraction and derivatization to yield naphthenic acids as their methyl esters, which were then analyzed by gas chromatography (GC) with flame-ionization detection (Herman et al. 1994). The oil sands industry has developed a standard method for naphthenic acids quantification that employs Fourier transform infrared spectroscopy for the analysis of extracted, underivatized naphthenic acids (Jivraj et al. 1995). A high-performance liquid chromatography (HPLC) method for quantification of naphthenic acids as their 2-nitrophenylhydrazine (2-NPH) derivatives was developed by Clemente et al. (2003) and modified by Yen et al. (2004). This method was based on protocols established by Miwa (2000) for quantifying carboxylic acids in food and beverages and has the advantage of permitting the analysis of aqueous samples (i.e. not extracted). Negative ion electrospray ionization mass spectrometry (MS) has also been used for measuring naphthenic acids concentrations (Headley et al. 2002, Lo et al. 2003).

Numerous MS methods have been designed or adapted for characterizing the molecular composition of naphthenic acids mixtures (reviewed by Clemente and Fedorak 2005; described in Section 1.5). Due to the inherent complexity of naphthenic acids mixtures, most MS methods employ soft ionization techniques, such as fluoride ion chemical ionization (Dzidic et al. 1988), fast atom bombardment (Fan 1991), atmospheric pressure chemical ionization, (Hsu et al. 2000) and electrospray ionization (Hsu et al. 2000, Lo et al. 2003), so that mass spectra are not further complicated by extensive fragmentation. Perhaps the most powerful MS method is electrospray ionization high-field asymmetric waveform ion mobility spectrometry (ESI-FAIMS) coupled with quadrupole and time of flight (QTOF) MS (Gabryelski and Froese 2003). In this method, ionized naphthenic acids undergo pre-separation by FAIMS before reaching the mass spectrometer. ESI-FAIMS-MS offers the advantage of minimal sample preparation, simply requiring that aqueous samples be diluted with methanol prior to analysis.

Unfortunately, the specialized instruments involved in this type of MS are not readily available to most researchers.

A GC-low resolution MS method (hereafter referred to as GC-MS) developed by St. John et al. (1998) and modified by Holowenko et al. (2002) was designed with accessibility in mind. In this method, naphthenic acids are derivatized to their *tert*-butyldimethylsilyl esters using *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) as illustrated in Figure 6.1, Scheme 1. Electron impact ionization results in fragmentation of the derivatized compounds to give characteristic [naphthenate + dimethylsilyl]$^+$ ions (Figure 6.1, Scheme 1). These ions are generally described as $[M + 57]^+$, where M is the nominal mass of the underivatized naphthenic acid and 57 is the molecular mass contributed by the dimethylsilyl group. Because the ions are singly charged, $[M + 57]^+$ values are equivalent to m/z (mass to charge) ratios detected by MS. Many $[M + 57]^+$ values that correspond to possible naphthenic acids have been tabulated (Clemente and Fedorak 2004) so that ions detected by the mass spectrometer can be easily assigned to n and Z numbers, as shown in Figure 6.1. The relative abundance (based on intensity measured by MS) of each $[M + 57]^+$ ion can then be plotted to show the naphthenic acids "fingerprint" of a particular sample (Holowenko et al. 2002). Some examples of naphthenic acids fingerprints of two OSPWs are shown in Chapter 1 (Figure 1.4).

The GC-MS method of St. John et al. (1998) has also been used for naphthenic acids quantification (Del Rio et al. 2006), but such application is not widely accepted because there is a substantial margin for error when using a single pure compound as a calibration standard for complex naphthenic acids mixtures. For example, Clemente and Fedorak (2004) showed that when approximately equimolar solutions of six model naphthenic acids were analyzed by GC-MS, the relative intensities measured for the resulting $[M + 57]^+$ ions were not identical. Thus, apparent naphthenic acids concentration could be affected by the pure compound selected as a calibration standard. Furthermore, GC-MS tends to overestimate the proportion of low molecular mass naphthenic acids due to the misassignment of fragments formed from higher molecular mass ions (Clemente and Fedorak 2004). This phenomenon would also impact quantification.

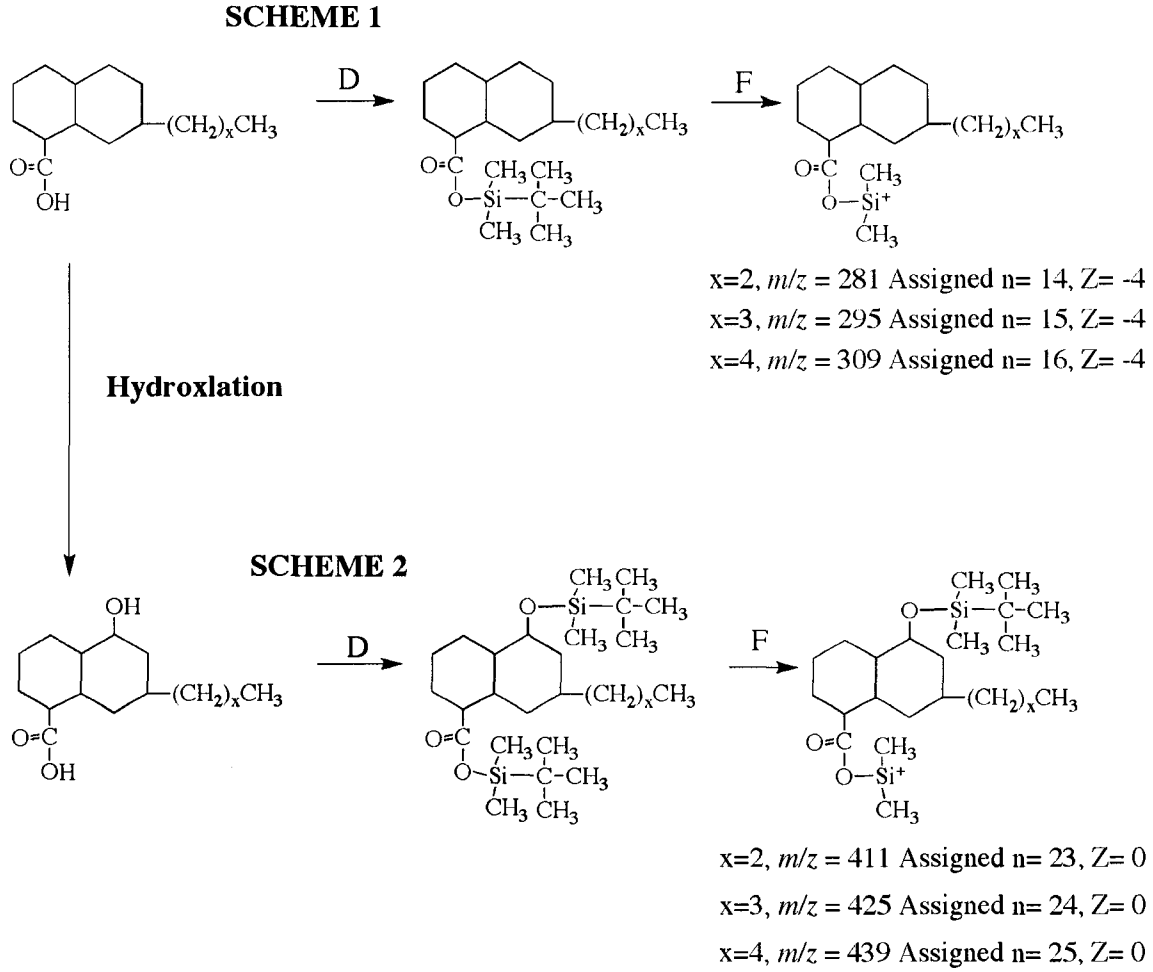


Figure 6.1 Hypothetical derivatization of bicyclic naphthenic acids with a side-chain of various lengths (x specifies the number of CH_2 units). For a non-hydroxylated naphthenic acid, Scheme 1 shows the product of derivatization (D) with MTBSTFA and the major ion formed after fragmentation (F) in the mass spectrometer. Based on *m/z*, the ion would be correctly assigned to Z = -4 with n = 14, 15, or 16, depending on the value of x. Derivatization of a possible product of hydroxylation would proceed via Scheme 2, resulting in misassignment of the ion to Z = 0 with n = 23, 24, or 25, depending on the value of x.

Although GC-MS analyses permit elucidation of some structural information about naphthenic acids mixtures, there are a multitude of isomers for each n and Z combination that cannot be differentiated. In addition to this limitation, Clemente et al. (2005) proposed that hydroxylated naphthenic acids, which could arise due to microbial oxidation, would be misassigned as high molecular weight naphthenic acids. Hydroxyl-

ated naphthenic acids possess two active hydrogens, meaning that derivatization can occur at two sites (Figure 6.1, Scheme 2). Thus, *m/z* of the resulting ion would no longer be equivalent to $[M + 57]^+$ and n and Z values assigned based on this value would be incorrect. For example, the major fragment ion of a hydroxylated naphthenic acid with n = 14 and Z = -4 would have *m/z* = 411 and would therefore be misassigned as a naphthenic acid with n = 23 and Z = 0 (Figure 6.1, Scheme 2). In a recent study, GC-high resolution MS was used to confirm the presence of three "double-derivatives" in a derivatized extract of oil sands naphthenic acids: $[C_{22}H_{43}Si_2O_3]^+$ $[C_{23}H_{45}Si_2O_3]^+$, and $[C_{24}H_{47}Si_2O_3]^+$ having exact masses of 411.2751, 425.2908, and 439.3064, respectively, were detected in the sample (Section C.3.2, Appendix C).

A newly-developed reverse-phase, capillary HPLC/QTOF-high resolution MS (hereafter referred to as HPLC/QTOF-MS) method for characterizing naphthenic acids is described in Appendix C. Briefly, solid-phase extraction (SPE) is used to recover naphthenic acids from aqueous samples which are then separated by HPLC as underivatized compounds and analyzed by QTOF-MS. The resulting mass spectra are screened for ions with exact masses corresponding to plausible naphthenic acids and the relative intensity of each recorded. These data can then be presented as three-dimensional plots of percent abundance as a function of n and Z; the same presentation style previously employed for visualizing GC-MS data (Holowenko et al. 2002, Clemente et al. 2004). Alternative data presentation methods, such as Van Krevelen diagrams, may also be used (Figure C.4, Appendix C).

A commercial naphthenic acids preparation (Merichem refined naphthenic acids) was analyzed by HPLC/QTOF-MS and the resulting three-dimensional plot was found to be very similar to the profile obtained by GC-MS (Figure C.S-4, Appendix C). However, when naphthenic acids from OSPW were analyzed, the profile generated from HPLC/QTOF-MS data was very different than the profile obtained by GC-MS (Figure C.3, Appendix C). A major difference was that HPLC/QTOF-MS did not detect any ions with $n > 21$, whereas 31% of the ions detected by GC-MS were assigned as $n \geq 22$. This observation further supported the hypothesis of Clemente et al. (2004): that many ions detected by GC-MS and assigned as $n \geq 22$ are artifacts arising from double-derivatization of hydroxylated, low molecular weight naphthenic acids (see Figure 6.1,

Scheme 2). These artifacts are not seen with HPLC/QTOF-MS because the naphthenic acids are not derivatized prior to analysis and, moreover, ions detected by HPLC/QTOF-MS are assigned to n and Z values based on exact mass rather than unit mass, as with GC-MS. HPLC/QTOF-MS can also be used to detect hydroxylated naphthenic acids based on the exact mass of these ions (see Figure C.4, Appendix C).

The HPLC/QTOF-MS method was designed to be semi-quantitative to facilitate monitoring biodegradation of specific naphthenic acids isomer classes, with the acknowledged limitation that accuracy could be compromised as naphthenic acids are degraded, changing the profile of the mixture (Section C.3.3, Appendix C). In the present study, HPLC/QTOF-MS was applied for studying biodegradation of a commercial naphthenic acids preparation (Merichem) and oil sands naphthenic acids in Syncrude OSPW. HPLC/QTOF-MS was also used to characterize an ozonated solution of Merichem naphthenic acids, which was predicted to contain hydroxylated naphthenic acids as oxidation products. This prediction was based on results from mechanistic studies with naphthalene, phenol, and chlorinated phenols that identified hydroxylated intermediates during ozonation (Hirvonen et al. 2000, Poznyak and Araiza 2005a, b). The ozonated sample was also analyzed by GC-MS for comparison.

6.2 Materials and methods

6.2.1 Biodegradation studies

Biodegradation of Merichem refined naphthenic acids (Merichem Chemicals and Refinery Services LLC, Houston, TX) and oil sands naphthenic acids in Syncrude OSPW was monitored in laboratory incubations with microorganisms obtained from an OSPW storage pond on the Syncrude lease (Syncrude Canada, Ltd., Fort McMurray, AB, Canada). Results for incubations with Merichem naphthenic acids were adapted from the biodegradation experiment reported in Appendix C. In that experiment, incubations were sampled on days 0, 2, 4, 8, 11, 14, 15, 21, and 28. Incubations with Syncrude OSPW were prepared and sampled as described in Section C.2.7 (Appendix C), except that the incubation period was 154 d and samples were taken on days 0, 42, 70, 98, 126, and 154. A sterile control was also prepared by heat-killing microorganisms in the OSPW by

autoclaving at 121°C, 100 kPa, twice for 20 min, with 24 h between treatments. Samples were extracted using SPE and analyzed by HPLC/QTOF-MS as described in Sections C.2.3 and C.2.4 (Appendix C). Quantification of Merichem naphthenic acids was based on the relative response (intensity) of ions compared to the intensity of the most abundant ion detected on day 0 (n = 13, Z = -2). Quantification of naphthenic acids in Syncrude OSPW was based on the relative response of ions compared to the intensity of an internal standard (tetradecanoic acid-1-^{13}C; purchased from Sigma-Aldrich Canada, Oakville, ON Canada), which was added before samples were injected into the HPLC. Absolute values for naphthenic acids concentrations were not determined, but changes in the relative response of each isomer class (and the entire mixture) during biodegradation enabled quantification in terms of percent increases or decreases. HPLC/QTOF-MS was performed by Mahmoud Bataineh and Xiumei Han (Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada).

6.2.2 GC-MS and HPLC/QTOF-MS analysis of ozonated Merichem naphthenic acids

An ozonated solution of Merichem refined naphthenic acids was produced in a previous ozonation experiment using an ozone dose of 28 mg/L (see Chapter 4, Section 4.2.1.3). In the present study, a subsample of this mixture was extracted using SPE and analyzed by HPLC/QTOF-MS as described in Sections C.2.3 and C.2.4 (Appendix C). The sample was also extracted using liquid-liquid extraction and analyzed by GC-MS according to the methods of Clemente et al. (2004) and Holowenko et al. (2002). A non-ozonated solution of Merichem naphthenic acids was analyzed by both methods as a control. Quantification was performed as described in Section 6.2.1 using tetradecanoic-acid-1-^{13}C as an internal standard for signal intensity. HPLC/QTOF-MS was performed by Xiumei Han (Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada).

6.3 Results and discussion

6.3.1 Biodegradation of Merichem naphthenic acids

Merichem naphthenic acids were incubated under aerobic conditions with microorganisms from an OSPW holding pond and biodegradation was monitored by HPLC/QTOF-MS. The naphthenic acids profile on day 0 (Figure 6.2) was very similar to profiles observed previously by GC-MS (Clemente et al. 2004; Figures 4.2 and 4.3, Chapter 4). The distribution of ions according to Z-series was comparable to results obtained by GC-MS, whereas the carbon number (n) distribution was shifted slightly higher. Specifically, with HPLC/QTOF-MS, ions were clustered around n = 13 to 14, whereas the most abundant ions detected by GC-MS were in the range of n = 11 to 13. After 2 d of incubation, there was little or no change in the proportion of isomers with $Z \leq -2$ (Figure 6.2). Conversely, the proportion of ions with Z = 0 decreased substantially within the first 2 d of incubation, indicating that aliphatic naphthenic acids were degraded preferentially. The naphthenic acids profile on day 4 (data not shown) resembled the profile on day 2 (Figure 6.2). On days 8, 11, 15, and 21, the naphthenic acids profiles were very similar to the profile observed on day 28. For this reason, only data for the day 28 sample is shown (Figure 6.2). By the end of the 28-d incubation period, the Merichem naphthenic acids were almost completely biodegraded, with less than 3% of the total initial naphthenic acids remaining in the incubations. Only 14 isomer classes were detected by HPLC/QTOF-MS in the day 28 sample, whereas the day 0 sample was comprised of more than 40 isomer classes (Figure 6.2). These findings are comparable to results obtained previously using GC-MS to characterize biodegradation of Merichem naphthenic acids (Clemente et al. 2004), with the exception that GC-MS (as applied here) does not provide quantitative information.

Biodegradation of individual isomer classes (i.e. ions with identical n and Z values) in the Merichem naphthenic acids preparation followed a sigmoidal pattern (Figure 6.3), except for the series with Z = 0, for which biodegradation displayed first order kinetics (data not shown). As indicated in Figure 6.3, there was a lag period of about 2 d before biodegradation of naphthenic acids with $Z \leq -2$ progressed rapidly. Most isomer classes were found to consist of both readily-biodegradable compounds, termed

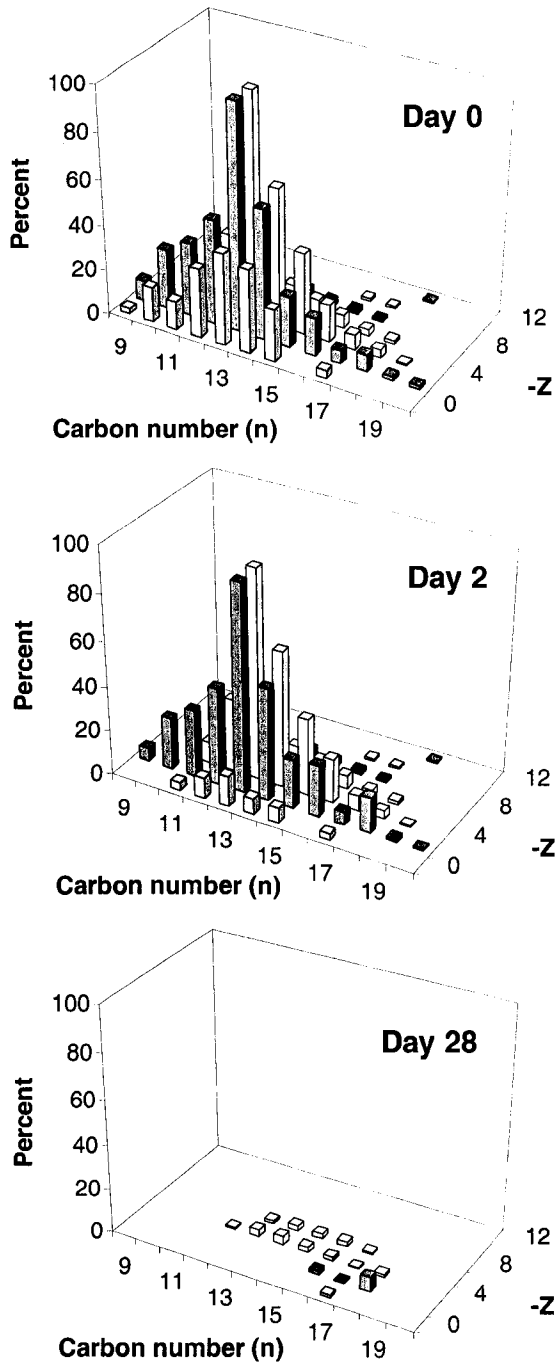


Figure 6.2 Three-dimensional plots showing the distribution of Merichem naphthenic acids (according n and Z) before (day 0) and after 2 and 28 d of incubation with microorganisms from a Syncrude OSPW pond. Less than 5% of the initial naphthenic acids remained on day 28. Data were generated by HPLC/QTOF-MS. Ion intensities were scaled relative to the intensity of the most abundant ion on day 0 (i.e. n = 13, Z = -2), which was considered to be 100%.

