Comparison of Biodegradation Kinetics of Geometric Isomers of Naphthenic Acids (NAs) in Athabasca River Water

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1. INTRODUCTION

1.1 Background

The Athabasca oil sands is one of the largest oil sands deposits in northern Alberta, Canada and covers some 42,000 km^2 surrounding Fort McMurray, Alberta (440 kilometers northeast of Edmonton) (ERCB 1991). The Athabasca oil sands deposit contains about 0.2×10^{12} m^3 of bitumen, the component of oil sands which is separated from the sand and other constituents in an extraction process. This extraction process produces large volumes of fluid wastes containing naphthenic acids (NAs) which are natural components of bitumen. These compounds are acutely and chronically toxic to aquatic organisms (CONRAD, 1998).

Petroleum acids or NAs are present naturally in petroleum, oil sands bitumen and crude oils. NAs are a complex mixture of mono- and poly- cyclic alkanes (predominantly cyclohexanes and cyclopentanes) that contain carboxylated aliphatic side chains of various lengths (CONRAD, 1998). NAs are available commercially only as a complex mixture of many different types of compounds. There is no pure model for NAs available (Herman *et al.*, 1994). The same group of researchers stated that approximately 20% of the organic compounds present in laboratory mixtures may be mineralized by microbial activity. By this mineralization, the acute toxicity of NAs is reduced to approximately half of the original level.

The separation process of bitumen from oil sands utilizes a caustic soda hot water floatation process which produces a waste product containing NAs. These NAs in a waste product are tested based on Fourier Transform Infrared Spectra. By performing a similarity comparison of commercial NAs and organic acids extracted from oil sands tailings, it is believed that NAs from tailing ponds are a major source

of acute toxicity (MacKinnon and Boerger, 1986). According to Fan (1991), the presence of NAs in refinery operating process units can cause corrosion problems and result in hazardous wastewater which leads to fish toxicity.

A recent study of toxicity of NAs derived from Athabasca oil sands (Roger, 2000) revealed that NAs have significant effects on the liver as well as the growth and hepatic function of Wistar rats. Therefore, NAs are proven to be a toxicant affecting mammals. No information is available on carcinogenicity, mutagenicity and tetratigenicity effects of NAs (CONRAD, 1998). Acute toxicity tests of NAs on rainbow trout (FTFC, 1995) in treated and untreated tailing pond water showed that the ability of trout eggs to hatch and for the young fish to survive in treated tailing pond water is substantially enhanced comparing with untreated tailing pond water. Little is known about the fate of NAs within plants.

The major commercial uses of NAs are applications of their metal salts. For instance, sodium naphthenates are used as emulsifying agents for the preparation of agricultural insecticides, additives for cutting oils, and emulsion breakers in the oil industry (Hatch and Matar, 1977). Calcuim naphthenate has been used as a hardening agent for protective coatings and plastic compounds. Lead, manganese, cobalt and zinc napthenates have been widely used as excellent paint driers (Encyclopedia of Chemical Technology, 1978). In addition, lead naphthenate is used as an extreme pressure agent for lubricating oils and zinc naphthenate is an antioxidant.

Previous studies (Lai *et al.*, 1996) have shown that microorganisms indigenous to oil sands tailings are able to degrade a commercial mixture of NAs and a mixture of organic acids extracted directly from oil sands tailings. Moreover, the biodegradation of NAs was influenced by many factors, for example, the effect of temperature, nutrient availability etc. (Lai *et al.*, 1996). Lai *et al.* (1996) also noted

that pH (range pH 7.3-8.5) and dissolved oxygen carbon (DOC) (range 3-10 mg/l) levels were found to have no significant effect upon the rate of biodegradation. Conversely, the addition of phosphate and increase/decrease of temperature levels were found to have significant effects on the rate of NAs degradation. The rate of biodegradation may likewise be affected by the chemical structure of the alkyl-substitution on the cycloalkane ring as well as the number of cycloalkane rings (Herman *et al.*, 1993).

1.2 OBJECTIVES

In view of the toxicity and prevalence of NAs in refinery wastewater from oil sands extraction, it is imperative that a better understanding of the factors affecting their persistence in aquatic environments be developed. This need is important since factors controlling possible selective degradation of components of NAs mixtures are not established. For example, it is not known whether such factors include the diversity of various NA mixtures or whether possible differences in degradation of geometric isomers have a significant effect on their bioavailability. If a study of different NAs compounds and geometric isomers could verify such factors and differences, one would have to consider possible cumulative effects since NAs are complex mixture of hundreds of compounds.

The specific objectives of this project were:

1. to determine the rate of biodegradation and half-lives of several representative NAs (4-methylcyclohexaneacetic acid (4MACH), 4-methylcyclohexanecarboxylic acid (4MCCH), and 3-methylcyclohexanecarboxylic acid (3MCCH)) in Athabasca River water under laboratory conditions and various imposed water quality conditions (including DOC, pH, and temperature adjustments);
2. to verify the order of NAs biodegradation kinetics; and

3. to compare the biodegradation kinetics in natural waters of *cis*- and *trans*-geometric isomers of several representative NAs.

2. LITERATURE REVIEW

The purpose of this literature review is to outline the physical and chemical properties of NAs including the chemical structure. Microbial degradation and metabolism of NAs are discussed as well as the factors influencing biodegradation. Several methods used for analysis of NAs in recent studies are also discussed in this chapter.

2.1 General Information

Naphthenic acids (NAs) or petroleum acids represent the carboxylic acids present in petroleum or crude oils. Attempts to characterize these acids date back prior to 1955. Since that time, NAs have been the topic of numerous studies extending over many years.

Petroleum is the world's primary source of energy and petrochemical feedstock. Although crude oil has few uses, it is refined to produce gasoline, jet fuel, kerosene, solvents and diesel as well as feedstocks such as ethylene, propylene, butane, isoprene and butadiene.

Alberta is the main producer of Canadian petroleum. Table 2.1 shows the total Canadian crude oil/equivalent production (m^3 per day) in 1999-2000. The average crude oil production of Alberta (~242,834 m^3 per day) is much larger than the average production from Saskatchewan, the second largest producer in Canada (~61,229 m^3 per day) (Oilweek, Dec, 2000).

Table 2.1. Total Canadian crude oil/equivalent production (m^3 per day).

	Jan-Jun '99	Jan-Jun '00
British Columbia	7,946	8,395
Alberta	242,022	243,646
Saskatchewan	57,503	64,955
Manitoba	1,564	1,691
Ontario	672	635
Northern Canada	4,528	4,255
Atlantic Canada	14,543	24,695
Total Canada	328,777	348,270

Source: Oilweek (December, 2000, pp. 34)

The Athabasca oils sand are a major source of crude oils in northern Alberta. Moore and Ramamoorthy (1984) reported that the natural deposits of bitumen in this area are as high as 812 billion barrels. These deposits permit the operation of major extraction plants with production of up to 190,000 barrels per day since the bitumen is located near the ground surface. The Athabasca River which flows through the oil sands area has been identified by Moore and Ramamoorthy (1984) as a river with a significant accumulation of hydrocarbons.

A recent study based on the Fourier Transform Infrared (FTIR) Spectroscopy method reported concentrations of NAs ranged from < 1 to 2 mg/l in the Athabasca River, to over 100 mg/l in fresh tailing water as shown in Table 2.2 (FTFC 1995, Suncor Inc. Oil Sands Group 1996).

Table 2.2. Expected NAs Concentration Ranges in Various Water Sources (FTIR Method).

Water Source	Expected Concentration Ranges (mg/l)
Background	
Steepback River	<1
Muskey River	<1
Clearwater River	<1
Athabasca River upstream of Fort McMurray	<1
Athabasca River downstream of Fort McMurray	<1 to 2
Process Water	
Tailing Pond Water	
1. Syncrude Canada Ltd.	90 to 110
2. Suncor Inc., OSG	75 to 90
Wastewater Effluent	
1. Syncrude Canada Ltd.	n/a
2. Suncor Inc., OSG	<1 to 5
Groundwater	<4 to 57

Source: CONRAD 1998; Suncor Inc. Oil Sands Group 1996; FTFC 1995

2.2 General Properties of Naphthenic Acids

2.2.1 Structural Formula

NAs are the most important group of acidic oxygenated compounds found in crude oils. The name, naphthenic acids, is derived from the early discovery of monobasic carboxylic acids in petroleum, with these acids being based on a saturated single ring structure. NAs are predominantly mono- and poly-cycloalkane (cyclopentane and cyclohexane) carboxylic acids with aliphatic side chains of various lengths

(CONRAD, 1998; Encyclopedia of Chemical Technology, 1978). The structural formula of NAs (Figure 2.1) shows the carboxylic group (COOH) at the end of aliphatic side chain (CH_2). R is a small aliphatic group, such as methyl, and *n* can range from 1 to 6. Furthermore, Seifert (1975) and Fan (1991) have placed the carboxylic acid group directly on the ring (*m* = 0) with R as the long aliphatic side chain. In Fig 2.1, cyclohexane is used as a representative cycloalkane, although cyclopentane, or a mixture of 5- and 6-carbon rings, may be present in NAs.