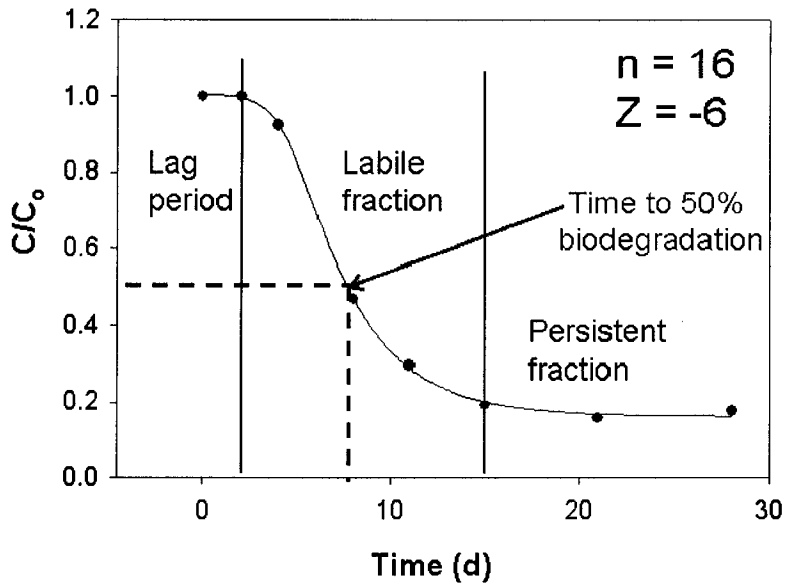


Figure 6.3 Normalized concentrations (C/C_0) of the n = 16, Z = -6 naphthenic acid isomer class plotted as a function of incubation time to illustrate the characteristic sigmoidal biodegradation pattern that was observed for all isomer classes in the Merichem naphthenic acids preparation with $Z \leq -2$.

the "labile fraction," and recalcitrant compounds, termed the "persistent fraction." For example, Figure 6.3 shows that the concentration of isomers assigned as n = 16, Z = -6 decreased by ~ 80% between days 2 and 15. Thus, ~80% of this isomer class was labile. The residual 20% did not decrease between days 15 and 28, and was therefore considered the persistent fraction (Figure 6.3).

Some isomer classes were completely labile (e.g. most of the Z = 0 and -2 families), whereas the persistent fraction of partly recalcitrant isomer classes ranged from 0 to 18%. The persistent fraction was thought to consist of highly alkylated or branched isomers, based on the results of Fedorak and Westlake (1981), who demonstrated that biodegradation of aliphatic and aromatic hydrocarbons was negatively influenced by the extent of alkylation. This hypothesis could not be tested directly because HPLC/QTOF-MS cannot resolve individual compounds. However, extracted ion chromatograms for three isomer classes (i.e. n = 15, Z = -6; n = 13, Z = -4; and, n = 13, Z = 0) showed a shift towards earlier retention times as the incubation period progressed (Figure C.5, Appendix C), and previous work by Gundersen (2001) demonstrated that shorter retention times are characteristic of more extensive alkyl branching of alkylphenols. Although the effect of

alkylation on elution of naphthenic acids has not been investigated, a linear internal standard (n =14, Z = 0) eluted at the extreme right of the chromatogram, as would be predicted by Gundersen's (2001) results.

The ability to quantitatively monitor biodegradation of individual isomer classes is a major advantage of HPLC/QTOF-MS over GC-MS. From plots like the one shown in Figure 6.3, it was possible to determine the time to 50% biodegradation (t_{50}) for the most abundant isomer classes with Z = -2, -4, and -6 (Figure 6.4). The t_{50} was also determined for the Z = 0 series using a first order kinetic model. It is clear from Figure 6.4 that t_{50} was not overly dependent on n, but was closely related to Z. Specifically, t_{50} increased with the degree of cyclization; observed t_{50} for the Z = 0 series was ~1 d, whereas t_{50} for cyclic isomers ranged from 3 to 8 d. This result was not unexpected, because microorganisms will generally degrade simpler organics before utilizing more complex substrates, such as polycyclic compounds.

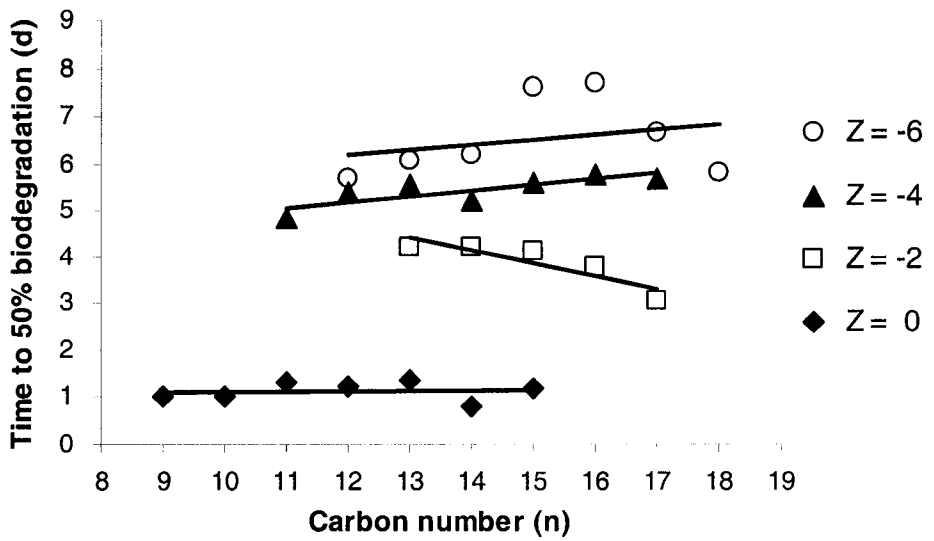


Figure 6.4 Length of incubation time required to reduce the concentration of individual naphthenic acid isomer classes by 50% based on quantitative HPLC/QTOF-MS. For isomers with Z ≤ -2, a sigmoid model (eg. Figure 6.3) was used to estimate the time to 50% biodegradation. For isomers with Z = 0, the time to 50% biodegradation was determined using a first order kinetic model.

An interesting pattern was observed for the Z = -2 series: t_{50} appeared to decrease with increasing n, but linear regression statistics showed that this negative slope was not significant (R=0.869 for P=0.05). A similar trend was seen within the Z = -6 series, where t_{50} of isomers with n = 17 and 18 was shorter than isomers with 16. In this case the slope of the line relating these three n values to t_{50} was significantly less than zero (R=0.9989 for P=0.05). Because HPLC/QTOF-MS cannot resolve individual compounds, it was not possible to determine conclusively what specific structural differences caused the isomers with lower n values to be less susceptible to biodegradation. However, it is possible that these isomer classes had a higher proportion of alkyl substituted isomers or isomers with more highly branched alkyl groups, making them more difficult to biodegrade.

6.3.2 Biodegradation of oil sands naphthenic acids

Biodegradation of naphthenic acids in nutrient-amended Syncrude OSPW was monitored by HPLC/QTOF-MS over a 98-d incubation period. The naphthenic acids profile on day 0 (Figure 6.5) was comparable to previous results obtained by HPLC/QTOF-MS (Figure C.3B, Appendix C) and very different than the profile observed previously by GC-MS (Figure C.3A, Appendix C). After 42 d, 88% of the total initial naphthenic acids remained in the incubations (Figure 6.5). This result supported the findings of a previous study in which HPLC quantification of naphthenic acids showed that, after 40 d, ~80% of the total initial naphthenic acids remained in incubations with Syncrude OSPW (Chapter 2). In the current study, incubations were maintained for an additional 114 d to determine whether a greater extent of biodegradation could be achieved with longer incubation (154 d in total).

After 70 d, 67% of the initial naphthenic acids remained in the incubations (data not shown) and after 98 d, 52% remained (Figure 6.5). HPLC/QTOF-MS did not show further decreases in the percent naphthenic acids remaining in the incubations on days 126 and 154 (data not shown). Although these results at first appeared to indicate that biodegradation halted in the incubations sometime between day 98 and day 126, the validity of these data became suspect after results for the sterile control on day 154 were considered (the day 126 sample for the sterile control was not analyzed). No

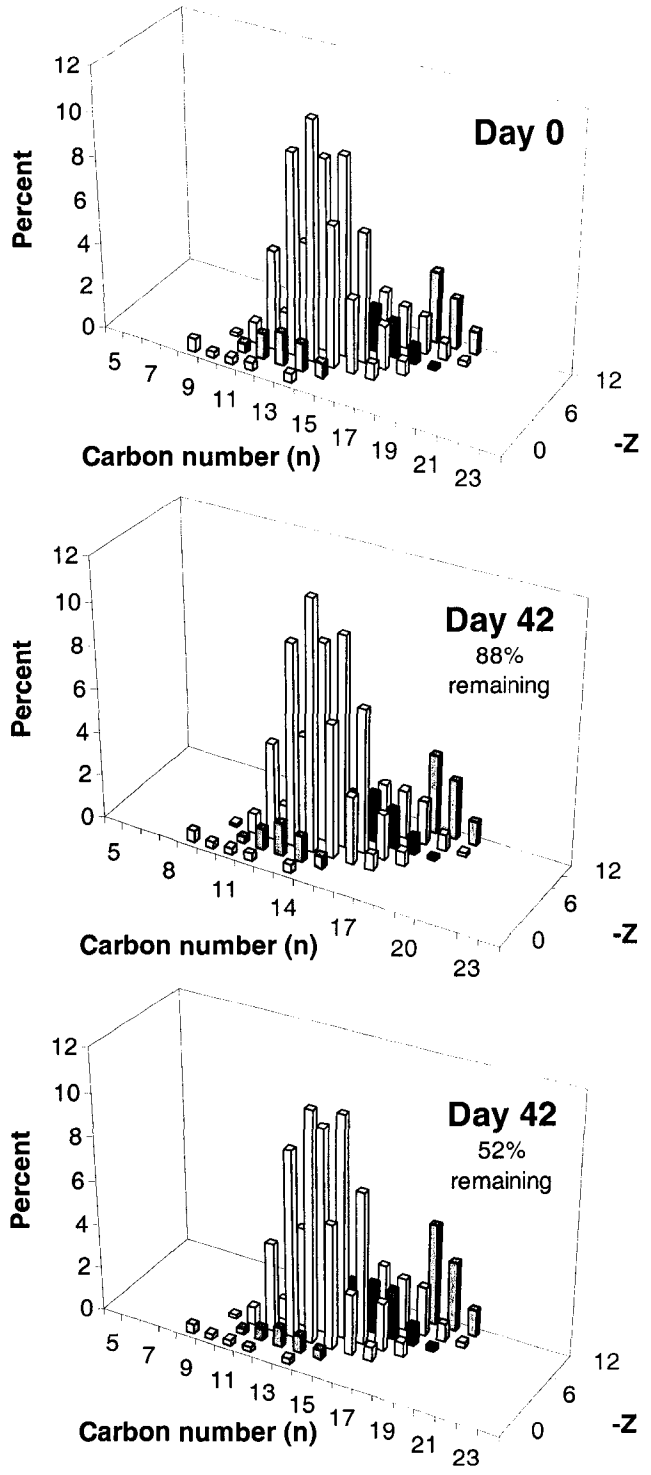


Figure 6.5 Three-dimensional plots showing the distribution of oil sands naphthenic acids in Syncrude OSPW (according n and Z) before (day 0) and after 42 and 98 d of incubation with microorganisms indigenous to the OSPW. The percentage of the total initial naphthenic acids remaining in the incubations is indicated. Data were generated by HPLC/QTOF-MS.

biodegradation was observed in the sterile control over the first 98 d of incubation, as evidenced by very little change in the relative intensity of ions compared with the internal standard (data not shown). However, there was an apparent increase in the percent naphthenic acids remaining on day 154. This increase was thought to be due to evaporation decreasing the liquid volume of the sterile control and, thereby, concentrating the dissolved naphthenic acids. The viable incubations would likely have experienced comparable evaporation during this period of the incubation. In this case, however, evaporation could have manifested as no apparent change in the percent naphthenic acids remaining (i.e. a leveling-off) if decreases due to biodegradation were effectively cancelled out by increases due to evaporation. Based on this it was concluded that the experiment should be repeated with control measures to limit evaporative losses in order to confirm the maximum extent of biodegradation of oil sands naphthenic acids. One way to do this would be using stoppered flasks. Despite the concern with data collected on days 126 and 154, results for the first 98 d of incubation were believed to be valid because the naphthenic acids concentration in the sterile control was stable during this period and kinetics were first order.

The appreciable decrease in total naphthenic acids (48%) measured at 98 d did not result in obvious changes to the naphthenic acids profile when data were plotted as shown in Figure 6.5. A similar trend was encountered when GC-MS data from previous biodegradation studies were plotted in this manner (Scott and Fedorak, unpublished results). Although this seemed to indicate non-preferential biodegradation of isomer classes within the oil sands naphthenic acids mixture, examination of the quantitative HPLC-QTOF-MS data obtained in the current study proved otherwise. Biodegradation of naphthenic acid isomer classes with Z = -2, -4, -6, and -8 followed a first order kinetic relationship (data not shown), as was observed for the Merichem Z = 0 series (see Section 6.3.1). The biodegradation pattern for oil sands naphthenic acids with Z = 0 could not be determined because the initial (day 0) abundance of isomers belonging to this series was too low. Biodegradation of isomer classes with Z = -10 and -12 was negligible compared to the extent of biodegradation observed for isomer classes belonging to higher Z-families. This trend is in agreement with the concept that microorganisms preferentially degrade simpler organic compounds before metabolizing complex substrates.

Half-lives for the most abundant isomer classes present in Syncrude OSPW were determined directly or extrapolated from the biodegradation rate over the first 98 d of the incubation period (Figure 6.6). As was seen in the biodegradation study with Merichem naphthenic acids (Figure 6.4), biodegradation of oil sands naphthenic acids was influenced by Z-series moreso than carbon number (n). However, half-lives for oil sands naphthenic acids ranged from 44 to 237 d, whereas t_{50} for Merichem naphthenic acids ranged from 1 to 8 d. These results support the findings of a previous study in which it was clearly demonstrated that commercial naphthenic acids (eg. Merichem), are more readily biodegraded than naphthenic acids in OSPW (Chapter 2). In that study, the observed recalcitrance of oil sands naphthenic acids was thought to be related to the hypothesized origin of these compounds as the persistent fraction of partially biodegraded petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Watson et al. 2002). More specifically, oil sands naphthenic acids were thought to be poorly biodegraded due to the overall complexity of the mixture and the presence of a greater proportion of high molecular weight naphthenic acids than were detected in

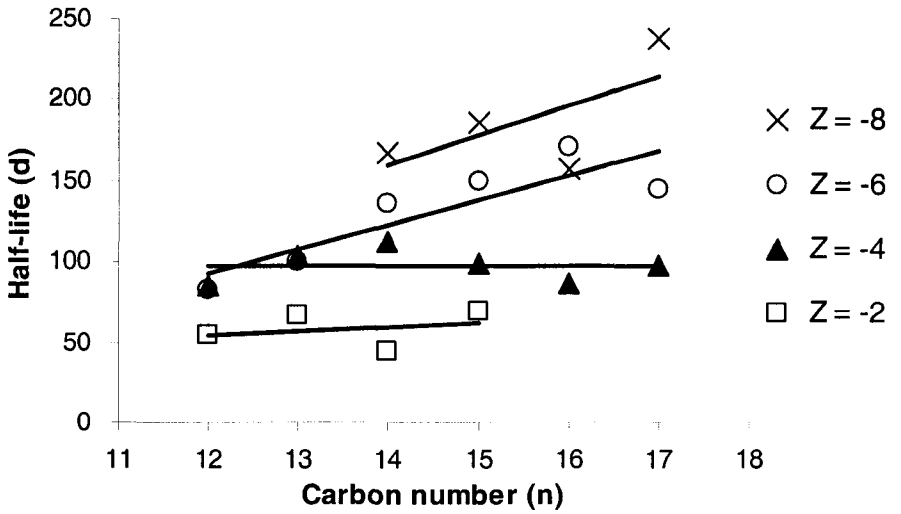


Figure 6.6 Biodegradation half-lives for the most abundant naphthenic acids isomer classes present in Syncrude OSPW. Half-lives for the Z = -2 series and isomers assigned as Z = -6 with n = 12, and Z = - 4 with n = 12 and 16 were determined directly from the biodegradation rate over the 98-d incubation period. Half-lives for all other isomer classes were extrapolated.

commercial preparations (Chapter 2). HPLC/QTOF-MS has now shown these high molecular weight naphthenic acids to be artifacts of the GC-MS method and identified them instead as low molecular weight, hydroxylated naphthenic acids (Section C.3.2, Appendix C).

The true distribution of oil sands naphthenic acids according to carbon number (Figure 6.5; day 0) is not very different from the distribution of commercial naphthenic acids (eg. Figure 6.2). However, oil sands naphthenic acids are characterized by a high proportion of isomer classes with $Z \leq -4$, whereas most naphthenic acids in the Merichem preparation are found in the $Z = 0$, -2 and -4 families. Thus, based on the true profile, the polycyclic character of oil sands naphthenic acids was thought to be one factor contributing to their poor biodegradability. However, specific characteristics of the chemical structures of oil sands naphthenic acids were thought to play a greater role in the recalcitrance of these compounds than cyclicity alone. This was hypothesized because, for all Z-series common to both naphthenic acids mixtures (i.e. $Z = -6$, -4, and -2), t_{50} or half-lives were 10- to 20-fold longer for the oil sands naphthenic acids than the Merichem naphthenic acids (Figure 6.4 and Figure 6.6). The discrepancy in biodegradability could not be attributed to molecular weight differences because the range of carbon numbers within each Z-series was comparable for the two naphthenic acids mixtures. Instead it was believed that that the oil sands naphthenic acids were less biodegradable due to extensive alkylation and branching of alkyl groups. Elution profiles obtained by HPLC/QTOF-MS provided some support for this explanation. extracted ion chromatograms showed that five isomer classes (arbitrarily selected) present in the oil sands naphthenic acids mixture eluted earlier than the same five isomer classes in the Merichem preparation (Figure C.1, Appendix C). As described in Section 6.3.1, shorter elution times are thought to be indicative of a higher degree of alkyl branching. Alkyl groups could have impeded biodegradation via steric hinderance. As an extension of the hypothesis that oil sands naphthenic acids are highly-branched, it was also hypothesized that the oil sands naphthenic acids might have a high proportion of tertiary carbons in the β position, resulting in poor susceptibility to β-oxidation.

6.3.3 GC-MS and HPLC/QTOF-MS analysis of ozonated Merichem naphthenic acids

A solution of Merichem naphthenic acids was treated with ozone (28 mg/L dose) in a previous experiment (Chapter 4). It was believed that the ozone decomposed under the slightly basic conditions (pH ~8) of this solution, forming hydroxyl radicals that then reacted with the naphthenic acids. However, the formation of hydroxyl radicals cannot be directly monitored and, therefore, the exact nature of the reaction could not be determined. For this reason, the reaction will simply be considered "ozonation." In the present study, the ozonated mixture and a non-ozonated control were analyzed by GC-MS and HPLC/QTOF-MS to examine changes in the naphthenic acids profile resulting from ozonation and data generated by the two methods were compared.

As described in Section 6.3.1, HPLC/QTOF-MS analysis of Merichem naphthenic acids generated similar results to those obtained by GC-MS (Figure 6.7). The overall distribution of naphthenic acids did not appear to change much with ozonation, but GC-MS showed the appearance of several high molecular weight isomers (n = 17 to 23) with Z = 0, -10, and -12. Similar findings were reported in Chapter 4. These ions were not detected by HPLC/QTOF-MS. It was proposed that the ions were in fact low molecular weight, hydroxylated naphthenic acids that had undergone derivatization as exemplified in Figure 6.1, Scheme 2. In this scheme, the presence of a second active hydrogen results in the addition of a second derivatizing group, inflating the molecular mass of the ion and, ultimately, resulting in it being misassigned to a high n value and incorrect Z series. The isomers with Z = 0, n = 21, 22, and 23 that were detected in the ozonated sample by GC-MS (Figure 6.7) could have arisen as shown in Figure 6.1, Scheme 2 if x were equal to 0, 1, or 2. Based on this, the true identities of the ions were predicted to be hydroxylated naphthenic acids with Z = -4, n = 12, 13, and 14.

To illustrate the possible origins of the other high molecular weight ions detected in the ozonated sample by GC-MS (i.e. those with Z = -10 and -12; Figure 6.7), hypothetical derivatization schemes are shown for an aliphatic naphthenic acid (Figure 6.8) and a monocyclic naphthenic acid (Figure 6.9), before and after hydroxylation. Figure 6.8 shows that a hydroxylated aliphatic naphthenic acid would be misassigned to the Z = -10 series. A hydroxylated monocyclic naphthenic acid would be misassigned to

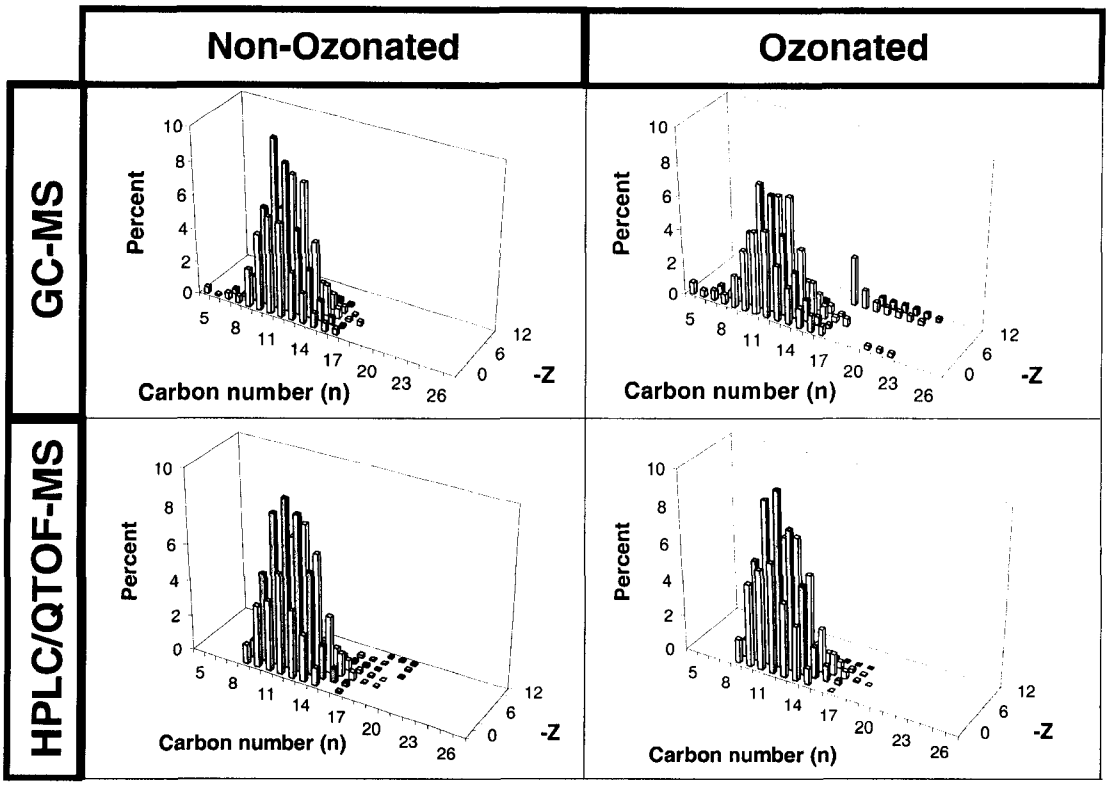


Figure 6.7 Three-dimensional plots showing the carbon number (n) and Z distribution of naphthenic acids in non-ozonated and ozonated samples of Merichem according to GC-MS and HPLC/QTOF-MS analyses. The sum of the bars in each panel is 100%.

the Z = -12 series, as shown Figure 6.9. The structures depicted in Figures 6.1, 6.8, and 6.9 are examples of plausible hydroxylated naphthenic acids belonging to the three isomer classes indicated. However, countless isomers are possible for any given n and Z combination.

HPLC-QTOF/MS showed a 61% decrease in total naphthenic acids with ozonation. Quantitative results could not be obtained by GC-MS, but naphthenic acids quantification by HPLC showed a decrease of only 17% (Chapter 4). The reason that the apparent decrease in naphthenic acids was much lower when determined based on HPLC data was attributed to the fact that hydroxylated naphthenic acids (hypothesized products of ozonation) would have been quantified as naphthenic acids whereas these compounds would have been excluded from the quantitative measurements made using HPLC-QTOF/MS data. In other words, the final naphthenic acids concentration measured by

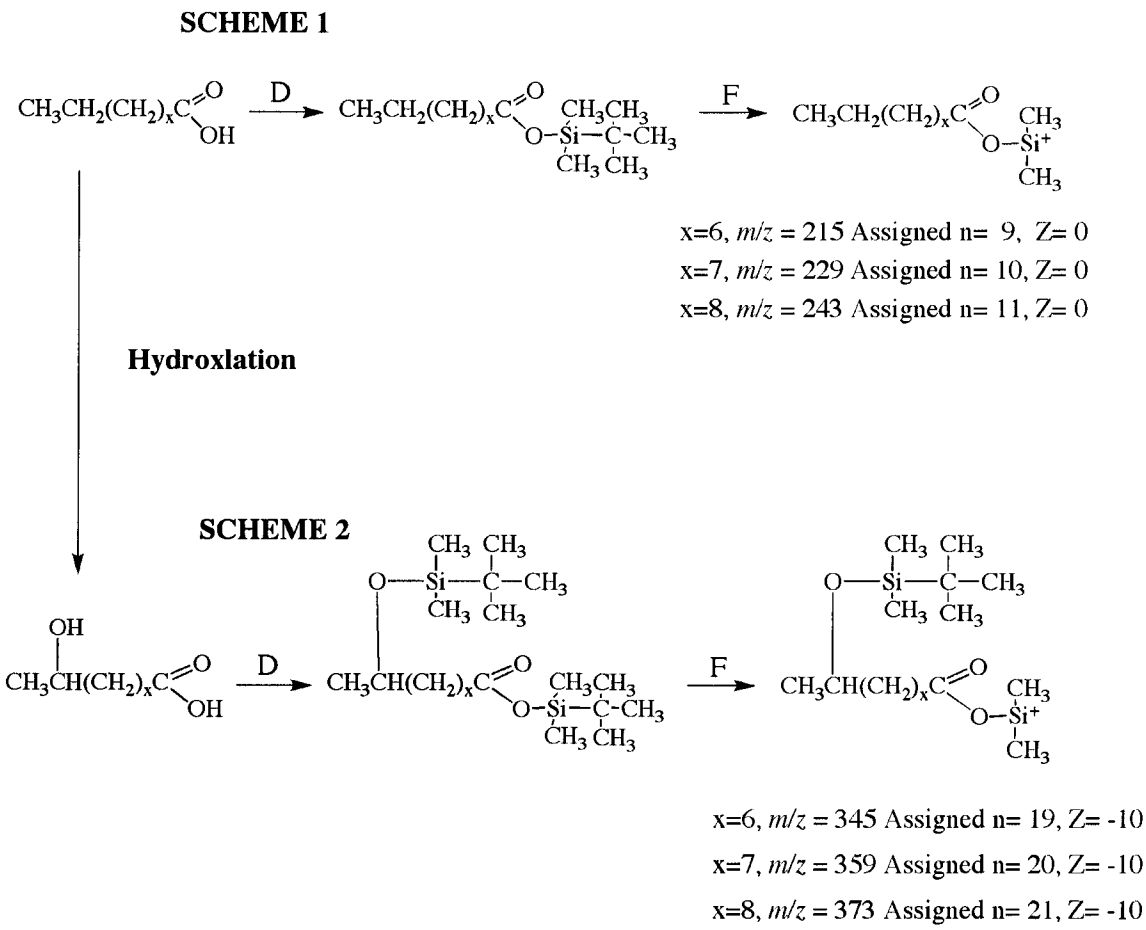


Figure 6.8 Hypothetical derivatization of aliphatic naphthenic acids with a side-chain of various lengths (x specifies the number of CH_2 units). For a non-hydroxylated naphthenic acid, Scheme 1 shows the product of derivatization (D) with MTBSTFA and the major ion formed after fragmentation (F) in the mass spectrometer. Based on *m/z*, the ion would be correctly assigned to Z = 0 with n = 9, 10, or 11, depending on the value of x. Derivatization of a possible product of hydroxylation would proceed via Scheme 2, resulting in misassignment of the ion to Z = -10 with n = 19, 20, or 21, depending on the value of x. These misassigned ions are present in the naphthenic acids profile by GC-MS shown in Figure 6.7.

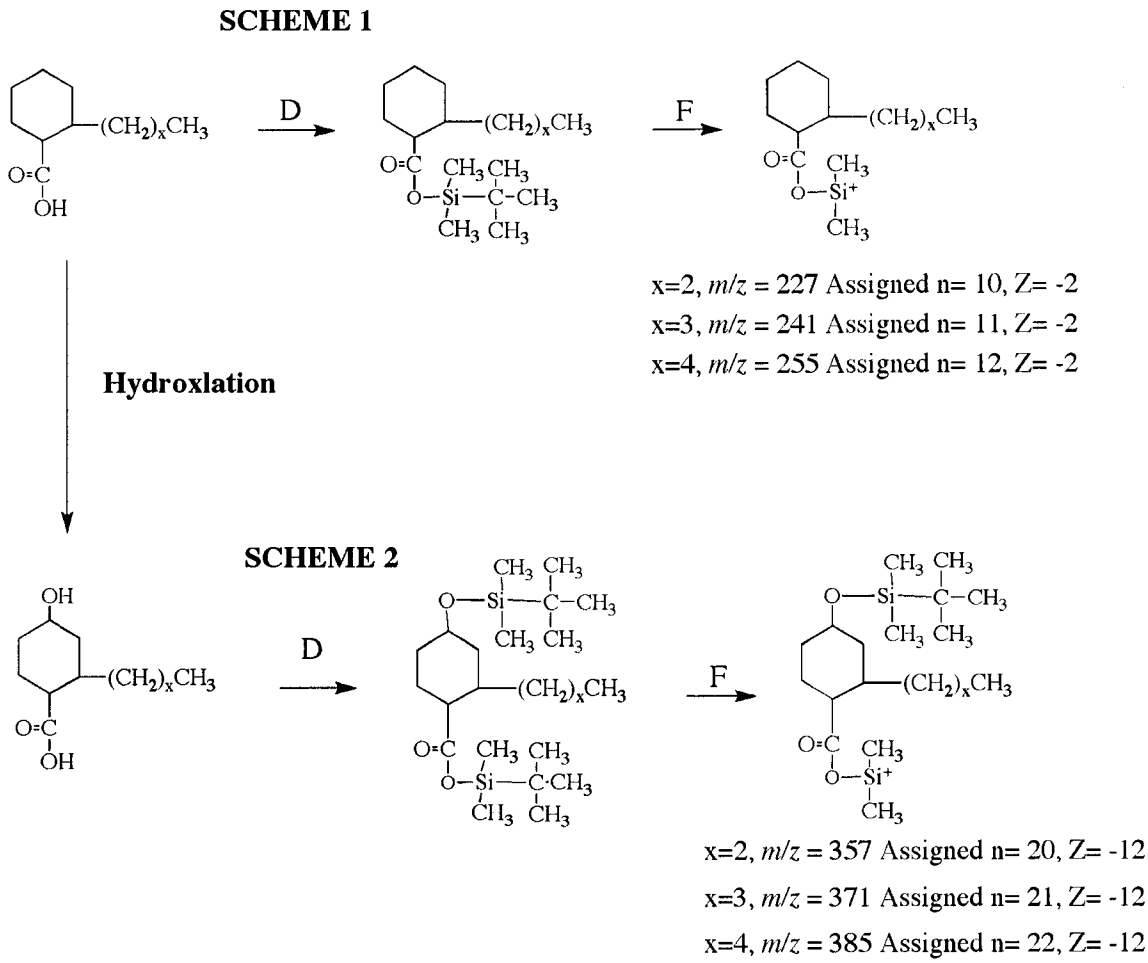


Figure 6.9 Hypothetical derivatization of monocyclic naphthenic acids with a side-chain of various lengths (x specifies the number of CH_2 units). For a non-hydroxylated naphthenic acid, Scheme 1 shows the product of derivatization (D) with MTBSTFA and the major ion formed after fragmentation (F) in the mass spectrometer. Based on *m/z*, the ion would be correctly assigned to Z = -2 with n = 10, 11, or 12, depending on the value of x. Derivatization of a possible product of hydroxylation would proceed via Scheme 2, resulting in misassignment of the ion to Z = -12 with n = 20, 21, or 22 depending on the value of x. These misassigned ions are present in the naphthenic acids profile by GC-MS shown in Figure 6.7.

HPLC was artificially high, resulting in a lower apparent decrease than what was calculated from the changes measured by HPLC-QTOF/MS.

To confirm the presence of oxidized compounds in the ozonated sample, HPLC/QTOF-MS data were screened for ions with exact masses corresponding to possible naphthenic acids plus the mass of one or more oxygen atoms. These ions were denoted M + 16, M + 32, and M + 48 and their distributions, according to n and Z, are illustrated in Figure 6.10. Ions identified as M + 64 were detected in five isomer classes (data not shown). Along with several other partially oxidized naphthenic acids, ions with exact masses corresponding to the plausible hydroxylated naphthenic acids shown in Figures 6.1, 6.8, and 6.9 were detected by HPLC/QTOF-MS (Figure 6.10A). Although the M + 16, M + 32, M + 48, and M + 64 ions were believed to be hydroxylated naphthenic acids, this could not be confirmed because other potential oxidation products, such as ketones, would have the same exact mass (i.e. the same chemical formula). For example, a hydroxylated naphthenic acid (M + 16) with n = 10, Z= -2 would have an exact mass of 186.1256, which is the same exact mass of the ketone product of a naphthenic acid with n = 10, Z = 0 (Table 6.1). The inability to differentiate isomeric oxidation products is an acknowledged limitation of the HPLC/QTOF-MS method. However, HPLC/QTOF-MS is preferable to GC-MS because the latter cannot even distinguish partially-oxidized naphthenic acids from high molecular weight naphthenic acids. No partially oxidized naphthenic acids were detected in the non-ozonated sample.

The relative abundance of oxidized products in the ozonated sample was estimated from the ratio of ion intensities to the internal standard. The sum total of these intensities was equivalent to ~15% of the total initial abundance of naphthenic acids in the non-ozonated sample. Thus, it was estimated that 15% of the naphthenic acids were converted to oxidized products during ozonation. Considering together the overall decrease in naphthenic acids (61%) and the percentage of oxidized products in the ozonated sample (~15%), it was estimated that ~46% of the naphthenic acids were mineralized or converted to products other than oxidized naphthenic acids. The oxidized products were primarily (80%) comprised of ions identified as M + 16 (hypothesized to be monohydroxylated naphthenic acids) (Figure 6.10A). Ions identified as M + 32 and

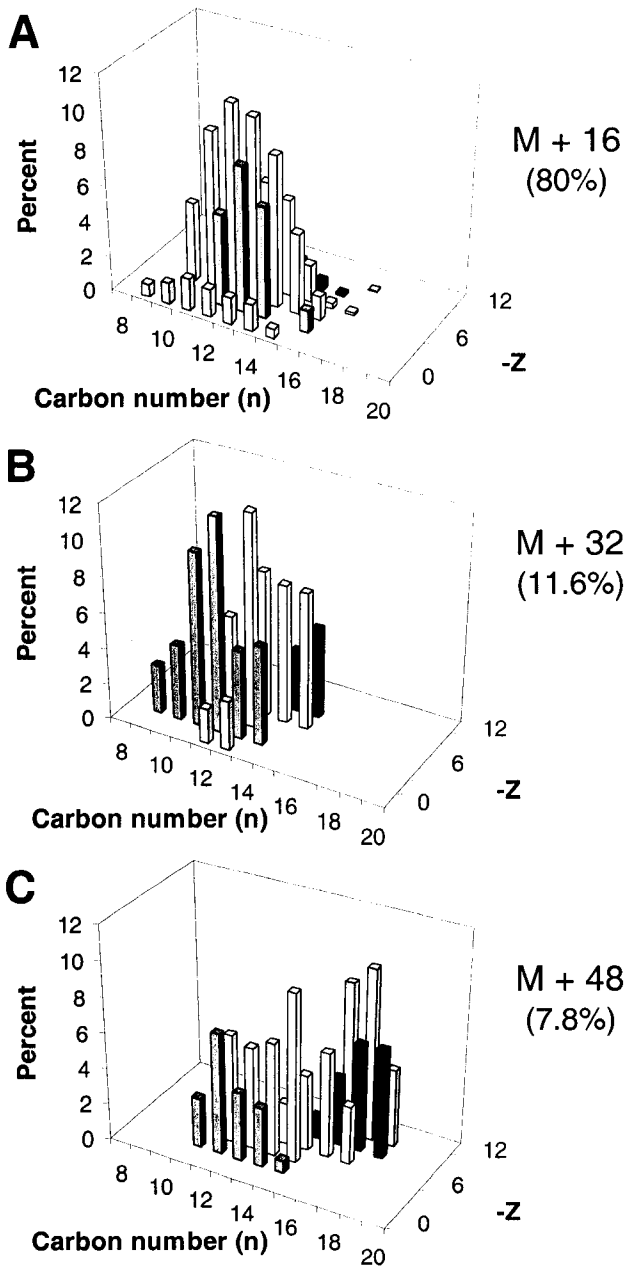
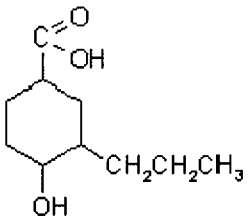
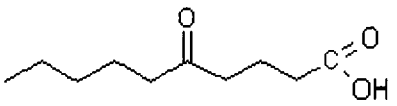


Figure 6.10 Three-dimensional plots showing the distribution of naphthenic acids with 1, 2, or 3 additional oxygen atoms (indicated as M + 16, M + 32, M + 48, respectively) in the ozonated Merichem sample. M corresponds to the molecular mass of the naphthenic acid and 16, 32, and 48 correspond to the mass contributed by the oxygen(s). These ions were believed to be mono-, di-, and tri-hydroxylated naphthenic acids. Percentages indicated in brackets are the percent of the total oxidized products contributed by the oxidation product shown. Approximately 0.6% of the oxidized products were M + 64 (not shown). Data were obtained by HPLC/QTOF-MS. The sum of the bars in each panel is 100%.

Table 6.1 Possible structures of two naphthenic acid oxidation products (M + 16) having the same chemical formula and exact mass but belonging to different Z-series and possessing non-identical functional groups (i.e. hydroxyl or ketone substitutions).

Structure	Formula	Exact mass	Z-series[a]
	$C_{10}H_{18}O_3$	186.1256	-2
	$C_{10}H_{18}O_3$	186.1256	0

[a] assigned based on structure shown, rather than based on comparison of *m/z* with tabulated Z values for naphthenic acids (Clemente and Fedorak 2004).

M + 48 comprised 11.6 and 7.8% of the oxidized products, respectively (Figure 6.10B, C). Less than 1% of the oxidized products were identified as M + 64.

A higher degree of oxidation (i.e. more additional oxygen atoms) was observed with increasing Z-series and, to a lesser extent, increasing carbon number (Figure 6.10). For example, ions identified as M + 16 were clustered around n = 11 to 14, Z ≥ -6, whereas ions identified as M + 32 were centered around the same n-values but there were very few Z = 0 isomers and a high proportion of Z = -6 and -8 (Figure 6.10A, B). Ions identified as M + 48 appeared in two clusters: one cluster was comprised of isomers with n = 11 to 15, Z = -2 and -4, whereas the other cluster was centered around n = 14 to 18 with Z = -6, -8, and -10 (Figure 6.10C). The Z = 0 series did not contain any ions identified as M + 48. It was hypothesized that aliphatic naphthenic acids (Z =0) that reacted with hydroxyl radicals may have been readily-mineralized and, therefore, fewer partially-oxidized products were observed for this Z-series. On the other hand, polycyclic naphthenic acids appeared to be more susceptible to multiple hydroxylation events (or other oxidative substitutions) but were less-readily mineralized, as evidenced by their persistence in the ozonated sample.