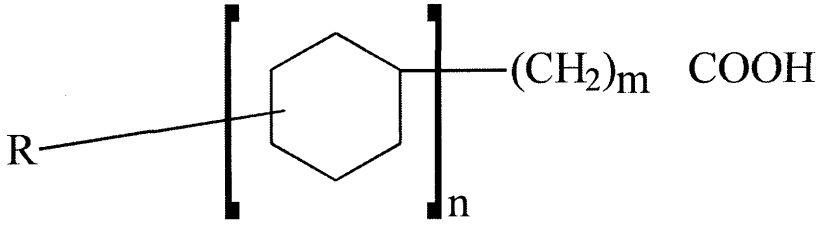


Figure 2.1. A structural formula for NAs modified from the Encyclopedia of Chemical Technology (1981).

There is a general formula, based upon CONRAD (1998), for NAs which is $C_nH_{2n+z}O_2$, where n represents the number of carbon atoms and z represents the number of hydrogen atoms. For example, NA can be a linear hydrocarbon chain (z=0), one ring (z=-2), and two rings (z=-4) etc. NAs consist mainly of monocyclic acids (z=-2) in the range of C-7 to C-12 (i.e. n=7 to 12) (Encyclopedia of Chemical Technology, 1995). Lai *et al.* (1995) also found that z=-4 predominated in oil sands tailing pond wastewaters of Suncor and Syncrude. Individual compounds within the grouping of NAs are expected to have varying physical, chemical and toxicological properties.

2.2.2 Physical and Chemical Properties

NAs are viscous liquids possessing a characteristic odor (Encyclopedia of Chemical Technology, 1978). The odor develops upon storage of refined acids.

No successful processes have been found for deodorization. The color of NAs ranges from pale yellow to dark amber (Encyclopedia of Chemical Technology, 1978).

Table 2.3 shows the physical and chemical properties of industrial use NAs. No information was found on toxicological characteristics such as carcinogenicity, mutagenicity and tetratigenicity of these compounds.

Table 2.3. Known physical and chemical properties of industrial use NAs. *Source: CONRAD* (June 1998)

Property	**Level**
Chemical formula	$C_nH_{2n+z}O_2$
Molecular weight of compounds of interest related to oil sands process affected waters	172 to 1000
Appearance	Brown or black liquid
Odor	Musty, hydrocarbon
PH	>10
Boiling point	Approximately 104 °C
Reid vapor pressure (psia = pounds per square inch absolute)	0.36 psia @ 21 °C 1.5 psia @ 46 °C 2.2 psia @ 54.5 °C
Specific gravity	1.00-1.10 @ 15.6 °C
Vapor Density (Air=1)	Not pertinent
Solubility in water	Soluble

Brient (1998) stated that NAs are mobile liquids with very low freezing points. They are completely soluble in organic solvents and oils. NAs behave chemically like

typical carboxylic acids with similar strength as the higher fatty acids; dissociation constants are in the order of 10^{-5} to 10^{-6}.

The boiling point of sodium naphthenates (i.e., sodium salts of NAs) is approximately 104 °C, while the freezing point is <-17.8 to 10 °C. Reid vapor pressure, the absolute vapor pressure of volatile crude oil and volatile nonviscous petroleum liquids, except liquefied petroleum gases (http://www.epa.state.oh.us/dapc/regs/3745-72/3745-72.html), ranges from 0.36 psia at 21 °C to 2.2 psia at 54.5 °C (CONRAD 1998; Drew, 1995).

Sodium naphthenates can react violently with strong acids and many organic compounds. The solution is extremely corrosive to all body tissues and harmful if inhaled. Over exposure requires first aid and medical follow-up. Hazardous compounds consist of 1 to 2 % sodium hydroxide (CAS No. 1310-73-2) and 2 to 10 % sodium naphthenate (CAS No. 617090-13-4) (CONRAD, 1998).

4-methy-1-cyclohexanecarboxylic acid, a 99% mixture of *cis* and *trans* isomers, is a colorless liquid. The melting point is approximately 16 to 17°C. The specific gravity is 0.960 (CAS No. 4331-54-8). 3-methy-1-cyclohexanecarboxylic acid, a 98% mixture of *cis* and *trans* isomers, is also a colorless liquid. The specific gravity is 0.989 (CAS No.13293-59-9). 4-methy-1-cyclohexaneacetic acid, a 97% mixture of *cis* and *trans* isomers, is a moist white solid (CAS No.6603-71-0). There is no information on physical properties of 4-methy-1-cyclohexaneacetic acid available from material data sheets.

2.2.3 Occurrences

Presently, NAs are believed to be a natural component of bitumen, although the elevated levels of NAs present in oil sands tailings may be the result of the alkaline

hot water extract process used to recover bitumen from the oil sands (MacKinnon and Boerger, 1986).

NAs are found in crude oils in much higher concentrations than fatty acids. In contrast to the fatty (aliphatic) acids, NAs are not common constituents of plant and animal fats (Davis, 1967). Although NAs occur in nearly all crude oil, not all crude oils contain sufficient quantities of usable acids to make recovery an economic process. Heavy crudes from geologically young formations have the highest acids content, and paraffinic crudes usually have low acid content (Encyclopedia of Chemical Technology, 1995).

NAs are rarely found in the environment as single compounds. They are typically found as complex mixtures of many different NAs (CONRAD, 1998). They may be naturally formed by: (a) the rearrangement of sedimentary organic material to form a component of petroleum reserves of crude oil and bitumen, or (b) the direct conversion of petroleum compounds by microbes and plants (National Research Council, 1975)

A concentration of 1.9 mg/l of NAs has been recorded in the main stem of the Athabasca River, a few kilometers upstream of Suncor's Tar Island Dyke (based on relatively few samples) (FTFC, 1995). Figure 2.2 shows the location of the sample site downstream of the oil sands deposits that are directly exposed to the Athabasca River near Fort McMurray, Alberta (FTFC, 1995). Table 2.2 supports the statement that NAs occur naturally in surface water.

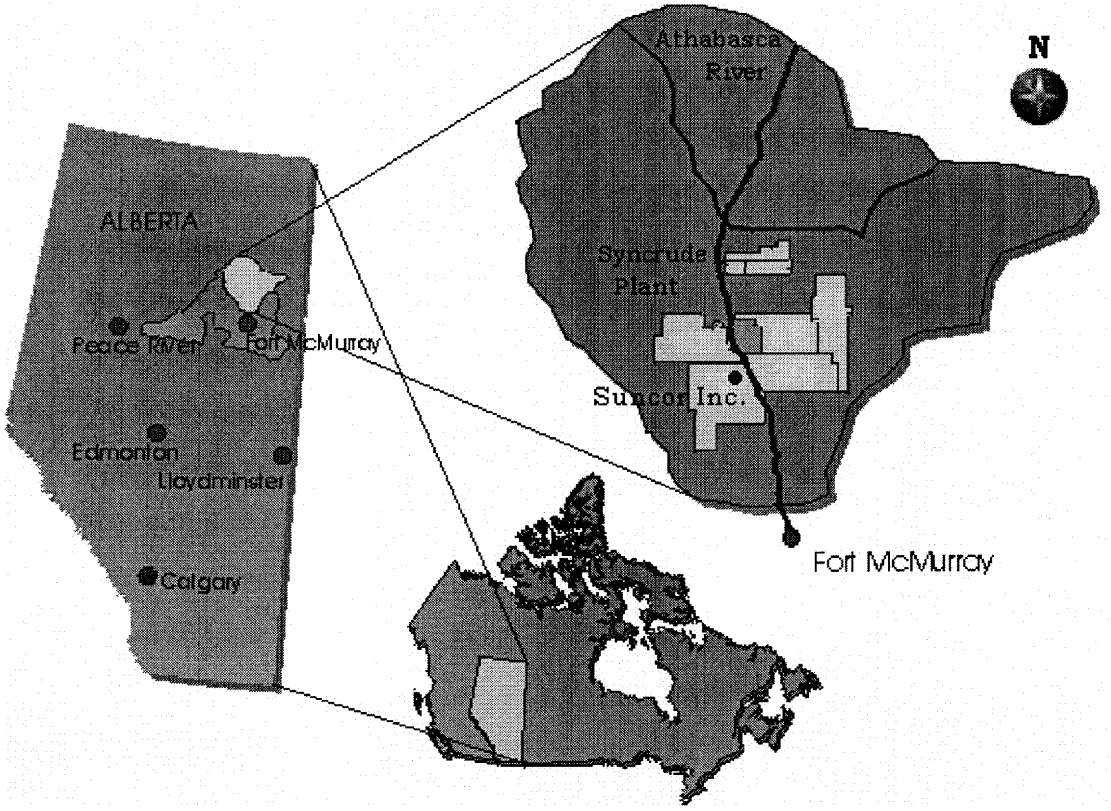


Figure 2.2. Locations of Suncor Inc., Syncrude Plant and Athabasca River.

Groundwater researchers (Golder and Klohn-Crippen, 1996) have found concentrations of NAs in the groundwater wells established on the Suncor Steepbank Mine site. The concentration of NAs found in these waters was in the ranges of <4 to 7mg/l in surficial aquifers, 8 to 36 mg/l in basal aquifers, and 47 to 57 mg/l in limestone aquifers. These distributions indicate that deeper aquifers may also contain concentrations of NAs.