Neither HPLC/QTOF-MS or GC-MS indicated that the oxidation of Merichem naphthenic acids occurred selectively (Figure 6.7). That is, no particular isomer class appeared to be preferentially degraded and almost all isomer classes that were detected in the non-ozonated sample were present, albeit at lower concentrations, in the ozonated sample. Furthermore, oxidized products were detected for almost every isomer class present in the non-ozonated sample (Figure 6.10). This non-specific degradation pattern is a stark contrast to the preferential biodegradation trends observed during microbial oxidation of Merichem naphthenic acids (Figure 6.4). One possible explanation for this discrepancy is that microbial enzymes are targeted to substrates with specific chemical structures or functional groups (Anfinsen 1973), whereas hydroxyl radicals are potent oxidizing agents that react non-selectively with organic compounds (Hoigne et al. 1985). Comparisons could not be made between the products of ozonation and microbial oxidation because no oxidation products were detected in the biodegradation experiment.

6.3.4 Summary

A recently-developed HPLC/QTOF-MS method (Appendix C) was applied for characterizing biodegradation and ozonation of naphthenic acids. The type of structural information that can be obtained by HPLC-QTOF-MS (i.e. n and Z values) is no more detailed than what can be gleaned from GC-MS, which has been the standard method of qualitative analysis since its development (St. John et al. 1998). However, the quantitative capacity of HPLC-QTOF-MS permits evaluation of changes to each naphthenic acid isomer class, elucidating preferential biodegradation patterns that can be predicted but not confirmed by GC-MS. Moreover, HPLC/QTOF-MS is superior to GC-MS because the assignment of ions to n and Z values is based on exact masses rather than unit mass. For this reason, HPLC/QTOF-MS is not subject to errors arising from "double derivatization" of partially oxidized (hydroxylated) naphthenic acids, whereas GC-MS analysis results in these ions being misassigned as apparent high molecular weight naphthenic acids with incorrect Z-values.

Partially oxidized naphthenic acids were found in a sample of OSPW but not in the Merichem commercial naphthenic acids preparation. A possible explanation for this relates to the hypothesized origins of naphthenic acids as partially-biodegraded petroleum

hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Tomczyk et al. 2001, Watson et al. 2002) and to the sources of these 2 naphthenic acids mixtures. Oil sands naphthenic acids come from bitumen, which is considered a mature or extensively biodegraded petroleum. Thus, it is plausible that even the naphthenic acids themselves could have undergone partial biodegradation in the formation. On the other hand, commercial naphthenic acids are typically prepared from petroleum sources that have not undergone extensive biodegradation (i.e. crude oil) (Brient et al. 1995), and, therefore, are not as likely to contain partially-oxidized naphthenic acids. Furthermore, the distillation process used to recover naphthenic acids for commercial applications (Brient et al. 1995) may inadvertently exclude hydroxylated compounds.

The results presented in this chapter demonstrate some potential applications of the newly-developed HPLC/QTOF-MS method for characterizing naphthenic acids. One disadvantage of the method is that data analysis is relatively labor-intensive compared to the GC-MS method, which was designed so that MS data could be exported directly into an Excel (Microsoft©) matrix that automatically assigns ions to n and Z values based on *m/z*. Currently, HPLC/QTOF-MS data must be screened manually for ions with exact masses corresponding to plausible naphthenic acids.

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7 SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

7.1 Summary

The purpose of this project was to examine the suitability of aerobic biodegradation as a treatment method to relieve the acute toxicity of oil sands process water (OSPW) and, if necessary, investigate alternative pre-treatment methods such as chemical oxidation with ozone. The toxicity of OSPW is primarily attributed to naphthenic acids (Headley and McMartin 2004, Clemente and Fedorak 2005). Therefore, removal of these compounds was a major target and determinant of treatment efficacy.

A previous study used solutions of commercial naphthenic acids as a surrogate for OSPW and assessed biodegradability of these mixtures using an inoculum of microorganisms from an OSPW storage pond that were enriched for growth on commercial naphthenic acids (Clemente et al. 2004). That study showed that two commercial naphthenic acids preparations were readily biodegraded in less than 2 weeks. It was hypothesized that naphthenic acids in OSPW would also be biodegraded if incubated under comparable conditions. Chapter 2 describes results that counter this hypothesis, showing instead that oil sands naphthenic acids were poor substrates for microbial biodegradation. In fact, only about 20% of the naphthenic acids in laboratory incubations of OSPW were degraded after 40 to 49 d of incubation. This was comparable to the average extent of mineralization observed by Herman et al. (1994) who monitored biodegradation of an extract of oil sands naphthenic acids in laboratory cultures. The study described in Chapter 2 also led to the discovery that commercial naphthenic acids are preferentially biodegraded when both commercial and oil sands naphthenic acids are mixed. This was determined by spiking OSPW samples with four different commercial naphthenic acids preparations and monitoring biodegradation by GC-low resolution mass spectrometry (hereafter, GC-MS) (Chapter 2). In all cases, total ion current chromatograms showed that the commercial naphthenic acids hump was degraded, whereas the oil sands naphthenic acids hump was persistent.

Initially differences in the biodegradability of oil sands naphthenic acids relative to commercial naphthenic acids was attributed the former having a greater proportion of high molecular weight and polycyclic compounds, which are typically less favorable substrates for microbial metabolism. In a subsequent study it was shown that the ions assigned as high molecular weight naphthenic acids based on GC-MS were actually artifacts caused by "double derivatization" of hydroxylated naphthenic acids (Appendix C, Chapter 6). Essentially, the presence of a second active hydrogen (the first being that of the carboxylic acid moiety) results in the addition of a second derivatizing group (i.e. *tert*-butyldimethylsilyl), increasing the mass of the derivatized compound and resulting in misassignment of the major fragment ion as a naphthenic acid of high molecular mass. These doubly-derivatized ions are also assigned an incorrect Z-value. Thus, distribution profiles of oil sands naphthenic acids generated by GC-MS are not accurate due to the presence of hydroxylated naphthenic acids in the mixture (confirmed by GC-high resolution MS, Appendix C).

The identification of hydroxylated naphthenic acids in OSPW was not surprising because naphthenic acids are thought to be remnants of partially biodegraded petroleum hydrocarbons (Tissot and Welte 1978, Meredith et al. 2000, Tomczyk et al. 2001, Watson et al. 2002) and hydroxylation is one mechanism of microbial oxidation. Furthermore, bitumen - the petroleum component of oil sands ore - is thought to be the residuum of extensively biodegraded crude oil (Tissot and Welte 1978). As would be predicted by this theory, bitumen contains a greater abundance of naphthenic acids than many conventional petroleums (Brient et al. 1995, CONRAD 1998, Clemente 2004). It is not surprising then that the acidic fraction of bitumen, having arisen due to biodegradation, also contains a large proportion of partially biodegraded (eg. hydroxylated) naphthenic acids (Appendix C, Chapter 6). On the other hand, commercial naphthenic acids preparations, which are typically recovered from distillates of conventional petroleum (Brient et al. 1995), do not contain hydroxylated naphthenic acids (Chapter 6). Therefore, GC-MS analyses of unaltered commercial naphthenic acids preparations are not subject to errors arising from the misassignment of doubly-derivatized ions (Appendix C, Chapter 6).

The recalcitrance of oil sands naphthenic acids clearly precluded the use of microbiological processes for treatment of OSPW and necessitated exploration of alternative treatment options. Chemical oxidation by ozone (ozonation) was tested in a single experiment with a prefiltered sample of OSPW (Chapter 3). Ozonation was conducted using proprietary diffusion technology manufactured by SEAIR Diffusion Systems Inc. (Edmonton, AB). The naphthenic acids concentration in the prefiltered OSPW was reduced to detection limits of both the HPLC and FT-IR quantification methods, and the final effluent was non-toxic according to the Microtox™ bioassay (Chapter 3). Thus, ozonation achieved the principal desired end-point of treatment: complete removal of acute toxicity. Unfortunately, implementing a treatment strategy that relies exclusively on ozonation is unlikely to be economically feasible for the oil sands companies due to the high cost associated with generating ozone (Ikehata and Gamal El-Din 2004, Quen and Chidambara Raj 2006). A more practical approach would involve coupling ozonation and biodegradation (i.e. using chemical oxidation as a pretreatment before microbiological treatment).

Ozonation of naphthenic acids was investigated in several additional experiments using solutions of a commercial naphthenic acids preparation (Merichem refined naphthenic acids) as a surrogate for OSPW (Chapter 4). These experiments were conducted using bench-scale glass reactors rather than the SEAIR reactor. After some preliminary experiments demonstrated that ozone could react with the naphthenic acids, solutions of Merichem were ozonated in semi-batch reactions (i.e. continuous bubbling of ozone for a set time-period) in order to generate partially oxidized mixtures for use in subsequent biodegradation studies. Reactions were stopped by quenching residual ozone with excess sodium thiosulfate, which turned out to have a negative impact on biodegradation. This effect was attributed to thiosulfate undergoing biological oxidation to sulfuric acid, creating unfavorable pH conditions in the incubations (Chapter 5).

A new ozonation protocol was developed in which Merichem naphthenic acids were mixed with limiting amounts of ozone, circumventing the need for an ozone-quenching agent (Chapter 4). Ozonated mixtures produced from these batch reactions had lower naphthenic acids concentrations and were less toxic than non-ozonated solutions of Merichem naphthenic acids. Because commercial naphthenic acids were known to be

readily biodegradable (Clemente et al. 2004, Chapter 2), it was hypothesized that the ozonated mixtures would be biodegraded more rapidly owing to partial oxidation during the pretreatment step. However, results showed that initial biodegradation rates in incubations with ozonated solutions were not significantly different from the biodegradation rate observed with a non-ozonated control (Chapter 4).

Another unexpected finding was that 25 to 30 % of the initial naphthenic acids remained in incubations with non-ozonated naphthenic acids. Previous studies had shown complete biodegradation of commercial naphthenic acids within 2 weeks (Clemente et al. 2004, Chapter 2). The only difference between the commercial naphthenic acids solutions used in the previous studies and the non-ozonated control used in this biodegradation study was the presence of phosphate buffer (30 mM, pH 8) in the latter. It was deemed highly unlikely that phosphate buffer had a detrimental effect on biodegradation, particularly because a previous study showed that addition of phosphate enhanced naphthenic acids biodegradation (Lai et al. 1996). On the other hand, it was discovered earlier in this project that phosphate buffer affects the HPLC method of naphthenic acids quantification (Appendix B). The procedure for preparing HPLC calibration standards was modified in an effort to circumvent quantification errors resulting from phosphate buffering. However, results from the biodegradation studies with ozonated and non-ozonated samples suggest that quantification of naphthenic acids in phosphate-buffered samples might still be inaccurate. Another concern was that the HPLC method of naphthenic acids quantification might be prone to errors resulting from double-derivatization of hydroxylated naphthenic acids, which were predicted products of the ozonation of Merichem. This is because the derivatizing agent used in the HPLC method (i.e. 2-nitrophenylhydrazine) reacts with active hydrogens and hydroxylated naphthenic acids contain more than one active hydrogen. Double-derivatization of hydroxylated naphthenic acids with 2-nitrophenylhydrazine was not confirmed and it is not known whether double-derivatization of naphthenic acids would actually affect quantification by HPLC.

Mineralization was monitored in one biodegradation study with ozonated samples so that these data could be compared with HPLC results (Chapter 4). It was found that more CO_2 was produced in incubations with ozonated naphthenic acids than non-

ozonated naphthenic acids. Perplexingly, the total amount of CO_2 produced in incubations with ozonated naphthenic acids was much greater than predicted based on the decrease in naphthenic acids concentration, as measured by HPLC. No plausible explanation could be found for this observation.

In the course of this project, collaborative research led to the development of an HPLC/QTOF-high resolution MS method (hereafter, HPLC/QTOF-MS) for characterization of naphthenic acids (Appendix C). This method offered the advantage of allowing quantitative measurement of changes to individual naphthenic acids isomer classes (i.e. n and Z groups). Moreover, ions detected by HPLC/QTOF-MS were assigned to isomer classes based on exact mass rather than unit mass as with GC-MS, which eliminated the potential for misassignment of ions.

Biodegradation of Merichem naphthenic acids and oil sands naphthenic acids was monitored in separate experiments by HPLC/QTOF-MS (Chapter 6). The time required to achieve 50% biodegradation (t_{50}) was determined for each naphthenic acids isomer class and the results reflected previous findings that demonstrated the superior biodegradability of commercial naphthenic acids (Chapter 2). Specifically, t_{50} for isomer classes in incubations with Merichem naphthenic acids ranged from 1 to 8 d, whereas t_{50} for isomer classes in incubations with oil sands naphthenic acids ranged from 44 to 237 d (Chapter 6). The incubation period for the latter biodegradation study was 154 d. Therefore, not all isomer classes were degraded to 50% of their initial abundance within the incubation period, and t_{50} had to be extrapolated based on data from the first 98 d of incubation. The recalcitrance of oil sands naphthenic acids was attributed to a large proportion of polycyclic compounds as well as high degrees of alkylation and alkyl-branching, although the latter could not be definitively shown by HPLC/QTOF-MS.

HPLC/QTOF-MS was also applied for the analysis of ozonated Merichem naphthenic acids (Chapter 6). Ozonation was predicted to generate partially oxidized products (i.e. naphthenic acids + one or more oxygen) such as hydroxylated naphthenic acids. Numerous oxidized compounds, belonging to various isomer classes, were detected by HPLC/QTOF-MS. Previous analyses of ozonated samples by GC-MS had shown the appearance of ions assigned as high molecular weight, polycyclic naphthenic acids but these ions were not detected by HPLC/QTOF-MS. The ions were subsequently

identified as doubly-derivatized hydroxylated naphthenic acids that had been misassigned in the same manner that hydroxylated naphthenic acids in OSPW were misassigned as high molecular weight naphthenic acids. Due to the ability of HPLC/QTOF-MS to differentiate between hydroxylated and non-hydroxylated naphthenic acids, the method was considered superior to GC-MS for analysis of ozonated samples.

7.2 List of major discoveries

For ease of reference, some of the major discoveries made during the course of this project are listed below. Where applicable, the related chapter or appendix is indicated in parentheses.

1. commercial naphthenic acids are more readily-biodegradable than naphthenic acids in oil sands tailings water (Chapter 2)
2. quantification of naphthenic acids in OSPW is affected by which commercial naphthenic acids preparation is used to prepare the calibration curve for HPLC analysis (Chapter 2)
3. ozone can react with commercial and oil sands naphthenic acids in aqueous solution, decreasing the concentration of the naphthenic acids and lowering toxicity of the effluent (Chapters 3 and 4)
4. thiosulfate, which is used to quench residual ozone, interferes with biodegradation of naphthenic acids (Chapter 5)
5. GC-MS characterization of oil sands naphthenic acids and partially-oxidized naphthenic acids results in misassignment of "doubly-derivatized" compounds as high molecular weight naphthenic acids, with an incorrect Z-value (Chapter 6, Appendix C)
6. naphthenic acids begin to precipitate at pH <8 (Appendix A)
7. phosphate buffer interferes with quantification of naphthenic acids by HPLC (Appendix B)
8. aeration to remove residual ozone causes foaming

7.3 Suggestions for future research

Ozonation of naphthenic acids in OSPW and the effect of ozonation on their biodegradability should be investigated more thoroughly. Trials could be conducted using actual OSPW, as suggested in Chapter 3, or using a surrogate solution prepared from an extract of OSPW. The latter would offer the advantage of being able to freely manipulate the initial naphthenic acids concentration. Several trials will likely be required to optimize the ozone dosage required to generate a mixture of partially oxidized oil sands naphthenic acids. Doses used in the experiments with Merichem naphthenic acids (Chapter 4) would be a good starting point. Oil sands naphthenic acids are less susceptible to biological oxidation than commercial naphthenic acids (Chapter 2) and the same may be true for chemical oxidation. However, the recalcitrance of oil sands naphthenic acids is thought to be due to extensive alkylation and alkyl branching; structural properties that have been shown to enhance the susceptibility of organic compounds to electrophilic attack by hydroxyl radicals (Rakovsky and Zaikov 1998). Based on this, oil sands naphthenic acids may actually be *more* susceptible to chemical oxidation by hydroxyl radicals than commercial naphthenic acids, which are not as branched. Alternative methods for generating hydroxyl radicals, such as H_2O_2/UV, and the effects of pH on oxidation efficiency could also be investigated in future studies.

The experimental protocols and analytical methods required for evaluating the effects of ozonation and for monitoring biodegradation of naphthenic acids during subsequent biological treatment have been previously established (Yen et al. 2004, Jivraj et al. 1995, St. John et al. 1995, Holowenko et al. 2002) or were modified or developed during the course of this project (Appendix B, C). However, the interference of phosphate buffer and the effect of hydroxylated naphthenic acids on quantification of naphthenic acids by HPLC should be resolved before pursuing more ozonation-biodegradation studies. It is recommended that the HPLC/QTOF-MS method of naphthenic acids characterization (Appendix C) be used for analysis of ozonated samples rather than GC-MS.