There is no information on the distribution of NAs concentrations at surface and subsurface soil sites on and near oil sands leases near the Athabasca River.

2.3 Biodegradation

Davis (1976) and several other references stated that NAs could be biodegraded by microbial communities isolated from refinery waste streams. Moreover, a recent study on the biodegradation of NAs by microbial populations indigenous to oil sands tailings showed that they were capable of degrading components of a commercial mixture of NAs and a mixture of organic acids extracted directly from oil sands tailings (Herman *et al.,* 1994). The indigenous bacteria included the species *Pseudomonas stutzeri* and *Alcaligenes denitrificans* in naphthenic acids sodium salts (Herman *et al.,* 1994).

Morales *et al.*, (1993) reported that the rate of biodegradation may be affected by the chemical structure of the alkyl-substitution on the cycloalkane ring. Another factor is the number of cycloalkane rings, although the same group of researchers could not test this factor because representative compounds are not commercially available. Fan (1991) concluded from his study that biodegradation does not appear to be affected by the characteristics of the NAs on a macro level. However, the specific carboxylic acid composition may be different.

NAs present within oil sands tailings have greater structural complexity than commercial compounds. However, studies have indicated the potential of oil sands tailings microorganisms to biodegrade both the alkyl side chain and the carboxylated cycloalkane ring components of NAs (Herman *et al.,* 1993). The same group of researchers also stated that biodegradation potential was reduced by methyl substitutions on the cycloalkane ring, although these compounds could be degraded with the addition of mineral nutrients.

Biodegradation rate constants in the laboratory studies are generally more rapid than those found in the field because the conditions in the laboratory are optimized

for microbial growth (CONRAD, 1998; U.S. EPA, 1982). Other factors affecting the rates of NAs biodegradation are salinity, temperature, dissolved oxygen, pH, nutrient availability, sediment structure and sunlight (CONRAD, 1998; U.S. EPA, 1982).

Mandelstam and McQuillen (1968) showed that temperature is a significant factor affecting the rate of biodegradation. Many bacteria species grow best in the range between 30°C and 37°C. Although most tolerate much lower or higher temperatures, only a few species (themophilic or cryophilic species) truly grow better at high or low temperatures.

2.4 Biodegradation Kinetics

Analysis of the biodegradation rate of NAs and order of the kinetics was determined in this study because no information was available in the literature. First-order substrate removal was assumed and confirmed by experimental results.

The easiest method of determining the order of a batch reaction is to substitute experimental data on the amount of material remaining at various times into the integrated forms of the various rate expressions, and then to solve for the reaction-rate constant. The equation that results in the most consistent K values is assumed to represent the order of the reaction. Alternatively, the order of a reaction can be determined by plotting the experimental data functionally, based on the integrated form of the rate expression. If a straight-line plot is obtained, it is assumed that the order of the reaction corresponds to the reaction plotted. For example, a first-order reaction rate law for a batch reactor can be written in the following equations (Pilling and Seakins, 1995):

$$dA/dt = -KA \quad (2.1)$$

Integrating (if $t_o = 0$), we find (2.2):

$$Ln[A] - Ln[A_o] = -Kt \quad (2.2)$$

$$Ln[A/A_o] = -Kt \quad (2.3)$$

Where: t_o, t are initial time (t = zero) and elapsed time of experimental measurement (hours), respectively,

A_o, A are concentration of NAs at zero time, and concentration of NAs at time t (mg/l),

K is the rate constant for the first-order biodegradation reaction ($hour^{-1}$).

Similar equations can be derived for reactions of other orders and some of them are shown in Table 2.4.

Table 2.4. Differential and integral forms of rate equations and half-life expressions.

Order	dA/dt	-Kt	$T_{1/2}$
0	-K	$(A-A_o)$	$A_o/(2K)$
1	-KA	$Ln(A/A_o)$	(0.693)/K
2	$-KA^2$	$(1/A_o)-(1/A)$	$1/(KA_o)$
3	$-KA^3$	$(1/2A_o^2)-(1/2A^2)$	$3/(2KA_o^2)$

The half-life or half-period ($t_{1/2}$) is a convenient way to quantify a reaction. This is the time the reaction takes for half of the original substance to disappear (Laidler, 1963) or the time at which the concentration of NAs was decreased to half its initial value, $0.5A_o$. For example, substitution in (equation 2.3) gives (equation 2.4) and shows that the half-life of a first-order kinetic is directly related to the first-order rate constant as shown in (equation 2.5).

$$Ln[0.5A_o / A_o] = -Kt_{1/2} = Ln(0.5) = -0.693 \quad (2.4)$$

$$t_{1/2} = 0.693/K \quad (2.5)$$

The results of half-life expressions in various reaction orders are shown in Table 2.4.

2.4.1 Effect of Temperature on Reaction Rate Constants

According to Pilling and Seakins (1995), rates of chemical reaction and rate coefficients are dependent upon the temperature. A majority of reactions have rate coefficients that follow the Arrhenius equation (equation 2.6).

$$k = Ae^{-(Ea/RT)} \quad (2.6)$$

where: k = reaction rate constant (variable units),

A = the pre-exponential factor or frequency factor (variable units),

Ea = the activation energy (J/mol),

R = the ideal gas constant (8.314 J/mol.K),

T = temperature (K).

To adjust the reaction rate constant from a base temperature the following may be derived from (equation 2.6).

$$k_{T1} = k_{T2}\theta^{(T1-T2)} \quad (2.7)$$

$$\theta = \exp^{(Ea/RT1T2)} \quad (2.8)$$

where: θ = temperature coefficient,

T1 = adjusted temperature,

T2 = base temperature,

K_{T1} = rate constant at adjusted temperature,

K_{T2} = rate constant at base temperature.

2.4.2 Rate of Bacterial Growth

Typically, to understand and define the growth of a particular microorganism, cells are placed in a flask in which the nutrient supply and environmental conditions are controlled. If the liquid medium supplies all nutrients required for growth and environmental parameters are optimal, the increase in numbers or bacterial mass can be measured as a function of time to obtain a growth curve (Maier *et al.,* 2000).

In batch culture, there is initially a low population of microorganisms and a high concentration of substrate. However batch culture eventually becomes a substrate-limiting reaction-rate system. The number of microbial cells varies with time depending upon the type of microbial population, concentration of substrate and environmental conditions (McMartin, 2000; Tarighian, 1999; Estrella *et al.,* 1993; Greer *et al.,* 1992).

Several distinct growth phases can be observed within a batch growth curve (Figure 2.3). These include the lag phase, the exponential or log phase, the stationary phase and the death phase. Each of these phases represents a distinct period of growth, and associated physiological changes as microorganisms shift from one phase to the next (Maier *et al.,* 2000).

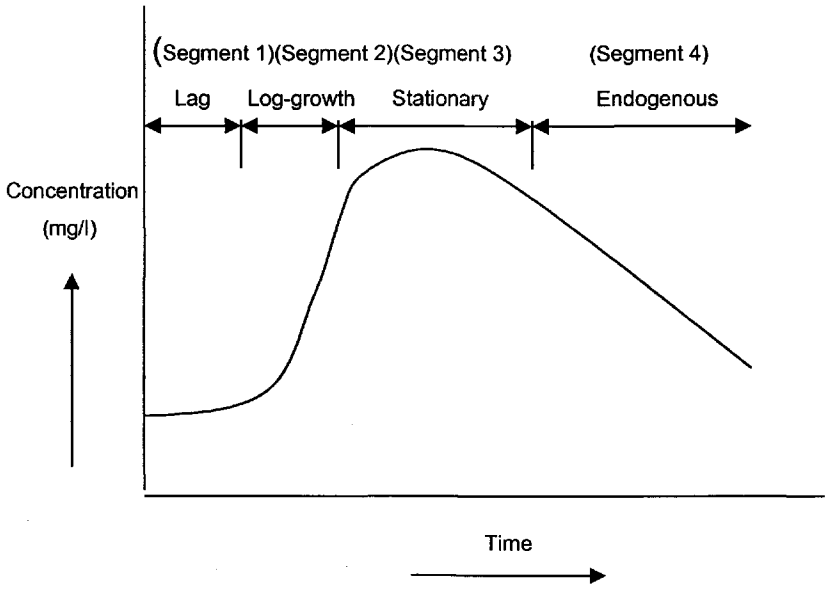


Figure 2.3. A typical batch growth curve for a bacteria population (Peavy *et al.*, 1985).

Growth curve or population curve can be illustrated in four phases:

1) Lag phase

In the lag phase microorganisms have to become acclimatized to their surrounding environment and to the food provided (Peavy *et al.*, 1985). The acclimatization period or the lag phase is represented in segment 1 on the curve. Moreover, the lag phase is due to the physiological adaptation of the cell to the culture conditions (Maier *et al.*, 2000).

2) Exponential or Log-Growth phase

Once growth has been initiated in segment 1, bacteria cells divide into segments that separate to become two new independent cells (Peavy *et al.*, 1985). The reproduction period or log-growth phase is represented in segment 2 of the curve. Reproduction by separations can be explained by the exponential equation:

$$N=2^{n-1} \qquad (2.9)$$

where N is the number of organisms produced from one individual after n regeneration times (Peavy *et al.*, 1985).