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APPENDICES

APPENDIX A

pH EFFECTS ON NAPHTHENIC ACIDS SOLUBILITY

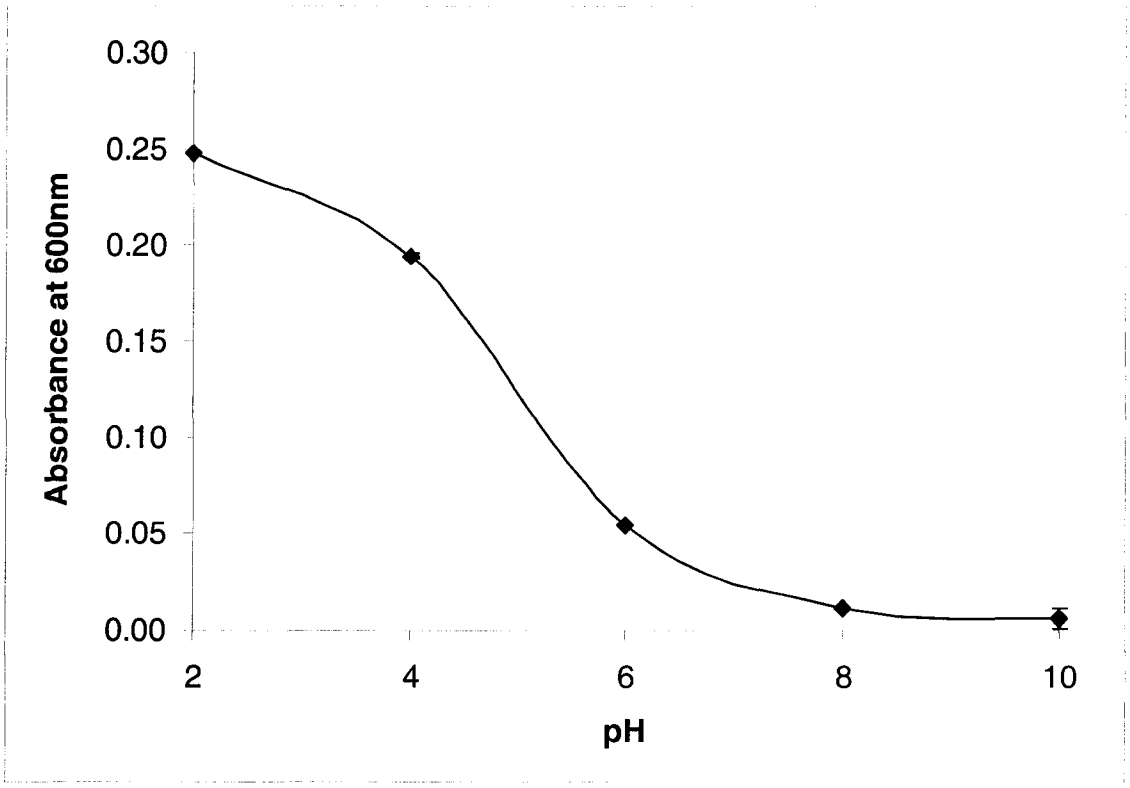
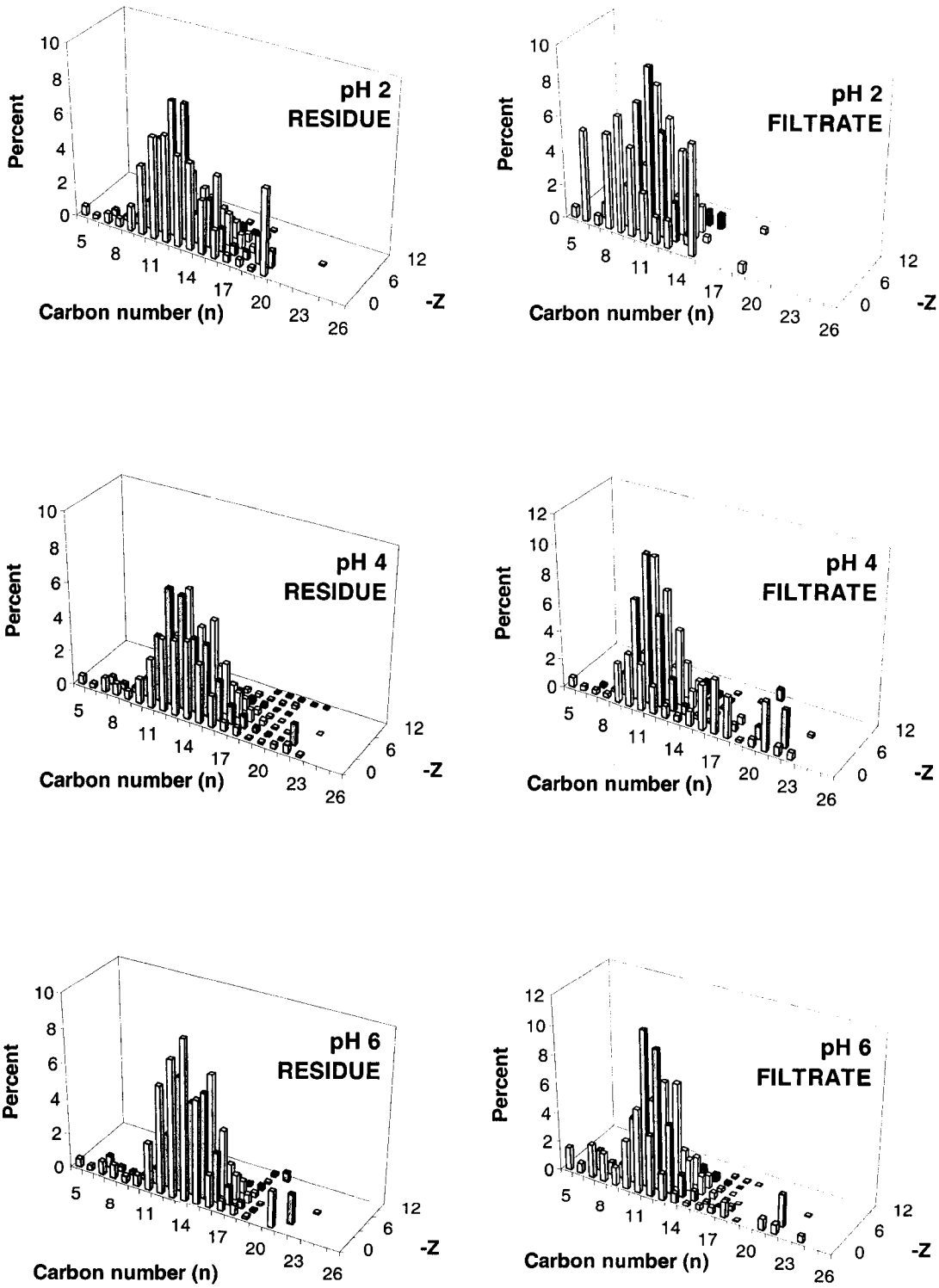


Figure A.1 Effect of pH on turbidity of a 150 mg/L solution of Merichem refined naphthenic acids.



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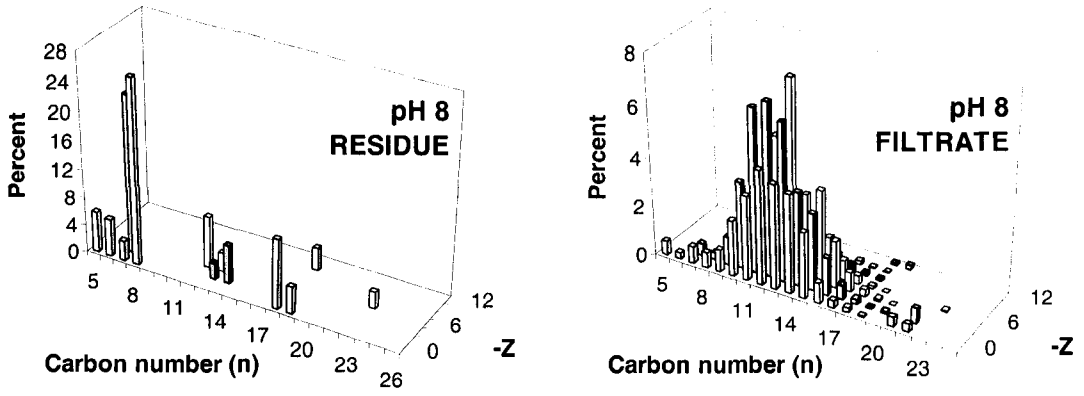

Figure A.2 Fractionation of Merichem naphthenic acids based on solubility at pH 2, 4, 6, and 8. Solutions of Merichem were prepared in buffers adjusted to the pH indicated and the solution was filtered through Millex-HA 0.45 μm filters (Millipore, Bedford, MA). Residues from each filtration were recovered from filters by washing with 0.1 M NaOH (pH 10). Filtrates and redissolved residues were extracted using the method of Clemente et al. (2004) and analyzed by GC-MS according to the method of Holowenko et al. (2002). A major ion with m/z = 281, corresponding to n = 14, Z = -4, was detected in the pH 8 residue. This ion was observed previously by Clemente and Fedorak (2004) and was identified as bleed from the GC column. Therefore, this ion was excluded from the three-dimensional plot for the pH 8 residue. Note that the y-axis scales are not identical for each panel.

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APPENDIX B

MODIFICATION OF THE HPLC METHOD

B.1 Background

HPLC quantification of naphthenic acids in the first sets of ozonated samples generated by David Wang and Dr. Rodney Guest (Civil and Environmental Engineering, University of Alberta) consistently yielded values that were lower than the target naphthenic acids concentration. Sample chromatograms had a different appearance than normal, with some early peaks shifting to later retention times and sometimes overlapping the naphthenic acids "hump." The latter contributed error to the total area of the naphthenic acids "hump," resulting in inaccurate quantification.

The original HPLC quantification method involves derivatization with 2-nitrophenylhydrazine (2-NPH) in the presence of 1-ethyl-3-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) under acidic conditions (Yen et al. 2004). These reagents are incubated with an aqueous sample of naphthenic acids for 20 min at 60°C, after which time the reaction is stopped by addition of potassium hydroxide (KOH) to raise the reaction pH to ~8. It was believed that phosphate buffer (30 mM, pH 8) in the ozonated samples may have affected pH of the reaction, resulting in inefficient derivatization, alteration of the absorption wavelength of derivatives, or a combination of both.

B.2 Methods, results, and discussion

Three sets of calibration standards were made up with Merichem naphthenic acids in different concentrations of phosphate buffer such that the final concentration of phosphate in each standard was either equivalent to the final concentration of phosphate buffer in the ozonated samples (30 mM; 1X) or 0.2X and 2X this amount. A set of control calibration standards were also prepared according to the original HPLC protocol (Yen et al. 2004).

Figure B.1 illustrates that phosphate buffer drastically influenced the quantification of naphthenic acids by HPLC. In the presence of "1X" phosphate buffer

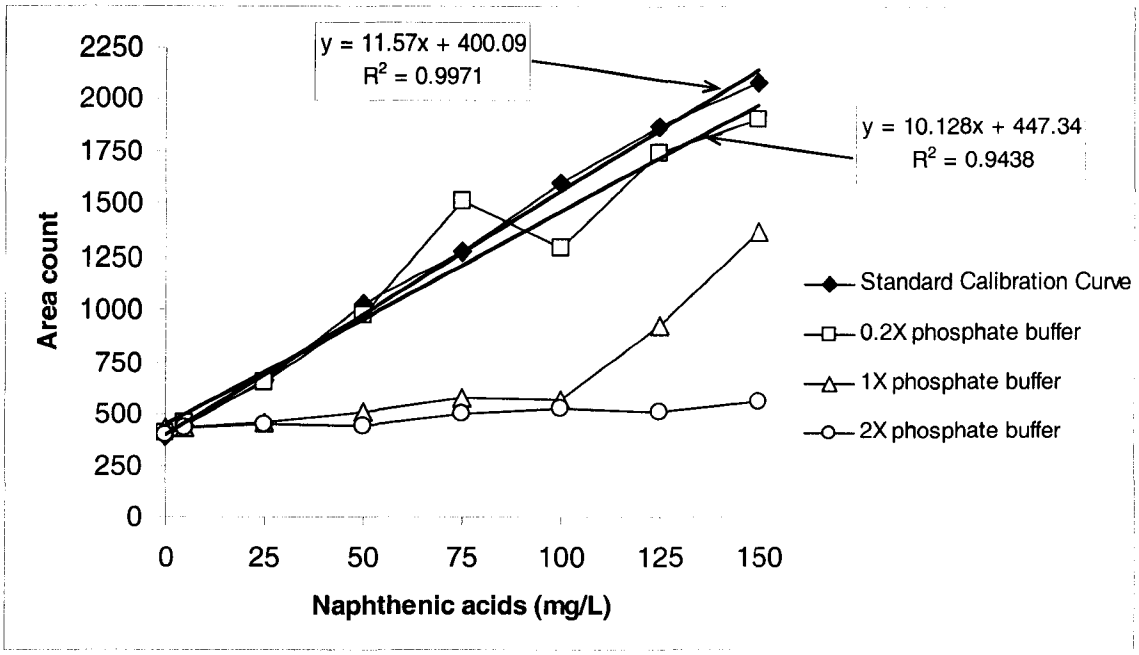

Figure B.1 Calibration curves from HPLC analyses of Merichem naphthenic acids standards prepared according to the protocol of Yen et al. (2004) (i.e. no phosphate buffer) and with phosphate buffer at three different concentrations. "X" represents the concentration of phosphate buffer used to prepare samples for the ozonation experiments (i.e. 30 mM). Trendlines were assigned for data that showed a linear relationship.

(30 mM) the lower detection limit for naphthenic acids increased from 5 mg/L (normal) to 100 mg/L. In the presence of "2X" phosphate buffer this effect was amplified to the extent that the increase in area over the whole calibration curve was less than 200 area counts, corresponding to a slope of 1, approximately 11 times less than the normal calibration curve. The slope of the calibration curve for standards prepared in "0.2X" phosphate buffer was 10, which was close to that of the standard calibration curve. The fact that the interference was less pronounced with lower concentrations of phosphate buffer lead us to believe the buffering problem might be overcome by manipulating reagent strengths. Rozlyn Young (Department of Biological Sciences, University of Alberta, Edmonton, AB) was engaged to investigate this possibility.

In order to gain a better understanding of how phosphate buffer was affecting the derivatization reaction, Ms. Young monitored the pH of Merichem naphthenic acids standards before and after addition of the derivatization reagents. She found that the pH

in standards with phosphate buffer (final concentration = 30 mM) was ~12 before reagent addition, which was comparable to the pH of non-buffered calibration standards. After reagent addition, pH in the phosphate-buffered standards dropped to between 7 and 8, which was 2 to 3 pH units higher than the pH of non-buffered standards after reagent addition. This discrepancy may account for the differences in slope shown in Figure B.1.

Ms. Young tested several modifications of the derivatization protocol, including increasing the concentration of hydrochloric acid used to prepare the 2-NPH solution and manually adjusting pH to ~5 after the addition of derivatization reagents to the phosphate-buffered (30 mM) standards. Although the latter modification was successful in generating a calibration curve of reasonable slope, adjusting the pH of every reaction was not a practical solution to the problem.

While working on this project, Ms. Young and I discovered a problem with the method for preparing calibration standards. The original protocol of Yen et al. (2004) involved making a concentrated stock solution of naphthenic acids in 0.1 M NaOH, followed by a ~1/5 dilution of the stock using double-distilled or Milli-Q water to yield a 500 mg/L solution of naphthenic acids in 0.02 M NaOH. Standards were then made up in 5-mL volumetric flasks as follows: 0 to 1.5 mL of the 500 mg/L naphthenic acids solution was mixed with 3 mL double-distilled or Milli-Q water and the volume balance was made up with 0.1 M NaOH. As a result, standards with the lowest naphthenic acids concentration received a larger volume of 0.1 M NaOH than standards with the highest naphthenic acids concentration. This inconsistency was rectified by substituting 0.02 M NaOH in place of 0.1 M NaOH to make up the volume balance in standards. The modification generated a good calibration curve (Figure B.2) with a slope that was comparable the slope generated using standards prepared according to the original dilution protocol (~13 area counts/mg/L).

Next, Ms. Young prepared phosphate-buffered standards using the modified dilution procedure. Making up buffered standards requires substituting 3 mL of phosphate buffer in place of the water used to prepare non-buffered standards. The calibration curve of the phosphate-buffered standards had a slope of 8.5 (Figure B.2), which was substantially better than previous slopes (Figure B.1), but still considerably

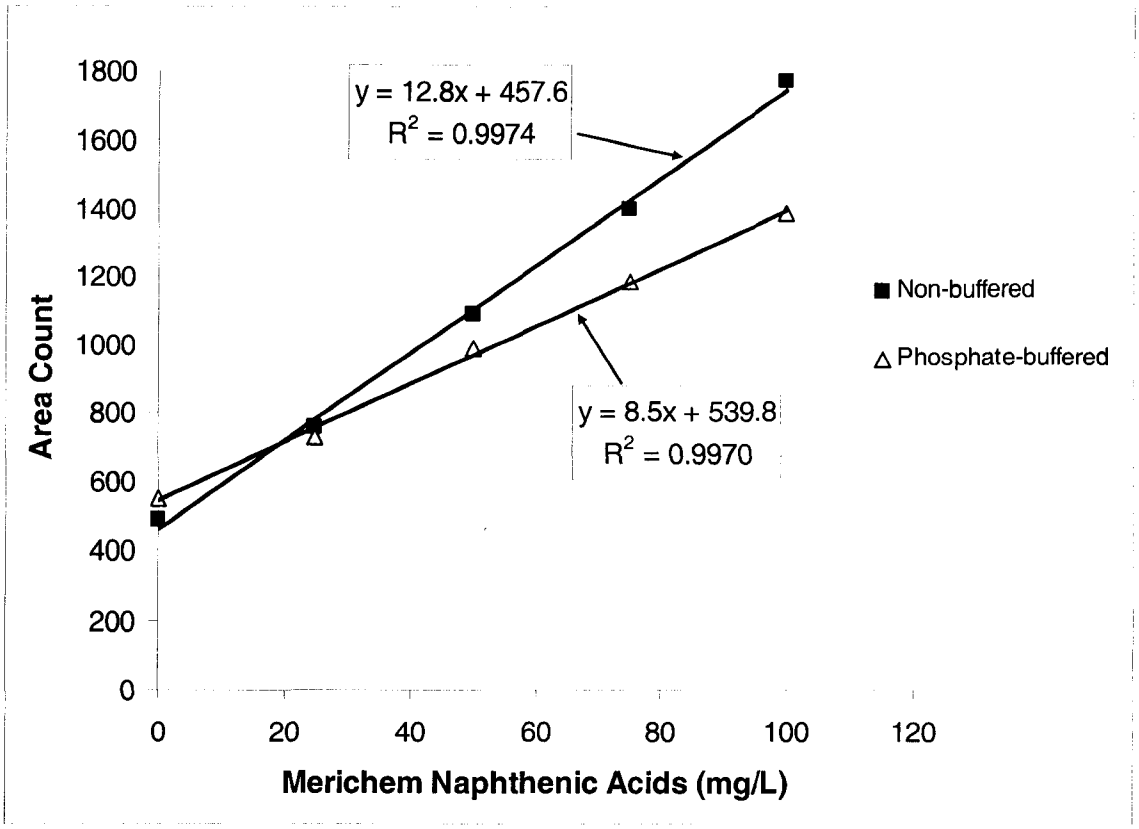

Figure B.2 Calibration curves for non-buffered and phosphate-buffered Merichem naphthenic acids standards prepared using the modified dilution procedure (i.e. dilution with 0.02 M NaOH).

lower than the slope for modified, non-buffered standards. Nevertheless, the calibration curve was reasonable enough to permit naphthenic acids quantification.

The modified dilution procedure has since been adopted as protocol for the preparation of non-buffered calibration standards. Likewise, phosphate-buffered calibration standards are prepared using the new dilution procedure and are employed for the quantification of all phosphate-buffered samples. Table B.1 shows the volumes of each component required for the preparation of calibration standards.

Table B.1 Volume of each component required for the preparation of calibration standards for HPLC quantification of naphthenic acids.

	Volume added to each standard (μL)			
Final conc.[a] (mg/L)	**500 mg/L working solution**	**0.02 M NaOH**	**MQ-H_2O *or* 50 mM phosphate buffer[b]**	**Total volume (mL)**
0	0	2000	3000	5
5	100	3900	6000	10[c]
25	250	1750	3000	5
50	500	1500	3000	5
75	750	1250	3000	5
100	1000	1000	3000	5
125	1250	750	3000	5
150	1500	500	3000	5
200	2000	0	3000	5

[a] final concentration of naphthenic acids; [b] dependent upon the sample matrix, MQ-H_2O or phosphate buffer are used to make up the volume balance of each standard; [c] the 5 mg/L standard was made up to 10 mL so that all volumes could be pipetted with the same pipetman (minimum volume 100 μL).

B.3 Literature cited

Yen, T.-W., Marsh, W.P., MacKinnon, M.D., and Fedorak, P.M. 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. J. Chromatogr. A 1033: 83-90

APPENDIX C

CAPILLARY HPLC/QTOF-MS FOR CHARACTERIZING COMPLEX NAPHTHENIC ACID MIXTURES AND THEIR MICROBIAL TRANSFORMATION♦

C.1 Introduction

Oil sands regions of northern Alberta, Canada, contain an estimated 1.7 trillion barrels of oil in the form of bitumen (MacLean 1998), representing the second largest deposit of crude oil in the world. A rapidly expanding industry extracts surface-mined bitumen from the sand using alkaline hot water, resulting in large volumes of aqueous tailings that must be contained on site due to toxicity and a no-discharge policy. The toxicity of these tailings has largely been attributed to naphthenic acids (Clemente and Fedorak 2005), a complex mixture of naturally occurring aliphatic and (poly-)alicyclic carboxylic acids having the general formula $C_nH_{2n+Z}O_2$, where n is the carbon number and Z is zero or a negative even number defining the hydrogen deficiency due to cyclization (e.g., 0, -2, -4, etc.). The structure of the many isomers within each isomer class (i.e., for each n and Z combination) is poorly understood. Naphthenic acids are regarded as persistent (Scott et al. 2005) and total concentrations in tailings ponds can range from 20 to 120 mg/L (Holowenko et al. 2002, Headley and McMartin 2004, Yen et al. 2004). Storage of tailings water represents a temporary solution but is a substantial cost to the industry, and the risk of large spills or of naphthenic acids leaching into surrounding aquatic environments (previously detected in groundwater; Headley and McMartin 2004, Clemente and Fedorak 2005) grows with the size of the industry. A more sustainable solution would be to reduce the toxicity of these aqueous tailings, thus, there is an urgent need to understand the fate of naphthenic acids under a variety of engineered scenarios.

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Naphthenic acid fate studies are currently hampered by a lack of adequate analytical methods (Headley and McMartin 2004) that can be rapidly applied to accurately and simultaneously determine naphthenic acid isomer class profiles, quantify degradation rates, and identify transformation products in complex environmental samples. To date, methods based on MS have provided the most useful data for characterizing naphthenic acid mixtures in water samples, including GC/MS or GC/GC/MS of volatile naphthenic acid derivatives (St. John et al. 1998, Jones et al. 2001, Holowenko et al. 2002, Clemente and Fedorak 2004, Hao et al. 2005) and direct liquid infusion analysis using atmospheric pressure ionization (API) MS (Fan 1991, Headley et al. 2002, Rudzinski et al. 2002, Lo et al. 2003, 2006) or high-resolution MS (HRMS) (Gabryelski and Froese 2003, Barrow et al. 2003, 2004) methods. None of these techniques has been applied to the analysis of naphthenic acid transformation products, but otherwise, each has its own intrinsic advantages and disadvantages in terms of rapidity, applicability, and accuracy. GC methods are sensitive and suffer few matrix effects but are time-consuming, and moieties originating from the volatile derivatives complicate mass spectral interpretation. Liquid infusion MS techniques are rapid, however, matrix effects severely hamper sensitivity and quantitative analysis (Barrow et al. 2003, 2004). Furthermore, for complex samples, the adequacy of unit resolution MS detectors is dubious because, for a homologous series of naphthenic acids normally encountered (e.g., n from 10 to 20, and Z from 0 to -12), an ion is expected to occur at every 2 *m/z* units in the mass spectrum, thus, the potential for misclassification is high from natural water constituents or oxidized naphthenic acid transformation products (e.g., hydroxylated naphthenic acids).

A method for the direct liquid chromatographic separation of naphthenic acids (i.e., without derivatization) has not previously been described, and we are unaware of previous applications of HPLC to the separation of large aliphatic or alicyclic acids (i.e., >C10) that would be suitable for MS detection of naphthenic acids or their transformation products. The intention of this work was to develop a high-resolution (i.e., mass resolution ~10 000), sensitive, specific, and quantitative capillary HPLC/QTOF-MS method that would be applicable to naphthenic acids and their transformation products to enable priority fate studies. The optimized and quantitative method is described and

compared to an established unit resolution GC/MS technique, oxidized naphthenic acid products are detected in the same chromatographic run, a new data visualization technique is adopted, and retention times reveal new knowledge on naphthenic acid fate in biodegradation studies.

C.2 Materials and methods

C.2.1 Chemicals and reagents

The six naphthenic acid model compounds, 1-methyl-1-cyclohexane carboxylic acid ($C_8H_{14}O_2$), *trans*-4-pentylcyclohexane-1-carboxylic acid ($C_{12}H_{22}O_2$), 2,2-dicyclohexylacetic acid ($C_{14}H_{24}O_2$), 2-hexyldecanoic acid ($C_{16}H_{32}O_2$), octadecanoic acid ($C_{18}H_{36}O_2$), and 5-β-cholanic acid ($C_{24}H_{40}O_2$), oxidized naphthenic acid model compounds, 12-hydroxydodecanoic acid, 12-hydroxyoctadecanoic acid, and 3-hydroxy-5-β-cholanic acid, the internal standard (also used as a model compound), tetradecanoic acid-1-^{13}C ($C_{14}H_{28}O_2$), and lock-mass compounds, pentafluoropropionic acid and nonafluoropentanoic acid were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Acetic acid, ammonium acetate, ethyl acetate, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Refined Merichem naphthenic acids were a gift provided by Merichem Chemicals and Refinery Services LLC (Houston, TX). Ultrapure water was prepared with a Milli-Q gradient A10 Ultrapure Water Purification System (Millipore, Billerica, MA).

C.2.2 Tailings water collection

A sample of tailings water was provided by Syncrude Canada Ltd. from the clarified zone of the "West In Pit" on January 16, 2006 and was stored at 4°C.

C.2.3 Water sample extraction

Sample preparation was performed by two independent methods and compared: liquid-liquid extraction (Clemente et al. 2004) and solid-phase extraction (SPE). For liquid-liquid extraction, tailings water (100 mL) was adjusted to pH 11 using 2 M NaOH and was centrifuged for 20 min (15000g) to remove suspended material. The supernatant

was recovered and the pH lowered to <2 using 2 M H_2SO_4. This was extracted three times with 25 mL of ethyl acetate containing 2% acetic acid, by volume, and the combined extract was washed with 10 mL of saturated NaCl solution and dried over anhydrous Na_2SO_4. This was then evaporated to dryness on a rotary evaporator, and the residue was transferred to a small vial in ethyl acetate and again taken to full dryness under a gentle stream of nitrogen.

SPE was based on a previous method (Headley et al. 2002) but was refined. Water samples (100 mL) were centrifuged for 20 min (15000g), and the pH was adjusted to pH 3 using formic acid. SPE cartridges (200 mg of Oasis HLB, Waters, Franklin, MA) were conditioned with 6 mL of ethyl acetate, 6 mL of methanol, and 6 mL of 0.1% formic acid, followed by extraction of the acidified sample at ~2 mL/min, and then by 3 mL of distilled water. Cartridges were dried under vacuum and analytes were eluted with 12 mL of ethyl acetate, which was subsequently evaporated to dryness under nitrogen at 35°C. The residue was dissolved in 60% methanol (0.1% formic acid) to a final volume of 1 mL for HPLC/QTOF-MS analysis.

C.2.4 HPLC/QTOF-MS

Chromatographic separation was performed using a micro-HPLC system (Series 1100, Agilent Technologies, Waldbronn, Germany) and a reversed-phase capillary column (Aquasil, 150 × 0.5 mm, 3 μm, Thermo). Injection volumes were always 3 μL. The mobile phases were as follows: eluant A, 10 mM ammonium acetate, eluant B, methanol (0.1% formic acid). The purpose of 0.1% formic acid was to protonate the naphthenic acids and, thus, to improve chromatographic retention on reversed phase. The flow rate was 10 μL/min, and a gradient elution program was used: 0-5 min 60% B, ramped to 70% B by 7 min, ramped to 100% B by 25 min, and held for 5 min before returning to initial conditions and equilibrating for 10 min before injection of the next sample.

Detection was performed in full scan (*m/z* 80-500 at rate of 1 scan/s) on an API QSTAR Pulsar i mass spectrometer (Applied Biosystem/MDS Sciex, Concord, ON, Canada) equipped with an ion spray source and running Analyst QS 1.1 software (Applied Biosystem, Foster City, CA). The system was operated in negative ion mode,

and the source and inlet parameters were optimized by infusion of analytes at 10 μL/min. Optimized parameters were as follows: ion spray capillary voltage of -4200 V, curtain gas flow rate 1.13 L/min, nitrogen nebulizer gas flow rate 4 L/min, DP1 25 V, DP2 15 V, and focusing potential -200 V. Mass calibration was performed with model naphthenic acid compounds (142-360 Da), and two perfluoroalkylcarboxylates in methanol were infused as lock masses during QTOF-MS data collection.

C.2.5 Method validation

Detection limits were defined as the mass of the analyte producing a signal-to-noise ratio of 3:1 and were determined for selected model compounds. Dynamic range was studied over 3 orders of magnitude (0.10-100 mg/L of refined Merichem) and instrumental precision was calculated by measuring peak area reproducibility (50 mg/L refined Merichem) for multiple injections within a day. Extraction recovery was calculated for model naphthenic acids and the refined Merichem commercial naphthenic acids by a 50-fold SPE enrichment.

Tailings water was used to independently evaluate the influence of matrix on the HPLC/QTOF-MS analysis and SPE recovery of model compounds. To assess instrumental matrix effects, a mixture containing 1.25 μg of each model naphthenic acid was spiked directly into two vials containing 1 mL of either 40% distilled water or 40% tailings water (Syncrude West In Pit), and 60% methanol. To assess the effects of matrix on SPE recovery, the same spike was added to duplicate 5-mL samples of either distilled water or tailings water and processed, as above, resulting in 10-fold enrichment (2.5 μg/mL final concentration). Matrix effects were assessed by comparing the instrumental response of matrix and matrix-free samples, after subtracting background response in the tailings matrix.

C.2.6 GC/MS and GC/HRMS.

For GC/MS analysis, the residue from liquid-liquid preconcentration was derivatized using *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (St. John et al. 1998) and GC/MS performed as previously described (Holowenko et al. 2002).

GC/HRMS was performed with an Agilent 6890 GC coupled to a Kratos MS50 mass spectrometer in EI ionization mode (Department of Chemistry, University of Alberta, Canada). Separation was achieved on a 30-m capillary ZB-5 column (Phenomenex, Torrance, CA, 0.25-mm i.d., 0.25 μm). Helium was used as carrier gas at a constant linear velocity of 3 cm/s, and the GC program was otherwise identical to GC/MS (Holowenko et al. 2002). Injector temperature was 300°C, and 1 to 5 μL of a derivatized sample was injected in pulsed splitless mode (207 kPa for 2 min). The MS system was tuned to a minimum resolution of 10 000 (10% valley) using perfluorokerosene as reference gas. Acquisition was performed in selected ion monitoring for ions corresponding to the formulas $C_{22}H_{43}Si_2O_3^+$, $C_{23}H_{45}Si_2O_3^+$, and $C_{24}H_{47}Si_2O_3^+$ (exact masses 411.2751, 425.2907, and 439.3064, respectively), specifically to confirm the presence of oxidized naphthenic acids.

C.2.7 Microbial biodegradation

Naphthenic acid biodegradation studies were conducted using established methods (Scott et al. 2005) for refined Merichem and Syncrude tailings water. Triplicate incubations were prepared for both sources of naphthenic acids in 500-mL Erlenmeyer flasks containing a final volume of 200 mL. For Merichem naphthenic acids, incubations had initial concentrations of 100 mg/L total naphthenic acids and were supplemented with 5%, by volume, modified Bushnell-Haas mineral salts medium (MMBH) (Wyndham and Costerton 1981). The inoculum was microorganisms from Syncrude tailings water. Tailings water incubations had initial concentrations of ~50 mg/L total naphthenic acids and were also supplemented with 5%, by volume, MMBH. All incubations were maintained under aerobic conditions on a shaker at room temperature (~21°C) for 28 d with intermittent sampling during this period. At each sampling time (day 0, 2, 4, 8, 11, 15, 21, 28), 3 mL was removed from each triplicate experiment and combined to minimize the number of analyses while generating pseudoaverage data. Methanol was added to each composite sample (two parts sample to three parts methanol), and these were stored at 4°C.

C.3 Results and discussion

C.3.1 HPLC/QTOF-MS method development

Seven model naphthenic acid compounds, representing a range of n and Z, were selected to optimize chromatographic separation and mass spectrometer parameters. Various reversed-phase capillary HPLC columns were initially tested (BetaBasic, Hypersil Gold, Zorbax Eclipse XDB, and Aquasil), and Aquasil produced the best separation and minimized tailing for the model compounds (Figure C.S-1, Supporting Information). Initial mobile-phase and injection solvent compositions were extremely important for achieving minimal peak widths, and the optimal composition was 60% methanol (0.1% formic acid) in water. Lower percentages of methanol resulted in massive peak tailing (e.g., >6 min) at all concentrations, even with 0.1% formic acid in the mobile phase and injection solvent. The pKa for naphthenic acids is between 5 and 6.5 thus, the analytes should be fully protonated under the mobile-phase conditions. The observed tailing may be interpreted as interaction of analytes with stainless steel surfaces (injector and the column) owing to their well-characterized corrosive properties on steel (Turnbull et al. 1998). Residual tailing was unavoidable under optimal conditions and may be due to additional hydrogen bonding with residual free silanols or C_2H_4OH moieties of the Aquasil stationary phase.

The optimized chromatographic method was then applied to refined Merichem and Syncrude tailings water (Figures C.1 and C.2). Whereas the peak widths for pure model compounds were ~1-2 min wide at base (depending on concentration) (Figure C.S-1, Supporting Information), peak widths for each naphthenic acid isomer class in a complex mixture were ~4 min in tailings water and refined Merichem (Figure C.1), owing to partial separation of the numerous isomers. Separation of naphthenic acids was influenced by Z-series and carbon number (Figure C.2). In general, increasing carbon number resulted in increased retention times, whereas increased cyclization (i.e., more negative Z) resulted in decreased retention. It was assumed that the least branched isomers within each isomer class peak eluted latest, and this was supported by the fact that the internal standard (linear C14, Z = 0) eluted at the extreme right of the extracted ion chromatogram for its respective isomer class peak. A similar trend, that increased

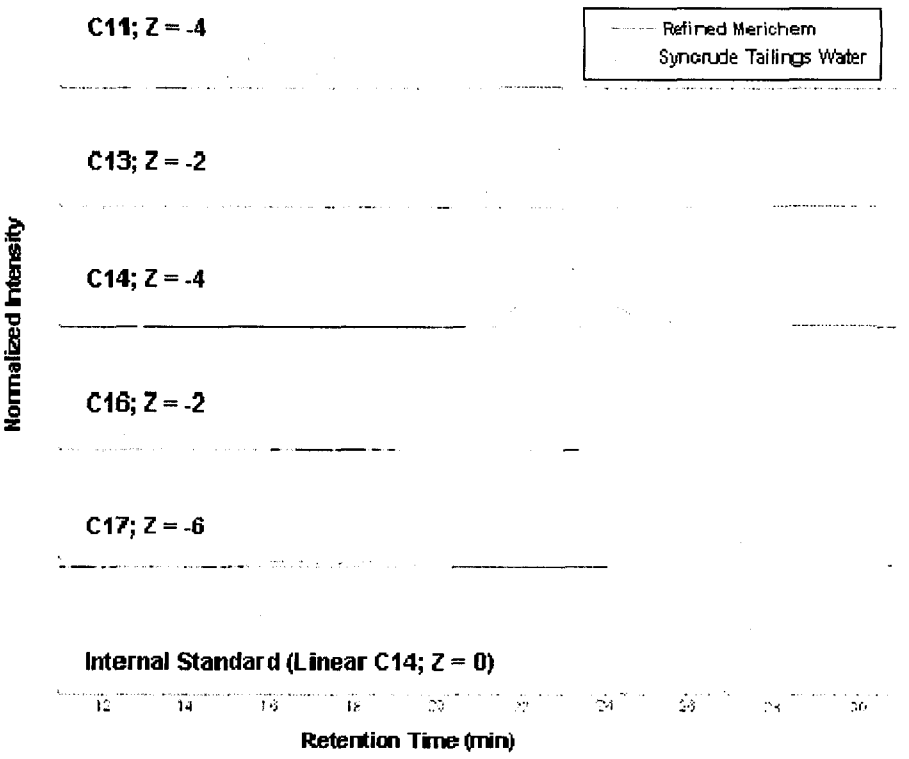

Figure C.1 Chromatographic behavior of naphthenic acids injected onto a capillary reversed-phase HPLC column (Aquasil) showing peak shapes and retention times of selected naphthenic acid isomer classes (selected ion chromatograms), and the internal standard, in Syncrude tailings water (pink chromatogram) and refined Merichem (blue chromatogram).

branching of an alkyl group resulted in shorter retention times, was noted for branched isomers of alkylphenols by reversed-phase HPLC (Gundersen 2001). Overall, it can be concluded that naphthenic acid carbon number, Z-series (i.e., cyclization), and degree of alkyl branching influence chromatographic retention by the current method and, thus, that the principles of separation include both hydrophobicity and Van der Waals forces. Most isomer classes in the complex mixtures eluted as broad humps, except for the Z = 0 series of refined Merichem, which displayed even broader humps containing several partially resolved peaks. The Z = 0 series was the latest eluting series, and thus, the improved chromatographic separation of isomers in this series can be attributed to their higher retention factors (i.e., k') on the C18 moieties of the stationary phase. Structural characterization or separation of all the isomers within each isomer class has never been achieved.

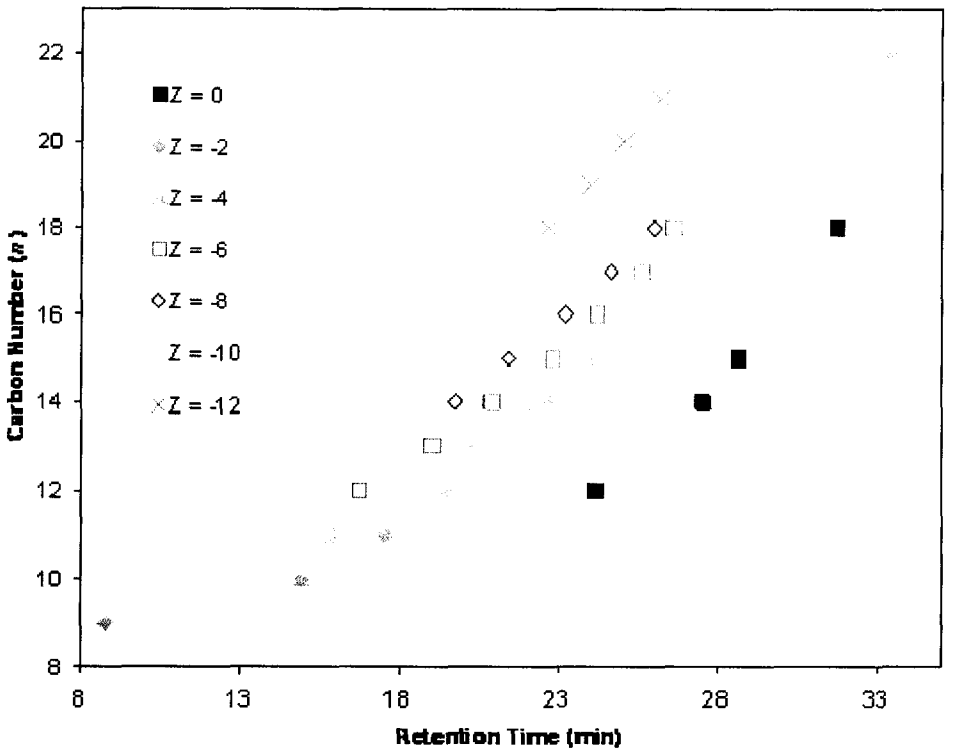

Figure C.2 Chromatographic behavior of naphthenic acids injected onto a capillary reversed-phase HPLC column (Aquasil) showing the retention times of all detected naphthenic acid isomer classes in Syncrude tailings water by Z-series and carbon number.

Although electrospray/high-field asymmetric waveform ion mobility spectrometry (FAIMS)/MS-MS shows great potential for characterizing individual naphthenic acid isomers (Gabryelski and Froese 2003) this is a laborious process that requires much more research before its benefits can be fully realized for structural elucidation in complex mixtures. The combination of FAIMS with HPLC/QTOF-MS is potentially a powerful combination but may be limited by the short duration over which compensation voltages (critical FAIMS parameter that enables isomer separation) may be scanned over the retention window of a typical chromatographic peak.

Chromatographic separation prior to QTOF-MS gave significant quantitative and qualitative advantages over direct infusion. Over the range of 100-2500 g/mL, calibration curves based on HPLC peak areas of model compounds had $R^2 > 0.98$, whereas calibration curves constructed by direct infusion (10 μL/min), based on peak intensity,

had R^2 between 0.74 and 0.88. Moreover, analytical sensitivity (slope of the calibration curves) was increased between 40- and 350-fold by HPLC compared to infusion. The qualitative advantages of the HPLC/QTOF-MS method come from the retention time information, not only as confirmation of detection but also by providing valuable information about the average amount of branching (i.e., alkyl substitution) within each isomer class. For example, the retention times of all isomer classes in Syncrude tailings water were shifted to earlier eluting retention times compared to the refined Merichem commercial mixture (Figure C.1). The retention time shifts were not an artifact of run-to-run variation as shown by the very small amount of shifting for the internal standard. Therefore, it was hypothesized that biodegradation of naphthenic acids favored the least branched isomers and that as tailings water aged the average distribution would shift toward a more highly branched, and earlier eluting, fraction. This hypothesis was tested in the subsequent section: Biodegradation of naphthenic acid isomer classes.