3) Stationary Phase

Stationary phase or segment 3 of the curve represents the time during which the production of new cellular material is approximately balanced by death and endogenous respiration (Peavy *et al.,* 1985). Although there is no net growth in the stationary phase, cells still grow and divide. Growth is simply balanced by an equal number of cells dying (Maier *et al.,* 2000).

4) Death Phase

Segment 4 of the curve or the final phase represents the death phase, which is characterized by a net loss of culturable cells. In the death phase, some individual cells are still metabolizing and dividing but more viable cells are lost than are gained (Maier *et al.,* 2000). The death phase is usually exponential although the rate of cell death is often slower than the rate of cell growth during the exponential phase.

2.5 Water Quality Guidelines

There is no information of ambient marine water or surface water quality guidelines for NAs in Canada or the United States. This is mainly due to a lack of sufficient chronic toxicity data regarding the long-term exposure of sensitive life cycle stages of marine or fresh water aquatic biota to NAs and their respective fractionated components.

Some acute toxicological data are available, such as the results of static toxicological assessments (i.e., LC_{50} 96 hour, LC_{50} 60 day bioassay) using commercial preparations of sodium naphthenate stock solutions. The individual substances or specific chemical components contributing to the principal toxicity of NAs as a group are unknown (CONRAD, 1998).

Based upon previous studies, the maximum permissible concentration of sodium naphthenates considered to be harmless for the fish species investigated was set at 0.8 mg/l (Dokholyan and Mogomedov, 1984). The same researchers also recommended that the acceptable level of sodium naphthenates in seawater was 0.15 mg/l.

2.6 Analytical Methodologies

As described earlier, NAs are complex mixtures of saturated aliphatic and alicyclic carboxylic acids of different molecular weights and structures. Conventional mass spectrometric techniques do not analyze NAs well for reasons related to their polarity and increasing non-volatility with increasing molecular weight (Morales *et al.,* 1993). As a result, gas chromatography is frequently used for NAs analysis.

2.6.1 Gas Chromatography Analysis

Gas chromatography (GC) is a highly versatile instrumental method of analysis which was first developed in 1951. Furthermore, Gibson (1984) concluded that GC is a routine separation and quantitation technique with a large variety of instrumentation available commercially.

2.6.1.1 Basic Components of Gas Chromatography

A schematic diagram of the components of a gas chromatography (GC) system is shown in Figure 2.4. The basic components of a GC system are a gas cylinder with reducing valve, a constant-pressure regulator, a port for the injection of the sample, a chromatographic column, a detector, an exit line, and recorder. In a typical GC system a liquid sample is vaporized followed by the separation of the

various gaseous components formed. The components can then be individually identified and quantitatively measured (Sawyer *et al.*, 1994).

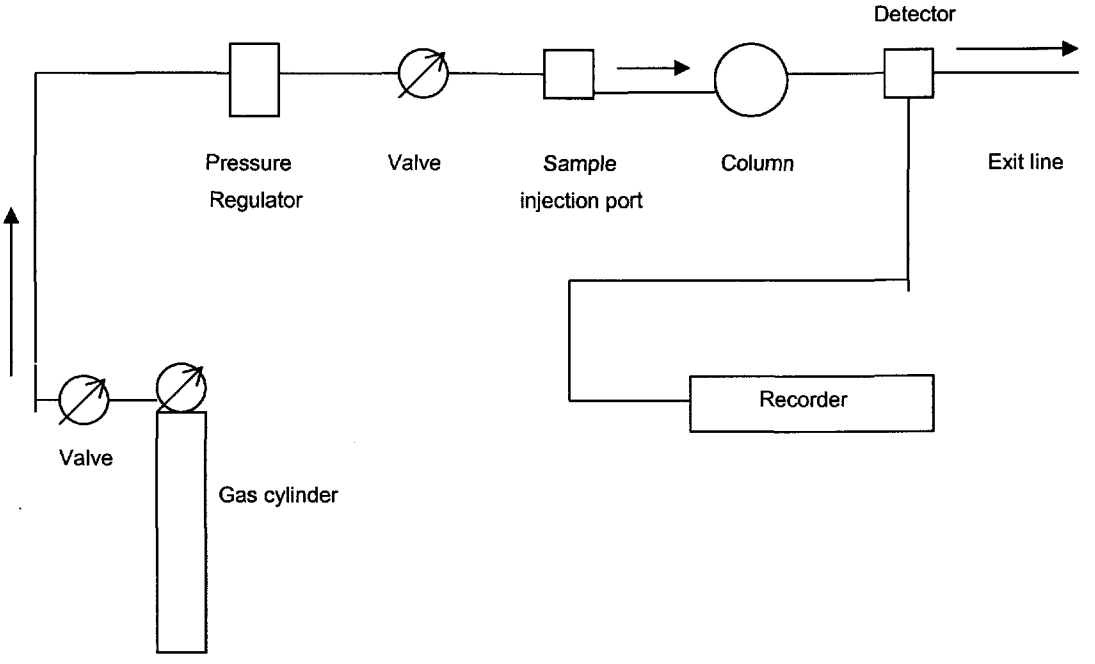


Figure 2.4. Modified schematic diagram of the components of a gas chromatograph (Sawyer *et al.*, 1994).

Sawyer *et al.* (1994) described the GC process as follows. A carrier gas such as hydrogen, helium, or nitrogen contained in a gas cylinder is continuously swept through the chromatographic column at a constant temperature and flow rate. A small amount of liquid sample is injected with a syringe into the sample port. The sample port converts the sample's components into gaseous state. The constantly flowing stream of carrier gas carries the gaseous constituents through the chromatographic column. The constituents travel through the column at different rates because different constituents will move at different rates, and each constituent will have a slightly different affinity (adsorption-desorption relationship) for the stationary phase with respect to the concentration in the mobile phase. For that reason, constituent gases emerge from the column at different times. Once

the separation within the column has been achieved, the detectors, which offer high sensitivity, precision and accuracy on every small amount of separated components, continuously generate electrical signals during the entire separation (http://www.sciencesolution4all.com/GC/index.htm). The response of the detector in the form of peaks for each component is fed into a strip chart recorder or microcomputer system.

Each peak represents a specific chemical compound or a mixture of compounds with the same rate of movement through the column. The time for each compound to emerge from the column is a characteristic of the compound and is known as its retention time. The area under the peak is proportional to the concentration of the compound in the sample (Sawyer *et al.,* 1994).

2.6.1.2 GC-Flame Ionization Detector (FID)

GC-Flame Ionization Detector (FID) is a common method for detecting organic constituents in the effluent from a gas chromatography column. According to Poole and Poole (1991), FID responds to the presence of nearly all organic compounds in gas chromatography effluent and is considered to be a general detector.

Sawyer *et al.* (1994) explained the principle of GC-FID as follows. Organic compounds yield ions and electrons when burned in a flame. This principle can be applied by measuring the current carried by charged particles (ions) when a potential of a few hundred volts is applied across the burner. A collector electrode is eluted from the chromatographic column and burned. Petroleum hydrocarbons in very small concentrations (mg/l) can be measured by using this detector.

2.6.2 Mass Spectrometry (MS)

MS is a powerful method for studying a sample at the molecular level. It can be applied to detect very low levels of specific compounds and elements. Moreover, it can provide the determination of masses with its high sensitivity and accuracy and provide more specific information per given amount of material than any other analytical technique (Encyclopedia of Physical Science and Technology, vol. 10, 1987).

Sawyer *et al.* (1994) explained the principle of GC/MS as follows. A mass spectrometer is an instrument that will separate charged gas molecules or ions. The analyzed substance is vaporized and converted to positive ions by bombardment with rapidly moving electrons. The ions formed are pulled from the gas stream by an electrical field. These ions are accelerated depending upon the type of instrument and are separated by their mass-to-charge ratio. A suitable detector can record the particles of different mass either qualitatively, quantitatively, or both. Headley *et al.* (2001) utilized electrospray ionization with mass spectrometric detection (ES/MS) to determine dissolved NAs in natural waters. This method proved to be a simple procedure for the quantitative analysis of NAs.

2.6.3 Fast Atom Bombardment Mass Spectrometry (FABMS)

In FABMS, an atom beam rather than an electron beam is used to bombard the sample in a non-volatile liquid matrix containing the analyte. FABMS is well known for its ability to analyze polar, non-volatile, and/or high molecular weight components. Fan (1991) demonstrated that FABMS is a simple and efficient approach for analyzing NAs, including the heavy and higher molecular weight components. However, according to Morales *et al.* (1993), FABMS is very powerful for quantitative analysis of polar, non-volatile compounds, but is not an ideal method for quantitative analyses of complex mixtures such as NAs.

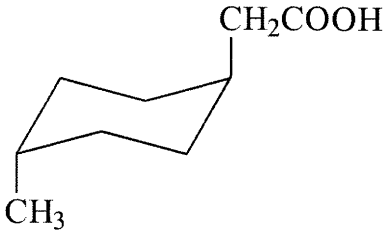
Lai *et al.,* (1996) reported that researchers at the University of Alberta have obtained semi-quantitative data by using FABMS on the relative abundance of different classes of NAs in oil sands tailings pond water. The method was used to characterize NAs based on the Z group type and the carbon number distribution (C_9 to C_{50}).