The QTOF detector was calibrated using model naphthenic acid compounds, and mass accuracy was always <20 ppm by external mass calibration, even at low concentrations. Mass accuracy was improved through correction of instrumental drift by use of lock masses. This was accomplished by teeing a reference solution, containing two perfluoroalkylcarboxylates in methanol (pentafluoropropionate, *m/z* 162.9824, and nonafluoropentanoate, *m/z* 262.9760), into the HPLC eluant. By this method, accurate mass errors for model naphthenic acids were reduced to <10 ppm and using this criterion in combination with retention time enabled unambiguous confirmation of naphthenic acid empirical formulas and of their transformation products in samples when limits were set to include only carbon, hydrogen, and oxygen atoms. This level of mass accuracy was adequate although Barrow et al. (2004) have reported better mass accuracy for naphthenic acid analysis using FTICR/HRMS (e.g., <1.14 ppm error).

The dynamic range of the method was studied between 0.1 and 100 mg/L (total refined Merichem concentration), covering the range of total naphthenic acid concentrations expected in tailings water. Over this range, R^2 for individual isomer classes were >0.99 (Figure C.S-2, Supporting Information, Z = -2 calibration curves shown), indicating that the method could be used quantitatively. Although it is impossible to quantify the absolute concentration of each naphthenic acid class in any sample (i.e.,

because no external standards are available, and the isomer composition within each carbon number and Z-series is unknown), the method linearity enables quantitative estimates of transformation rates. An acknowledged limitation is that, as complex naphthenic acid isomer mixtures are degraded, the resultant isomer composition may be altered and the average electrospray efficiency may be affected, likely causing a small analytical bias that cannot currently be controlled.

To increase instrumental sensitivity, and to simplify mass spectral interpretation, electrospray source parameters were optimized to minimize in-source fragmentation of model compounds, and fragmentation was not detectable for any isomer class in a refined Merichem commercial mixture as determined by spectral deconvolution (Spectralworks AnalyzerPro). Instrumental detection limits, determined for model compounds, varied between 24 and 110 pg on-column for most compounds except for the model compound $C_8H_{14}O_2$ (n = 8, Z = -2,) which was less sensitive (528 pg), presumably due to lower retention and broader peak shape (Table C.1, Figure C.S-1, Supporting Information). For all naphthenic acids in refined Merichem, instrumental precision was lower than 4% rsd for multiple injections within the same day.

The level of sensitivity and precision was adequate for application to detection of naphthenic acids in tailings water, but to further increase the sensitivity of the method to lower abundance isomers, or to monitoring of background surface and groundwater samples, a 50-fold SPE preconcentration step was briefly evaluated for quantitative recovery. Few preconcentration methods have been reported, and none have evaluated recovery of specific naphthenic acid isomer classes, but Rogers et al. (2002) reported ~85% extraction efficiency for total naphthenic acids in tailing pond water using liquid-liquid extraction and FT-IR analysis, and Headley et al. (2002) reported 107% recovery using SPE (ENV+ cartridges) of naphthenic acids from river water and liquid infusion MS. Oasis HLB cartridges were used to concentrate 50 mL of water spiked with model compounds, and it was noted that relatively large volumes (>10 mL) of solvent were necessary to elute the wide range of compounds tested, thus requiring a subsequent concentration step. However, using methanol or acetonitrile as the elution solvent resulted in unacceptable evaporative losses for the lower molecular weight naphthenic acids (<45% recovery), whereas ethyl acetate was found to be optimal for full elution and

Table C.1 Instrumental detection limits, SPE recovery, and matrix effects for model compounds by HPLC/QTOF-MS.

		% Recovery (s.d.)[b]		Tailings Water Matrix Effect	
Model Compound	**IDL[a] (pg)**	**Absolute**	**Internal Standard Corrected**	**Direct Analysis %[c]**	**10-fold SPE Matrix % (n=2)[d]**
$C_8H_{14}O_2$, Z=-2	528	61(8)	90(8)	67	80
$C_{12}H_{22}O_2$, Z=-2	110	76(5)	112(2)	99	70
$C_{14}H_{24}O_2$, Z=-4	24	84(2)	126(9)	79	35
$C_{16}H_{32}O_2$, Z=0	66	62(2)	92(6)	98	102
$C_{24}H_{40}O_2$, Z=-8	33	60(5)	89(9)	108	137

[a] mass injected at instrument detection limit (S/N =3), [b]spiked level was 20 μg/L and the value represents the mean and standard deviation of n=4 determinations. [c] diluted with 60% methanol. [d] in presence of 10-fold SPE matrix diluted with 60% methanol.

also for minimizing evaporative losses. The absolute recovery of model compounds ranged from 60 to 84% by the optimized method and ranged from 89 to 126% when recovery was corrected using ^{13}C-tetradecanoic acid spiked prior to SPE (Table C.1). The optimized SPE/HPLC/QTOF-MS method performed even better for the complex mixture of naphthenic acids in refined Merichem, whereby recoveries ranged from 81 to 94% without internal standard correction. The method was also compared directly with an established liquid-liquid extraction method (Clemente et al. 2004) and the SPE method resulted in 11-30% higher recovery for all naphthenic acid ions detected in Syncrude tailings water when both concentrates were analyzed by HPLC/QTOF-MS (Figure C.S-3, Supporting Information).

Matrix effects can have undesirable impacts on electrospray quantification by reducing or enhancing the ionization efficiency of analytes in complex matrixes relative to standards in pure solvent. For example, using the same instrument as the one here, but without chromatographic separation, Gabryelski and Froese (2003) reported matrix effects for infused naphthenic acid mixtures that impaired sensitivity and any quantitative applications. To assess matrix effects by the current HPLC/QTOF-MS method, tailings water (diluted with methanol to give 60% v/v methanol) was spiked with the six model

compounds at individual concentrations of 2.5 ppm, and the instrumental response was compared to an equivalent injection of the same concentration in pure methanol (and by correcting for background response in tailing water). Accuracy in the matrix ranged from 79 to 108% for all model compounds, except for the C8 model compound, which suffered 33% suppression (i.e., 67% accuracy) compared to the no-matrix control (Table C.1). Fortunately, naphthenic acids having less than 10 carbons were not detected in tailings water by this method, thus, significant matrix effects are not expected for the routine application of this method to direct analysis of tailings water. However, using the SPE method to preconcentrate tailings water 10-fold resulted in both suppression (e.g., 35% apparent recovery for $C_{14}H_{24}O_2$) and enhancement (e.g., 137% apparent recovery for $C_{24}H_{40}O_2$) of selected ion signals compared to the no-matrix control, thus, matrix effects should not be discounted when SPE preconcentration is used. The bulk of the matrix responsible for suppressing signals by direct infusion is believed to be removed early in the HPLC analysis because large negative peaks were visible in many extracted ion chromatograms of tailings water between 3 and 6 min.

C.3.2 Characterization of naphthenic acid isomer class profiles in refined Merichem and tailings water

In an interlaboratory study, data from an established GC/MS technique were compared to data produced by HPLC/QTOF-MS for refined Merichem (Figure C.S-4, Supporting Information) and tailings water (Figure C.3). For refined Merichem, the profile produced by HPLC/QTOF-MS was very similar to the profile produced by GC/MS. This was somewhat fortuitous because the relative abundance of naphthenic acids is very much a function of source parameters (Lo et al. 2003), thus, an identical distribution is not necessarily expected. By HPLC/QTOF-MS, the most intense ions in refined Merichem were for the Z = -2 series (41%), followed by Z = -4 (32%) > Z = 0 (20%) > Z = -6 (5.8%) > Z = -8 (1.7%) > Z = -10 (0.3%), and these were all centered around n = 13 or 14 (Figure C.S-4B, Supporting Information). This is very similar to GC/MS except that ion abundance by the latter method was centered around n = 11 to 13 (Figure C.S-4A, Supporting Information). No naphthenic acids were observed beyond n = 20 in any Z-series by either method, and the most obvious, although minor, difference

between the two methods was the absence of naphthenic acids below n = 7 by HPLC/QTOF-MS, whereas GC/MS detected ions at n = 5, 6, and 7 in the Z = 0 series.

More substantial differences were observed when comparing profiles of tailings water (Figure C.3). Overall the GC/MS data appeared far more complex than by HPLC/QTOF-MS. A sample of "Syncrude tailings water" was previously analyzed by infusion FTICR/HRMS (ultrahigh resolution) by Barrow et al. (2004), and although numerical data were not explicitly presented, their qualitative characterization is indiscernible from the HPLC/QTOF-MS profile (Figure C.3A). The most intense masses by HPLC/QTOF-MS were for Z = -4 (36%) and Z = -6 (36%), followed by Z = -12 and -10 (7.8%) > Z = -2 (6.7%) > Z = -8 (5.6%) > Z = 0 (0.4%). By contrast, the GC/MS profile showed that the most abundant masses were for Z = 0 (21%) > Z = -6 (20%) > Z = -4 (17%) > Z = -2 (16%) > Z = -8 (10%) > Z = -12 (9%) > Z = -10 (7%), and many abundant ions were observed at higher and lower carbon numbers, including the prominent "C22+ cluster" (30%), that were absent by HPLC/QTOF-MS and have been noted previously by GC/MS (Holowenko et al. 2002). Although the source of samples is different, this GC/MS profile for Syncrude tailings water is similar to tailings water profiles, published elsewhere, produced by unit resolution infusion techniques such as infusion API/MS or GC/MS methods (Holowenko et al. 2002, Hao et al. 2005, Lo et al. 2006, Frank et al. 2006) in that many prominent high (>C22) or low (<C9) molecular weight ions are detected.

By HPLC/QTOF-MS, a variety of ions were detected in the low molecular weight naphthenic acid range that did not fit the exact-mass criteria, and thus, the potential for misclassification by unit resolution MS techniques is high from arbitrary matrix interferences. The absence of the C22+ cluster by HPLC/QTOF-MS could not be explained by poor liquid chromatography or sensitivity for high molecular weight naphthenic acids given that one of the model compounds was C24, Z = -8 (Table C.1, Figure C.S-1, Supporting Information). Alternatively, a hypothesis was tested that naphthenic acids appearing in the C22+ cluster by GC/MS were artifacts caused by double derivatization of hydroxylated naphthenic acids - previously presented as a possibility by Clemente et al. (2004). To confirm this possibility, the GC/MS sample was further analyzed by GC/HRMS to look specifically for three selected high-abundance

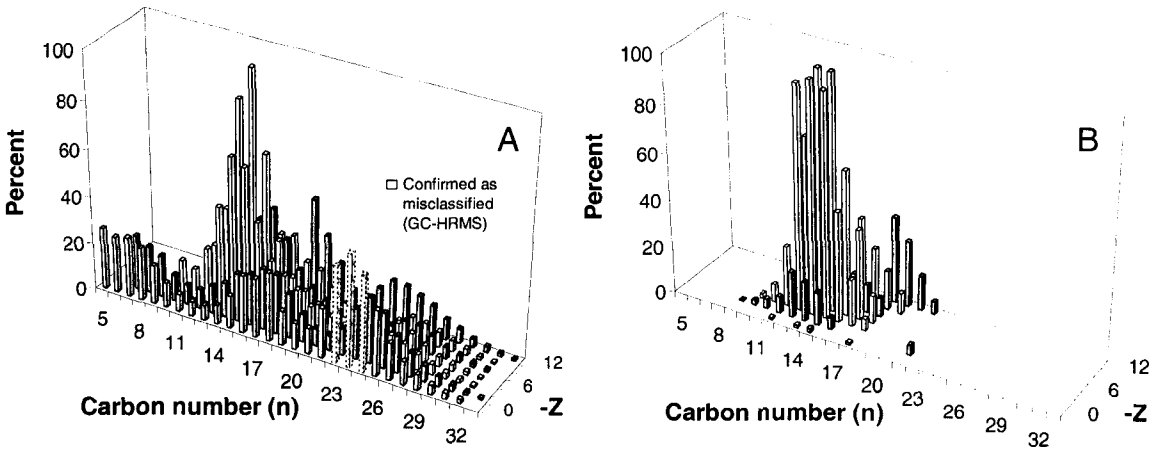

Figure C.3 Three-dimensional plots of relative ion intensity versus carbon number (n) and Z-series for an extract of Syncrude tailings water analyzed by (A) an established GC/MS technique and (B) the new HPLC/QTOF-MS method. The hatched bars in (A) represent ions that were confirmed by GC/HRMS to be artifacts from double-derivatized hydroxylated naphthenic acids.

naphthenic acid isomer classes in the C22+ cluster detected by GC/MS (highlighted in Figure C.3A). All three of these ions, previously assumed to be C23, C24, and C25 (Z = 0) naphthenic acids, had accurate masses consistent with fragment ions of hydroxylated naphthenic acids containing two *tert*-butyldimethylsilyl derivatives ($[C_{22}H_{43}Si_2O_3]^+$, $[C_{23}H_{45}Si_2O_3]^+$, and $[C_{24}H_{47}Si_2O_3]^+$, corresponding to exact masses of 411.2751, 425.2908, and 439.3064, respectively). Therefore, the true identities of these three species were most likely the hydroxylated C14, C15, and C16 (Z = -4) naphthenic acids, and it is likely that many other misassignments by unit resolution GC/MS contribute to the differences between Figure C.3A and B (See subsequent section). Given these findings, unit resolution MS methods should only be used for profiling purified commercial naphthenic acid mixtures, and HRMS techniques should be employed if the goal is to accurately profile tailings water or background environmental samples.

C.3.3 Detection of transformation products and data visualization

Hydroxylated naphthenic acids are hypothesized to be products of biotransformation and, thus, were key analytes assessed by the new method. Separation of three hydroxylated naphthenic acid model compounds (12-hydroxydodecanoic acid,

12-hydroxyoctadecanoic acid, 3-hydroxy-5-β-cholanic acid) showed earlier retention time compared to their parent naphthenic acids by between 5 and 7 min, but chromatographic properties, including peak shapes and relative retention times, were otherwise similar to naphthenic acids. We then targeted detection of hydroxylated $[M - H + O]^-$ and dihydroxylated $[M - H + 2O]^-$ naphthenic acid ions in Syncrude tailings water and several novel species were confirmed (Figure C.4). Although the Z-series is displayed for the oxidized naphthenic acid products (Figure C.4), a technical limitation is that it is not possible to unambiguously confirm the chemical nature and, hence, Z-series, of the oxidized products. This is because, for example, the hydroxylated mass of a C10, Z = -2 parent naphthenic acid would have the same exact mass (i.e., same empirical formula) as the ketone product of a naphthenic acid having the same carbon number but in the higher Z = 0 series, and in a complex mixture it is not possible to know which naphthenic acid was the parent compound from which the detected product was derived. None of the three hydroxylated naphthenic acids confirmed by GC/HRMS were detected by HPLC/QTOF-MS, however, it is expected that the GC/HRMS method is more sensitive than the HPLC/QTOF-MS method, owing to narrower peak widths by GC.

A conventional approach for presenting complex naphthenic acid data is to display three-dimensional plots of carbon number versus Z-series versus ion intensity as shown in Figure C.3. However, to make better use of the transformation product information available by HPLC/QTOF-MS, a new graphical approach was necessary. Thus, the use of van Krevelen diagrams (VKDs) (Kim et al. 2003) was adapted. Such diagrams were originally used in geochemistry to study the evolution of coal or oil samples but have recently been applied to characterization complex organics (Wu et al. 2004) or fulvic acid mixtures (Reemstma and These 2005). This approach involves plotting, for each empirical formula detected, the molar ratio of H/C versus O/C (two-dimensional, Figure C.4), versus chromatographic peak area (three-dimensional) (Figure C.S-5, Supporting Information). Plotted in two dimensions, this approach graphically separated naphthenic acid congeners into their respective Z-series (top to bottom) and arranged each homologous Z-series by increasing carbon number (from right to left) (Figure C.2). The oxidation products can be plotted on the same axes based only on their H/C and O/C ratios, it is not necessary to know whether the true identity is a ketone (+14)

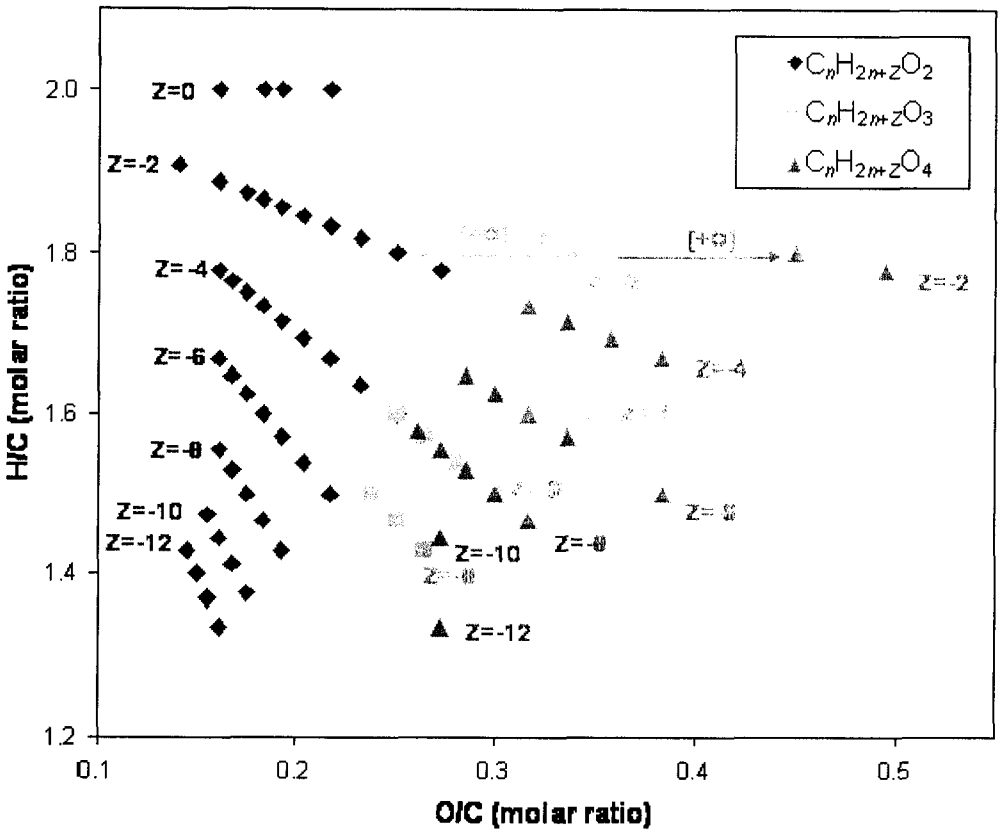

Figure C.4 HPLC/QTOF-MS derived Van Krevelen diagram showing parent naphthenic acid compounds and their oxidized products in Syncrude tailings water. An example is shown for the double oxidation of C_{10}, Z = -2, whereby the oxidized products appear immediately to the right from the parent naphthenic acid (assumes products are a hydroxy and dihydroxy).

or an alcohol (+16) product, a limitation noted in the previous paragraph. In two-dimensional VKDs, a hydroxylated product would be shifted horizontally to the right from its precursor, and an example is shown for this in Figure C.4 for a single and double hydroxylation of a parent naphthenic acid (C10, Z = -2). By GC/MS, the mass of this monohydroxylated C10, Z = -2 ion would be misconstrued as a high molecular weight naphthenic acid (C20, Z = -12), and a schematic explanation for this is presented in Figure C.S-6 (Supporting Information). This high molecular weight naphthenic acid was absent in the HPLC/QTOF-MS profile (Figure C.3B) but contributed 1% to total naphthenic acids by GC/MS (Figure C.3A) for Syncrude tailings water. Another example

of the necessity for HRMS to avoid erroneous conclusions in biodegradation studies, even when working with commercial mixtures can be illustrated with data previously presented in Clemente et al. (2004). In their biodegradation study with refined Merichem, the n = 10, Z = -2 isomer class initially accounted for 5% of total naphthenic acids. These were consumed by day 7, whereas the n = 20, Z = -12 isomer class, which was initially absent, emerged on day 7 to account for 2% of total naphthenic acids. Several other high molecular weight naphthenic acids that were initially absent also appeared on day 7 by GC/MS. Presumably the appearance of the n = 20, Z = -12 isomer class represented hydroxylation of some of the n = 10, Z = -2 naphthenic acids, rather than evolution of novel naphthenic acids. This will be studied in future work by HPLC/QTOF-MS.