2.7 *cis-trans* Isomerism

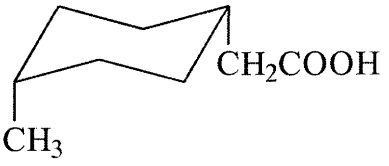
Cis-trans isomers of a molecule have the same order of attachment of substituent chains but a different arrangement of the molecule in space due to the presence of either a carbon ring or a carbon-carbon double bond (Brown, 1997). Figure 2.5 shows the *cis-trans* isomers of the NA compounds used in this study.

The prefix *cis* (Latin: on the same side) is used to indicate that the substituents are on same side of the ring; the prefix *trans* (Latin: across) is used to indicate that they are on opposite sides of the ring (Brown, 1997). There is no information regarding the effect of *cis-trans* isomers on the rate of biodegradation of NAs.

4MACH

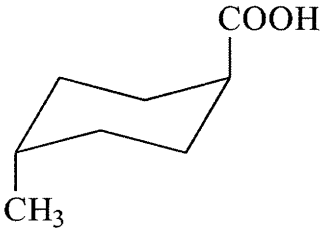


[A] (a) *trans*-isomer

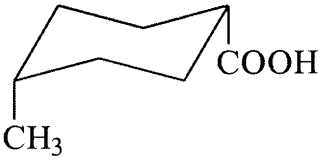


(b) *cis*-isomer

4MCCH

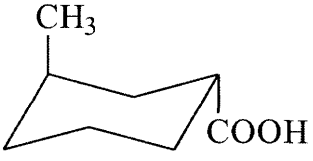


[B] (a) *trans*-isomer

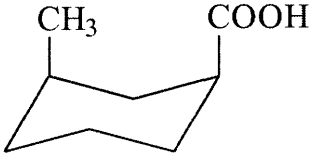


(b) *cis*-isomer

3MCCH



[C] (a) *trans*-isomer



(b) *cis*-isomer

Figure 2.5. *Trans*- and *cis*- isomers of [A] 4-methycyclohexaneacetic acids (4MACH), [B] 4-methycyclohexanecarboxylic acids (4MCCH) and [C] 3-methycyclohexanecarboxylic acids (3MCCH).

3. MATERIALS AND METHODS

3.1 Sampling

Water samples of approximately 10 liters each were collected from the Athabasca River near Fort McMurray in plastic containers (Figure 3.1). These containers were first carefully cleaned and rinsed with distilled water several times. The water samples were collected a depth of 0.2 meter below the surface (Figure 3.2). At this depth, it was expected that significant aerobic microbial communities and minimal suspended solids would be present. The samples were collected in September 2000, 2 kilometers downstream from Suncor Inc. and 2 kilometers downstream from Syncrude Canada Ltd., as shown in Figure 2.2. The water sample were transported at ambient temperature from the site to the National Hydrology Research Center (NHRC) within 24 hours and stored at 4°C for 6 months.

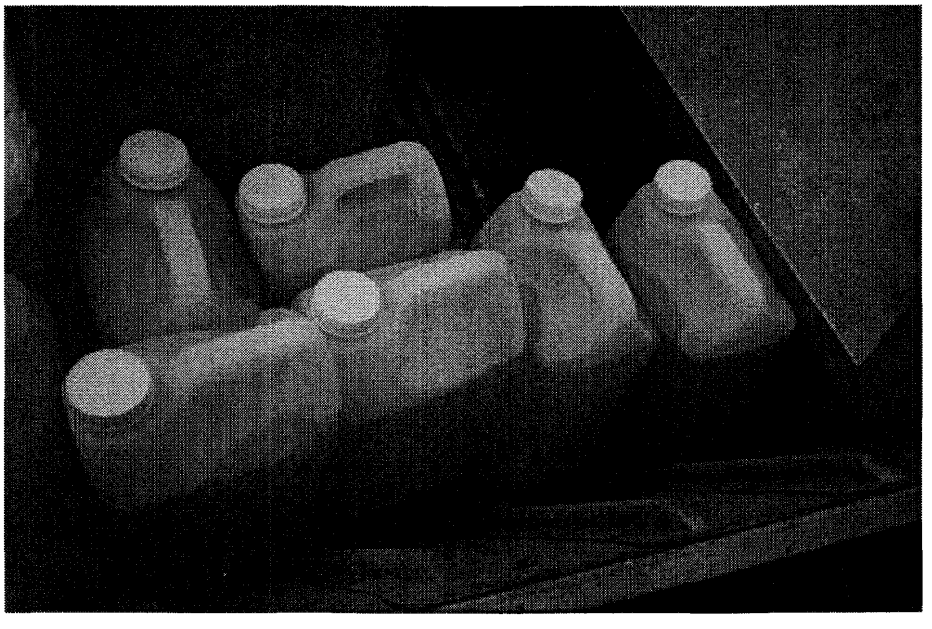


Figure 3.1 Bottles used to contain river water samples used in the study.

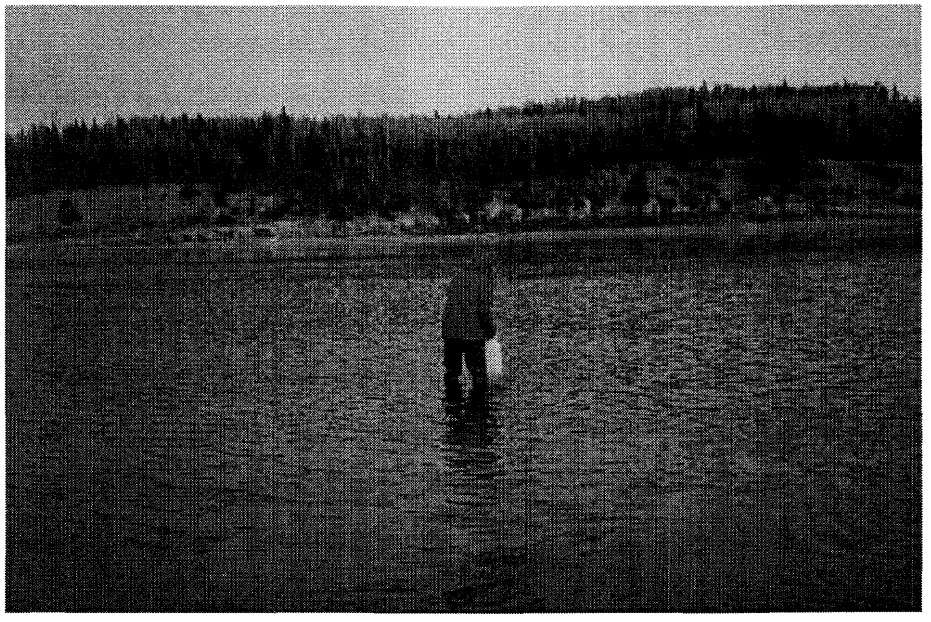


Figure 3.2 Sampling site at the Athabasca River, Fort McMurray downstream from Suncor Inc. and Syncrude Canada Ltd.

3.2 Water Characterization

The Athabasca river receives inputs of organic materials from forest drainage and natural oil sand deposits, and more recently from industrial mining activity. Oil sands mining and extraction may contribute organic compounds from several sources, such as refinery waste, coke storage runoff, etc., and may also contribute natural compounds from the drainage of surface and groundwaters (Strosher and Peake, 1978; Nix, *et al.*, 1981).

Terrestrial & Aquatic Environmental Managers Ltd. (1993) reported the results of chemical analyses of water samples collected from the Athabasca river on 22nd October 1992. In general, the pH recorded from all samples ranged from 8.29 to 8.46, while dissolved organic carbon concentration ranged from 2.3 to 3.9 mg/l. Water temperature based upon the climatic condition on the day of sample

collection was found to be between 7°C and 9°C. Recorded total organic carbon and dissolved oxygen were 2.9 to 4.0 mg/l and 11.2 to 11.4 mg/l, respectively.

3.3 Materials

Representative NA compounds were chosen on the basis of commercial availability. A preliminary test was conducted on each compound to determine its solubility in the water samples. *Cis* and *trans* 4-methylcyclohexaneacetic acids [CAS No. 6603-71-0], *trans* 4-methylcyclohexanecarboxylic acids [CAS No. 13064-83-0], *cis* and *trans* 4-methylcyclohexanecarboxylic acids [CAS No. 4331-54-8] and *cis* and *trans* 3-methylcyclohexanecarboxylic acids [CAS No. 13293-59-9] were obtained (Sigma-Aldrich Canada Ltd., Oakville, ON)

Humic acid was obtained from Aldrich Chemical Company (Milwaukee, WI). For the microbial growth study, R_2A agar was obtained from Sigma Chemical Company (St. Louis, MO).

Dichloromethane (DCM) and hexane (analytical grade) were obtained from the Fisher Scientific (Fair Lawn, NJ). BF_3-methanol (Boron Trifluoride) was obtained from Supelco (Bellefonte, PA). These chemicals were used in the sample extraction.

3.4 Experimental Methodology

3.4.1 Experimental Plan

Four experiments of 4 to 6 weeks duration each were conducted to evaluate the biodegradation kinetics of geometric isomers of NAs in Athabasca river water. A

primary consideration in the experimental design was the availability of space and equipment.

Biodegradation of NAs was observed in several 125 ml Erlenmeyer flasks washed and double autoclaved prior to each experiment. All flasks were sealed with foil covered latex-stoppers (see Figure 3.3). The flasks acted as batch bioreactors in which the rate and extent of NAs biodegradation could be observed under several water quality conditions. Three different structural groups of NA compounds were studied (see Figure 2.5). Experiments were conducted at three temperatures, 10°C, 20°C and 30°C. The experiments at 10±1°C and 30±1°C were monitored in model I-35LLVL Pervical controlled environment chambers (Boone, IW) (Figure 3.4) without light. Dissolved organic carbon (DOC) content was adjusted from ambient conditions for several experiments by adding 5 mg/l of humic acid (HA). Three adjusted pH levels were used in the experiments (pH 7, pH 6 and pH 3.5). pH adjustments were made by the addition of concentrated hydrochloric acid (HCl). Once the amended or non-amended river waters were added to the flasks, the bioreactors and controls were placed in a fume hood where each experiment was monitored until completion.

4MACH was added at 9 mg/l and 7.5 mg/l in the first and second experiments. 4MCCH was added at 9 mg/l in the third experiment, whereas 3MCCH was added at 12 mg/l in the fourth experiment. These concentrations were selected based upon preliminary tests conducted prior to the start of each experiment. These preliminary tests were used to approximately determine the maximum solubility and biodegradation potential of each NA.

Figure 3.3 Experimental set up in a fume hood under minimal light conditions and room temperature (20±2°C).

In order to avoid possible photodegradation, all bioreactor flasks were placed in a fume hood with minimal light (20±2°C), and incubated in a controlled environment chamber with no light (10±1°C and 30±1°C).

Each bioreactor was manually shaken once before collecting samples for NA analysis. A 100µl sample was pipetted from the bioreactor into a volumetric tube for analysis. Daily sample collections were conducted in the first experiment, and three days per week (Mondays, Wednesdays, and Fridays) in the last three experiments. To avoid possible photodegradation, two bioreactors for microbial analysis were taken out of the fume hood 2-3 times per week for a period not exceeding 20 minutes in each experiment. PH analysis of each experiment took place in the fume hood for approximately 15 minutes. Therefore, no flask was removed from the fume hood for a period exceeding 20 minutes in all experiments.

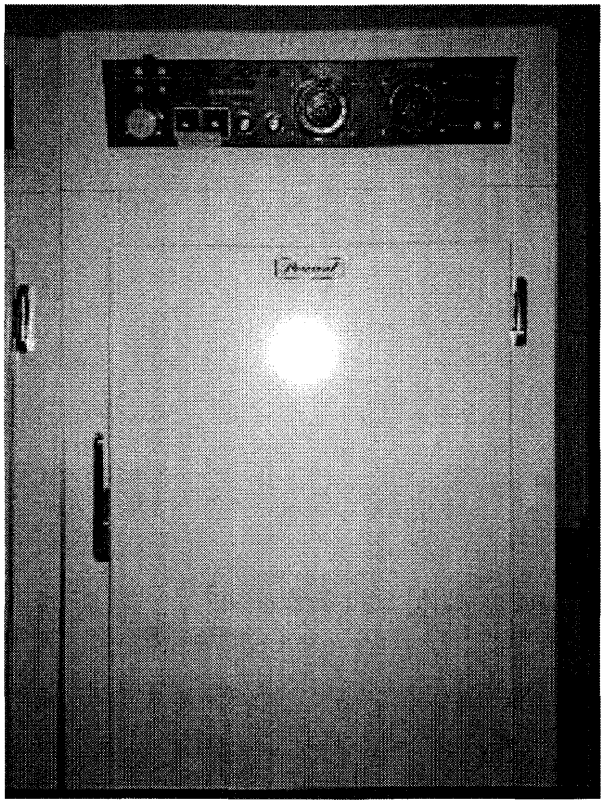


Figure 3.4 Controlled environment chamber.

3.4.2 Sample Preparation

The water quality conditions for each experiment are presented in Table 3.1. Each 125 ml Erlenmeyer flask contained 120 ml of river water plus any prescribed amendments. In the first and fourth experiments, the batch experiments on each water quality combination were conducted in triplicate, while in the second and third experiments, the batch experiments were conducted in duplicate. The number of replicate reactors used in each experiment depended upon the availability of flasks.

Table 3.1 Water quality conditions for each experiment.

EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4
4MACH(9 mg/l)	4MACH(7.5 mg/l)	4MCCH(10 mg/l)	3MCCH(12 mg/l)
1a) Non-amended* (base condition) pH 8.8	2a) pH decreased to 3.5* from base condition	3a) Non-amended* (base condition) pH 8.7	4a) Temp. at 30°C from base condition pH 8.8
1b) 5 mg/L of HA added* to base condition	2b) Temp. at 30°C from base condition pH 8.7	3b) pH decreased to 6* from base condition	
1c) pH decreased to 7* from base condition	2c) Temp. at 10°C from base condition pH 8.6	3c) Temp. at 30°C from base condition pH 8.8	
1d) pH to 7; 5mg/l of HA* added from base condition		3d) Temp. at 10°C from base condition pH 8.7	

* at room temperature (20±2°C)

The effect of extreme pH i.e. pH 3.5 was monitored in the second experiment, whereas the effect of small pH changes (pH 7 and pH 6) was monitored in the first and third experiments. pH values were adjusted by adding small volumes of concentrated hydrochloric acid (HCl) to reduce the pH from the base condition. Temperatures were adjusted by using the controlled environment chambers to increase or decrease temperature from ambient room temperature. On average, the recorded pH values under base condition for all experiments ranged from 8.5 to 8.8, while base condition of ambient room temperature was 20±2°C.

In experiment 1b) and 1d) 5 mg/l of granular HA from the Aldrich Chemical Company (Milwaukee, WI) was added to the water sample to increase the DOC level from the base DOC concentration. The base DOC concentration was not measured. The combination of pH adjustment and HA added (pH 7 plus 5 mg/l HA added from base condition) was also examined in first experiment.

A model 5210 Ultra Sonic Bath (Branson, Danbury, CT) was employed for 2 hours to dissolve granular 4MACH in the river water samples in the first two experiments. Liquid 4MCCH and 3MCCH were dissolved in raw river water samples by mixing for 2 hours using a magnetic stirrer. Tests were conducted prior to each experiment to determine maximum solubility of each compound.

The number of bioreactors required for each experiment was determined by the number of water quality conditions tested multiplied by the number of replications. Since triplicate batch reactors for each of the water quality combinations shown in Table 3.1 for the first and fourth experiments were prepared under the imposed 4MACH and 3MCCH concentrations, a total of 12 and 3 bioreactors were monitored during each experiment, respectively. Duplicate batch reactors for each of the water quality combinations (see Table 3.1) of the second and third experiments were prepared under the imposed 4MACH and 4MCCH concentrations, therefore a total of 6 and 8 bioreactors were monitored during each experiment, respectively.

3.4.3 Controls and Blanks

Static controls were evaluated under the same NA dosages and water quality conditions as the batch bioreactors. The controls were used to determine the extent of NAs volatilization and/or sorption to the glass bioreactors and latex

stoppers, as well as assess the possibility that the minimal light source may be responsible for loss of NAs from the bioreactors.

In the first two experiments controls were prepared by double-filtering water samples through 0.7 and 0.2 μm triple-autoclaved filters (Millipore filter units) (Millipore Corporation, Bedford, MA) to ensure no microbial survival. The controls for the last two experiments used unfiltered double-autoclaved river water to ensure no microbial survival.

Blank samples (i.e. those not dosed with NA) were monitored during each experiment under the sample water quality conditions as the bioreactors. All of the blank samples were analyzed by GC-FID simultaneously with the bioreactors to ensure that no cross-contamination of NAs occurred during sampling procedures.

In addition, the blank samples in the first three experiments were evaluated two or three times per week for microbial growth. This allowed verification that NA biodegradation in the bioreactor experiments was the result of substrate-induced microbial growth. The microbial growth in the raw river water in blank reactors was expected to be much lower than that in the NAs amended river water in the bioreactors.

3.5 Analytical Methods

3.5.1 pH

A calibrated ISFET model IQ 125 pH meter (Scientific Instrument Inc., San Diego, CA) was employed to measure the pH of the water in each experiment. Two pH levels were monitored in the first three experiments, including the base condition pH (8.7) and the amended level obtained by the addition of concentrated

hydrochloric acid. The effect of moderate pH (7) was examined in the first experiment, while extreme pH (3.5) was examined in the second experiment. pH 6 was examined in the third experiment. pH was measured at the start and end of each experiment.

The pH probe was rinsed with Milli-Q® water and dried between measurements. The meter was calibrated with a set of three standard solutions ranging between 4 and 10 standard units.