It is germane to note that although it is only a semiquantitative estimate of their relative abundance, naphthenic acid profile VKDs plotted in three dimensions enable the relative importance of each isomer class to be considered, as shown for refined Merichem (Figure C.S-5, Supporting Information). However, this may not have significant advantages over the traditional three-dimensional plots of n versus Z-series and intensity.

C.3.4 Biodegradation of naphthenic acid isomer classes

To validate the new quantitative and selective HPLC/QTOF-MS method, isomer class-specific biodegradation was studied for refined Merichem and tailings water in a 28-d biodegradation study. Over the course of the study, no noticeable degradation was observed for any isomer class in the tailings water incubations, confirming the known recalcitrance of naphthenic acids in tailings water (Scott et al. 2005). However, substantial degradation was observed in refined Merichem incubations. The rate of transformation in refined Merichem was dependent on Z-series and, to a lesser extent, on carbon number. Quantitative structure-reactivity relationships will be discussed in greater detail in a future publication, however, some qualitative results are presented here for three randomly selected isomer classes (Figure C.5), and Figure C.5C also demonstrates the improved isomer separation that was common to the Z = 0 series. While the extent of biodegradation was complete for some compounds in only a few days (Figure C.5C), a significant fraction was still detectable after 28 d for other isomer classes (Figure C.5A and B). For each isomer class, the average retention time of the remaining fraction shifted

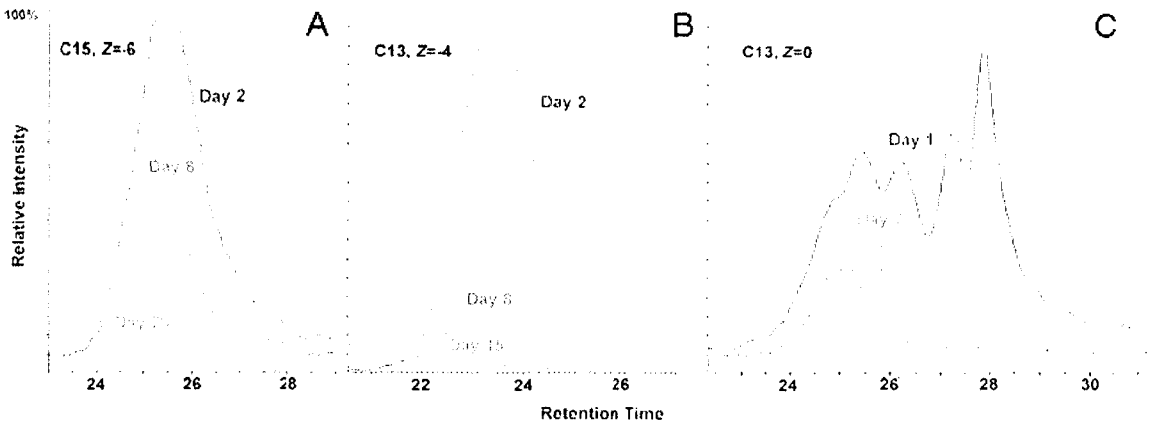

Figure C.5 Extracted ion chromatograms for three arbitrary naphthenic acid isomer classes in refined Merichem throughout a 28-d biodegradation study. The time of the sample collection is shown for each, and a shift toward earlier retention times is evident in each circumstance. The improved resolution of alkyl-substituted isomers in the Z = 0 class is also evident from the multiple peaks and wider width at base.

to earlier retention times as biotransformation continued, suggesting that the more recalcitrant fraction was indeed more highly alkyl-substituted (e.g., branched), as hypothesized. This is analogous to results from biodegradation studies with hydrocarbons. For example, increasing numbers of alkyl groups on naphthalene or phenanthrene decrease biodegradability, and the highly branched isoprenoid pristane is more resistant to biodegradation than n-alkanes (Fedorak and Westlake 1981). These experimental observations provide a plausible explanation for the shifted distribution in Syncrude tailings water relative to refined Merichem (Figure C.1) and also provide an explanation for why refined Merichem naphthenic acids were so readily biotransformed while no significant degradation was evident for tailings water naphthenic acids. These results lead to the hypothesis that tailings water microorganisms preferentially deplete the least alkyl-substituted fraction, leaving behind a persistent, highly branched fraction typical of what was observed in Syncrude tailings water (Figure C.1). Analysis of fresh (nonrecycled) tailings water and of tailings ponds of various ages may confirm this. The stark difference in biodegradability of commercial naphthenic acid mixtures relative to tailings water naphthenic acids has previously been shown using GC/MS (Scott et al. 2005) but a plausible explanation for this has only now become clear following the

application of this more specific and highly qualitative method.

Analysis of refined Merichem biodegradation samples did not reveal any oxidation products (e.g., M + 16, M + 14, or M + 32) at detectable levels over the course of the study. Significant mineralization (~60% as CO_2) was observed by Clemente et al. (2004) for refined Merichem biodegradation under a similar experimental setup, thus, the absence of oxidation products is not an unexpected finding here. The source of oxidized naphthenic acids in tailings water, therefore, remains unknown, but it is expected that microbial oxidation of the more recalcitrant isomer fractions may follow pathways different from the fraction that was biodegraded in this 28-d experiment. Furthermore, only the aqueous fraction was analyzed (cells were centrifuged out), and thus, intracellular metabolites may have been missed, or alternatively, carbon from naphthenic acid transformation products may have been covalently incorporated into cell biomass.

C.4 Supporting information

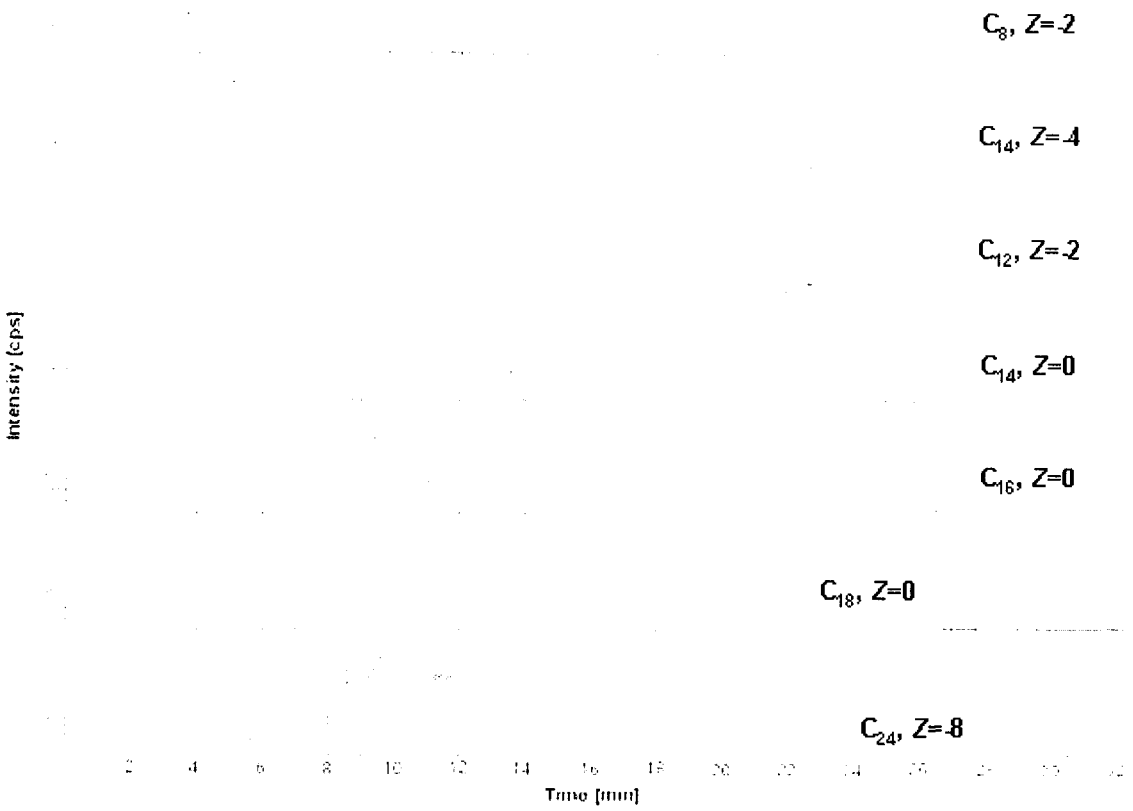

Figure C.S-1 HPLC/QTOF-MS chromatogram of mixed naphthenic acids model compounds.

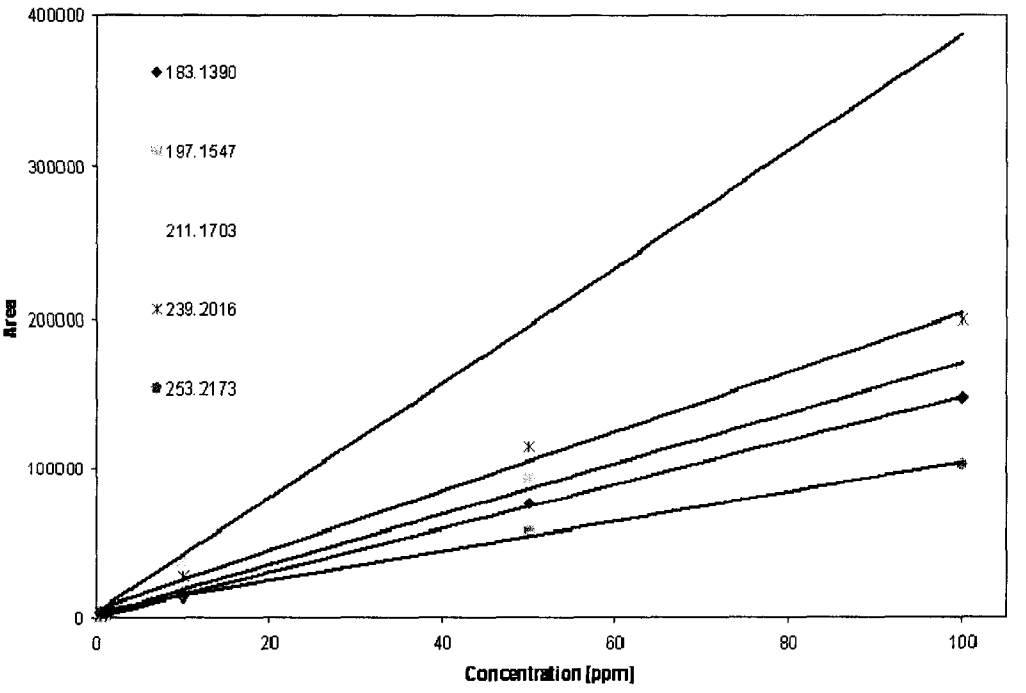

Figure C.S-2 Example calibration curves for Z=-2 naphthenic acids in refined Merichem. The x-axis is based on total concentration of the refined Merichem complex naphthenic acids mixture.

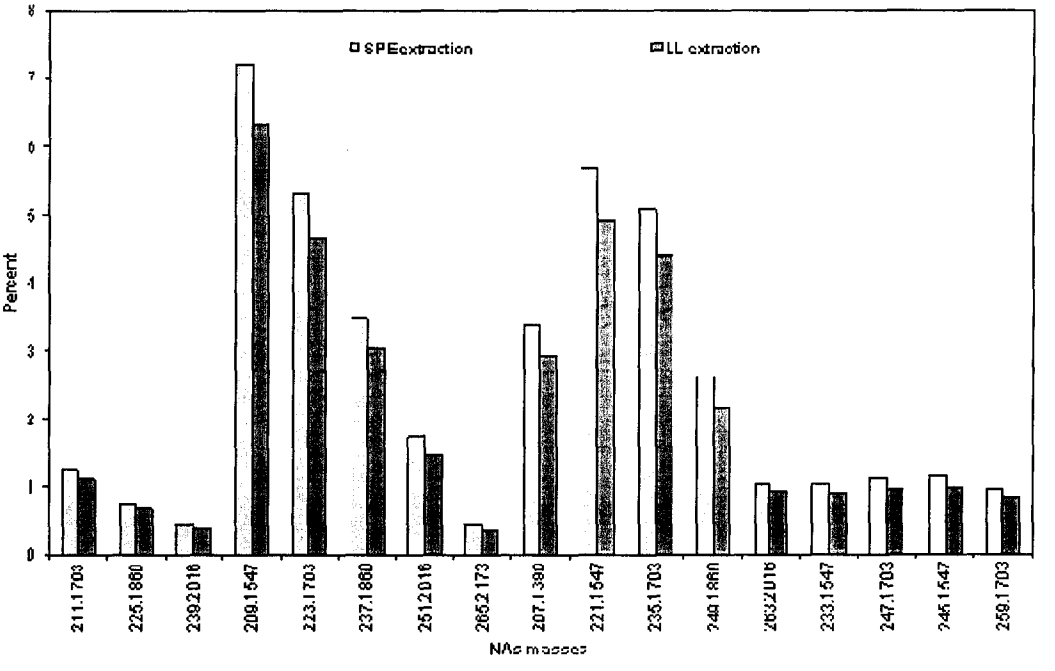

Figure C.S-3 Comparison of HPLC/QTOF-MS response for Syncrude tailings water preconcentrated by solid phase extraction on HLB (SPE) or by liquid-liquid extraction.

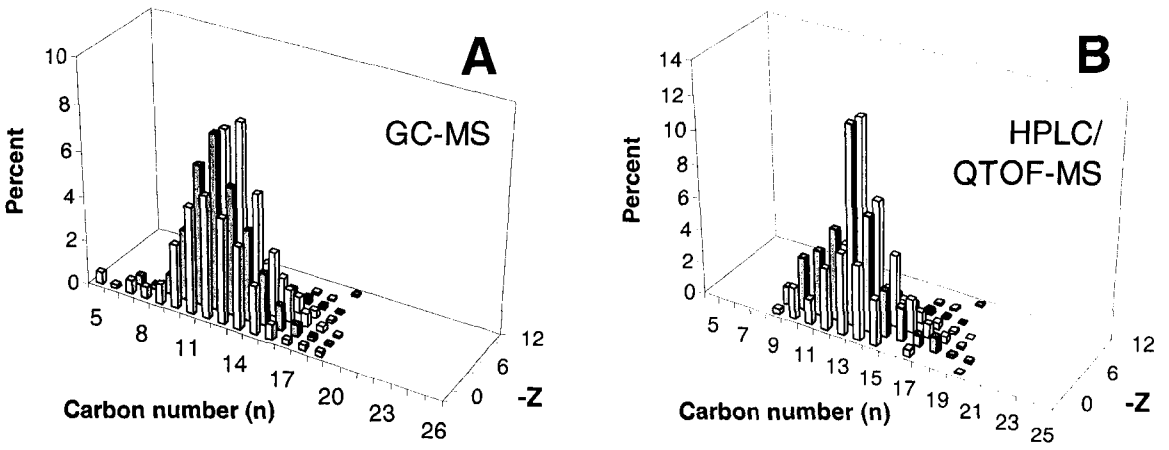

Figure C.S-4 Three-dimensional plots of naphthenic acids in refined Merichem naphthenic acids determined by GC-MS (A) and HPLC/QTOF-MS (B). The sum of the bars in each panel is 100%.

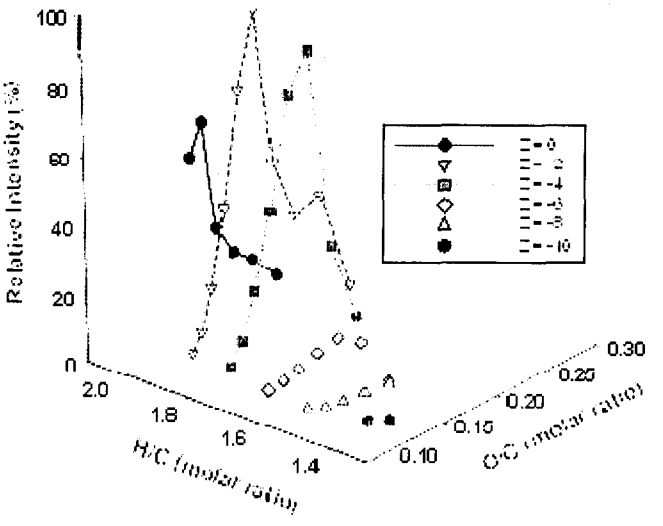

Figure C.S-5 Profile of naphthenic acids in refined Merichem determined by HPLC/QTOF-MS and plotted as a Van Krevelen diagram.

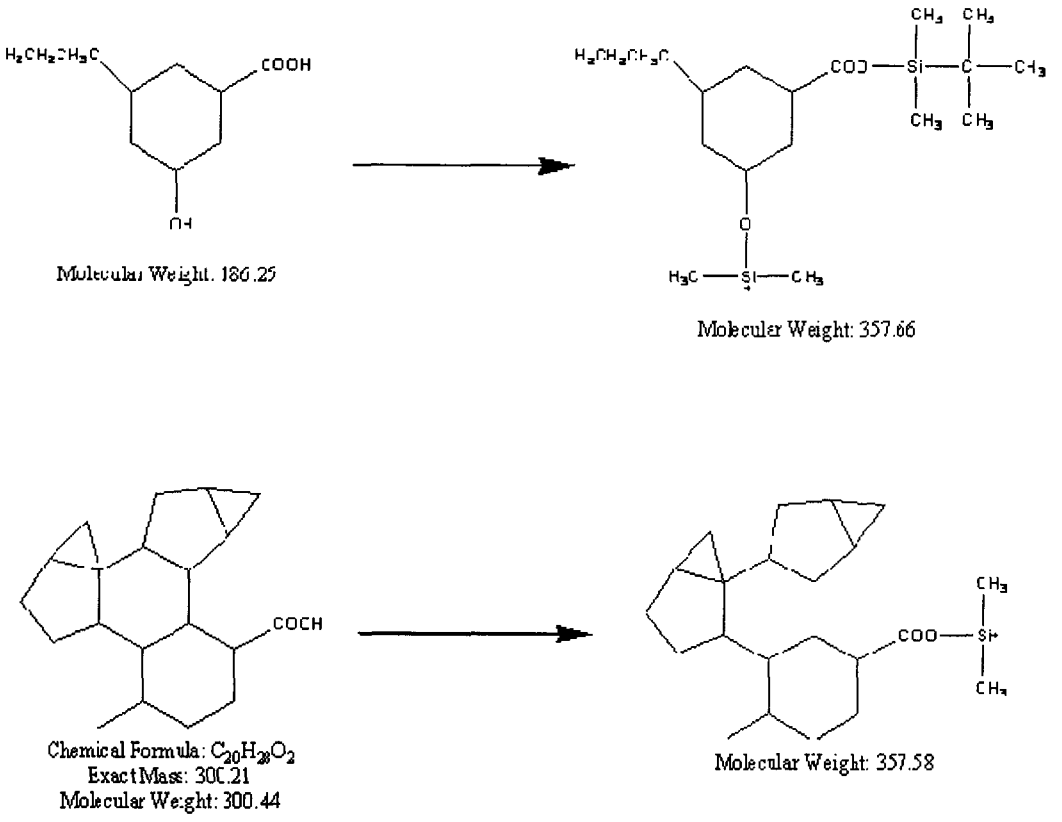

Figure C.S-6 Schematic of how a hydroxylated C10, Z=-2 naphthenic acid may be misclassified as a high molecular weight naphthenic acid. Both derivatives have the same unit mass, but can be distinguished using HRMS.

C.5 Literature cited

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