3.5.2 Microbial Analyses

The heterotrophic plate count (HPC) is a procedure for estimating the number of living heterotrophic bacteria in water. Heterotrophic bacteria are a mixed population of aerobic bacteria which utilize organic compounds as an energy source. Colonies may arise from pairs, chains, clusters or single cells, all of which are included in the term "colony-forming units" (CFU) (APHA, 1998).

The microbial populations were monitored in the first three experiments. The fourth experiment was excluded because of the limited volume of water samples available. One set of imposed NAs samples and blank samples were used to study the typical time progression of heterotrophic population in the experiments.

Replicate plates for enumeration of bacterial populations were made in a dilution series by the spread plate method twice per week for each four-week experiment. In preparing the plates, sample volumes were transferred by using an autoclaved pipette. Three dilutions were tested for each sample. A blank sample and one representative NAs biodegradation sample were also plated in each instance. The aim was to have at least one dilution between 30 and 300 colonies in the plate count. Only one plate having 30 to 300 colonies was considered in determining

the plate count (APHA, 1998). Counts of all plated microbial populations were performed after a five- to seven-day period at room temperature.

R_2A agar (Difco Laboratories, Detroit, MI) was prepared at the beginning of each experiment. Powdered R_2A agar was dissolved in fresh distilled water at the concentration specified. Each media bottle of agar was then autoclaved on the liquid cycle at a temperature of 121°C for 40 minutes.

The neck of the bottle was flamed, and at least 10 to 12 ml liquefied medium maintained at 44 to 46°C was carefully poured into each dish by gently lifting the cover just high enough to pour. Plates were allowed to solidify for a period of up to 30 minutes before being stacked and re-sealed in the plastic bags.

Distilled water autoclaved at a temperature of 121°C for 40 minutes was used for sample dilutions. Dilutions of the microbial communities were prepared in 9 ml of autoclaved distilled water. Volumes of 1 ml from each water sample were pipetted into the first dilution blank using buffered, autoclaved pipette tips. The solution (water sample plus dilution blank) was vortexed and 1 ml from the first inoculated dilution blank was added to the second. This process was repeated until the fourth inoculated dilution blank. A 0.1 ml aliquot of each dilution solution was plated to a labeled agar plate using an autoclaved pipette tip. Microbial populations were spread over the agar plates with a flamed metal spreader. The metal spreader was cleaned by dipping into ethanol, then dipping into fire to flame excess ethanol from the spreader.

According to the APHA method (1998), all plates were incubated for a duration of 5 to 7 days at a temperature of 20±2°C to obtain the highest counts (see Figure 3.5). Each plate was counted manually. Duplicate plates were counted and results were averaged.

Figure 3.5 Plate samples in the incubator

3.5.3 GC Analysis

Analyses were performed using a Varian 3500 capillary gas chromatograph (Georgetown, ON) (Figure 3.6) equipped with a flame-ionization detector (FID) and a split/splitless injector. The injector temperature was set to 250°C with a purge time of 1.0 min. A SP5 capillary column was utilized having a length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 µm. The helium carrier gas feed at a linear velocity of 41.2 cm/s. The column temperature program was 50°C for 1.0 min, ramp to 280°C at 10°C/min and held for 0.5 min. The detector temperature was 320°C. Quantification of the GC peaks was achieved using HP GC ChemStation software (Rev. A. 06.03 [509]) (Hewlett-Packard) utilizing external standards for calibration. Calibrations were checked daily to confirm linearity in the 2-10 ppm range. Working standards and stock solutions were stored in amber glass bottles at 4°C.

Prior to each series of NA analysis, a calibration curve was prepared by running four standards (2, 4, 8, and 10 ppm). Four fresh standards were taken from stock solutions for each calibration prior to GC analysis. Standards and stock solution were stored in amber glass bottles at 4°C. New standards were prepared for each experiment.

Standard methods were used to prepare a calibration curve by plotting area response against concentration of the standards. A calibration factor was calculated, as the ratio of concentration to area response was constant over a wide range of concentrations. The concentration of unknown water samples was determined by comparing the GC response to the standards within the linear range of the calibration curve. Typical error associated with the measurements was approximately ±0.1%.

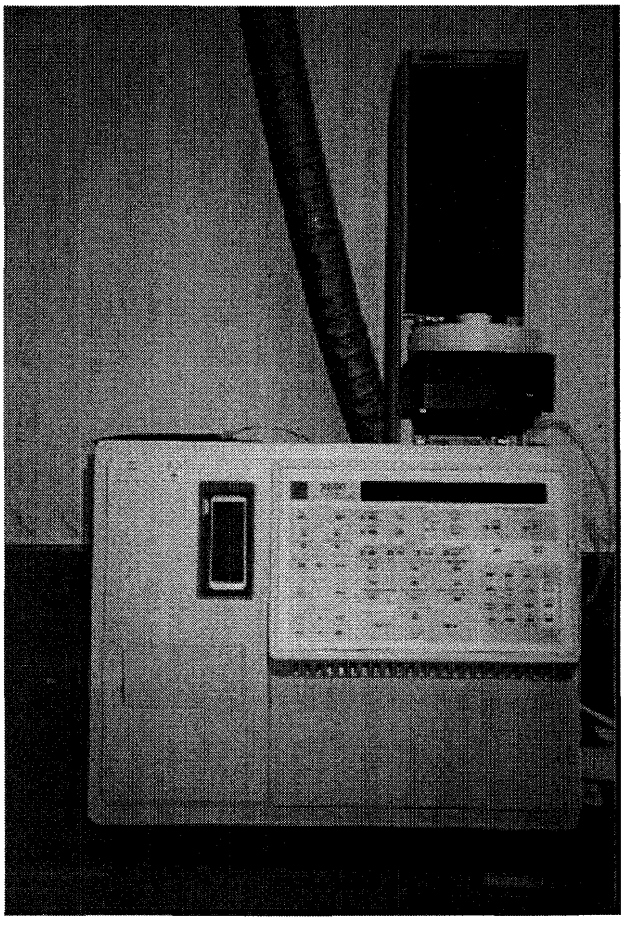


Figure 3.6 GC-FID equipment used for NAs quantification.

3.5.4 Extraction

A 1 ml water sample from the bioreactors was adjusted to pH 2-3 using HCl. DCM was used to extract NAs from the water sample. A volume of 2 ml of DCM was added to the acidified sample, the vial capped and then vortexed. The lower layer of DCM containing NAs was transferred to a micro reaction vessel, two further serial extractions were performed and the extracts combined to ensure all of the NAs in the water sample were transferred. This combined extract was then evaporated using a gentle stream of nitrogen gas to a volume of 2 ml.

The NAs were transferred from the combined extract to hexane by extracting three times with 2 ml of hexane and then concentrating by evaporation to a volume of 1 ml. This step was conducted to prepare the NAs sample for GC analysis and ensure all of NAs sample was transferred. For GC analysis, methyl esters of the NAs were formed using derivatization with BF_3-Methanol. In brief, 1.5 ml of BF_3-methanol solution was added to the sample extract, and the headspace purged immediately with nitrogen gas. The reaction was accelerated by heating this liquid mixture at 70°C for 2 hours, using a water bath (Grant Instruments Ltd., Cambridge, England).

Once the reaction vial was cool to touch, 1 ml of hexane and 1 ml of distilled water were added to the solution. This liquid mixture was vortexed vigorously and the upper solvent layer, containing the derivatized NAs was carefully transferred to a volumetric tube. Two serial extractions of the hexane addition were performed, the extracts combined and adjusted to a final extraction volume of 2 ml. An aliquot of 0.5 ml was transferred to a micro vial for subsequent GC analysis. To avoid possible degradation of samples from the vials, all samples including controls and blanks were analyzed as soon as possible (generally within 24 hours) by GC-FID.

4. RESULTS AND DISCUSSIONS

The microbial action in the four bioreactor experiments resulted in first-order kinetic decomposition of the NAs in Athabasca river water. Results of the experiments are presented and discussed in this chapter. The effects of pH, temperature, organic carbon content, and imposed NAs content were quantified using a first-order biodegradation rate constant (K). Calculations of half-life values are also discussed as well as the effects of geometric isomerism upon biodegradation.

4.1 Microbial Analysis Results

The results of this study show that micro-organisms indigenous to raw Athabasca river waters are capable of degrading NAs components within a commercial mixture of NAs.

Duplicate plate counts of water samples taken from filtered or autoclaved control reactors in each experiment showed no living microbial population in all control flasks. This evidence is clearly displayed from the comparison of amended NAs in each water quality condition and each control sample.

In response to the addition of the substrate, heterotrophic population increased exponentially and then declined to a relatively static level over the duration of the experiment. The same microbial community in the blank samples did not mirror the exponential growth response. Therefore, it is concluded that the heterotrophic populations in the river water were able to utilize the NAs as a carbon source. This ability is clearly evident from the difference in microbial growth observed for river water amended with NAs compared to the blank samples. A plot of the

microbial growth in blanks (raw river water) and NA amended river water supports this statement (see Figure 4.1).

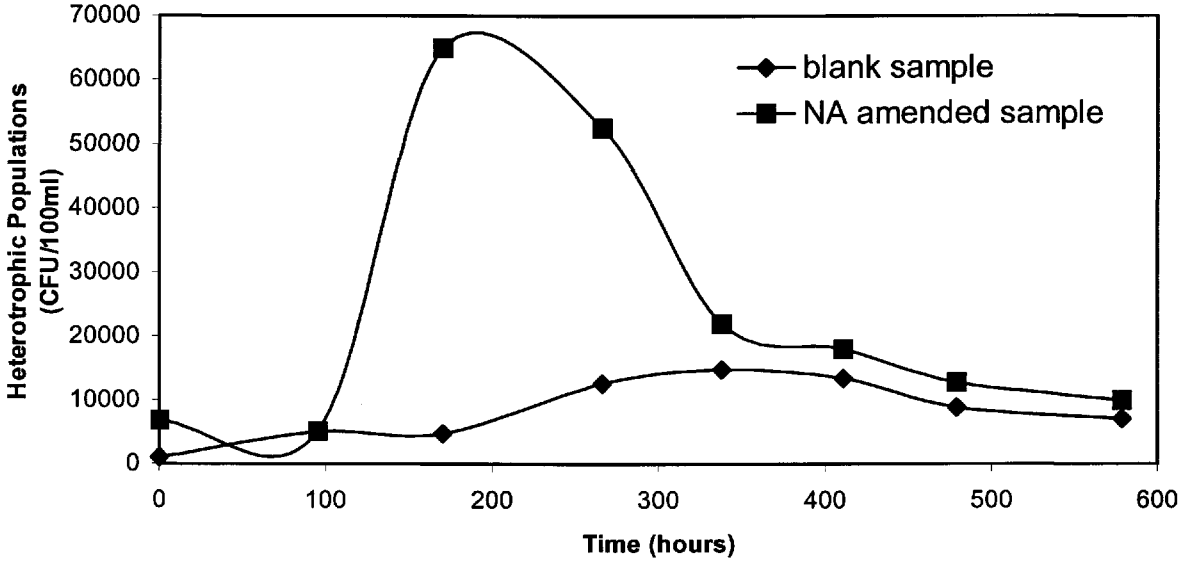


Figure 4.1 Comparison of growth of heterotrophic bacteria in blanks versus NAs amended raw river water in the first experiment.

Table 4.1 showed the results of microbial enumeration in blank and NAs amended raw river water during the first to third experiments.

Table 4.1 Results of microbial enumeration in blank and NAs amended raw river water during the first to third experiments.

Time (hours)	Blank raw river water	NAs amended raw river water
1st experiment	Heterotrophs (CFU/100ml)	Heterotrophs (CFU/100ml)
0	9.75×10^2	6.70×10^2
95.3	5.05×10^2	5.15×10^2
170.3	4.75×10^2	65.00×10^3
266	12.55×10^2	52.50×10^3
337.9	14.75×10^2	22.00×10^3
411.2	13.40×10^2	18.00×10^3
479.2	8.85×10^2	12.80×10^3
578.5	7.00×10^2	10.00×10^3

Continued Table 4.1

Time (hours)	Blank raw river water	NAs amended raw river water
2nd experiment	Heterotrophs (CFU/100ml)	Heterotrophs (CFU/100ml)
0	9.40×10^2	2.00×10^2
71	1.42×10^2	4.68×10^2
167	1.67×10^2	2.14×10^3
240	1.73×10^2	3.34×10^3
407.3	1.25×10^2	3.52×10^3
502.3	5.55×10^2	3.73×10^3
575.3	1.95×10^2	3.15×10^3
671.3	1.94×10^2	3.10×10^3
3rd experiment		
0	2.00×10^2	1.26×10^2
94.3	4.68×10^2	5.02×10^2
142.3	2.13×10^2	2.86×10^3
261.6	3.34×10^2	3.69×10^3
309.6	3.33×10^2	3.60×10^3
429.6	3.30×10^2	3.26×10^3
477.9	3.72×10^2	2.84×10^3
597.2	3.10×10^2	2.83×10^3
645.2	3.10×10^2	2.69×10^3

4.2 Biodegradation of Naphthenic Acids Results

4.2.1 GC-FID Analysis

Herman *et al.* (1993) reported that a mixture of *cis*- and *trans*- isomer of NA (2-methylcyclohexanecarboxylic acids) appeared as two adjacent peaks in gas chromatographic analysis. In the GC-FID analysis in this study, the isomers of the three NA compounds also appeared as two adjacent peaks. The *cis*- and *trans*- isomers of the NAs were well resolved from each other as shown in Figure 4.2. The *trans*- isomers eluted before the *cis*- isomers for 4MACH, whereas the order was reversed for 4MCCH and 3MCCH.

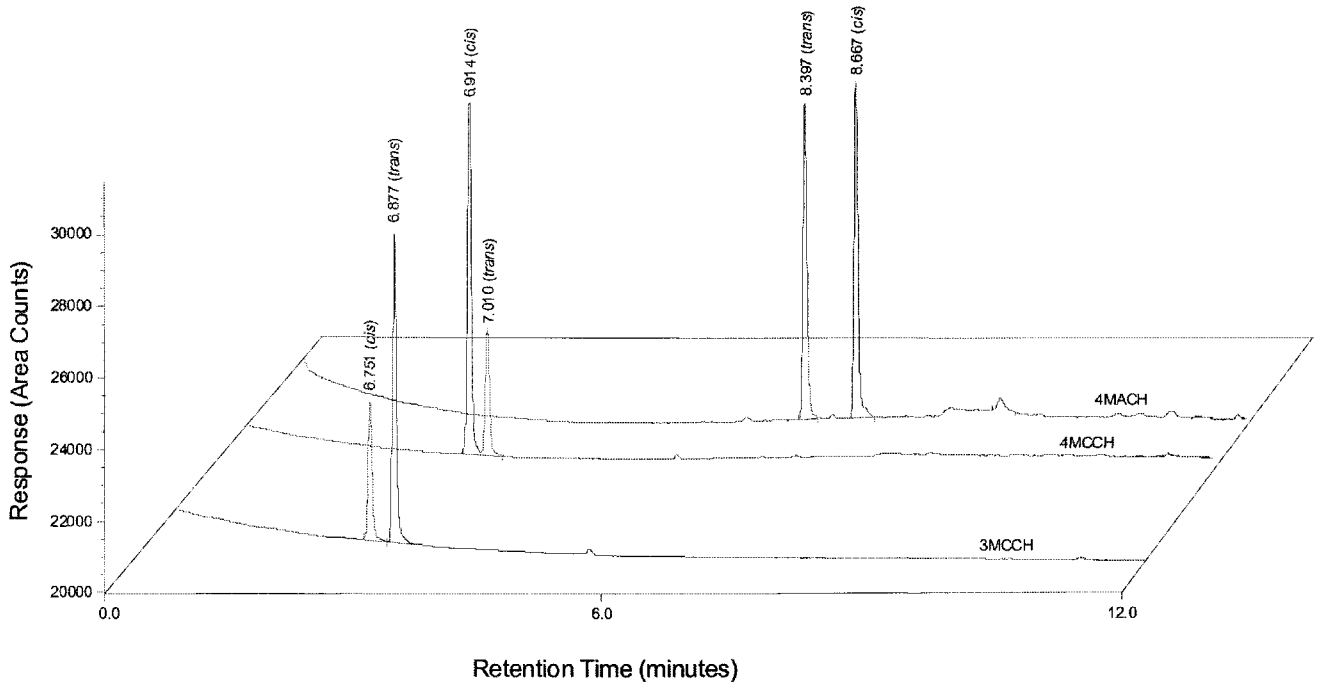


Figure 4.2 Representative GC/FID chromatograms of the three NAs investigated.

The results obtained from the GC-FID analysis were used to identify the presence of NAs in each experiment and to determine their concentrations. These results were obtained by comparing measurement results to standards within the linear range of a calibration curve. Standard methods were used to prepare a calibration curve by plotting area response against concentration of analytes in the standards. Calibrations were checked daily to confirm linearity.

4.2.2 Controls and Blanks

All of the control and blank samples were analyzed by GC-FID simultaneously with the bioreactors. Analysis of blank samples in each experiment indicated the absence of NA concentration. Therefore, no cross-contamination of NAs occurred during the sampling procedures or through volatilization of the substrate.

NAs are generally non-volatile (Morales *et al.,* 1993). Therefore, volatilization from a water environment is not expected to play an important role in removal of NAs. As such losses of NAs from either volatilization or sorption to the glass bioreactor surfaces were not considered to be a major factor in the reduction of NAs in any of the reactors. Control samples confirmed this hypothesis.

An example chromatograph for 4MACH control samples at the beginning (t=0 hour) and at the end (t=502.7 hours) in the first experiment is shown in Figure 4.3. The peaks indicate the presence of 4MACH in the water samples. *Trans*- 4MACH in the first experiment eluted from the control samples at the beginning and end of the experiment at approximately 8.558 min and 8.542 min respectively, while *cis*-4MACH eluted from the controls at approximately 8.830 min and 8.814 min. The area under each peak was integrated and compared to prepared calibration curves to calculate the concentration of 4MACH. The concentration of each 4MACH isomer is shown above the peaks in Figure 4.3. From these chromatographs and similar plots for the other experiments, it is concluded that there was negligible loss of mass in the control samples.

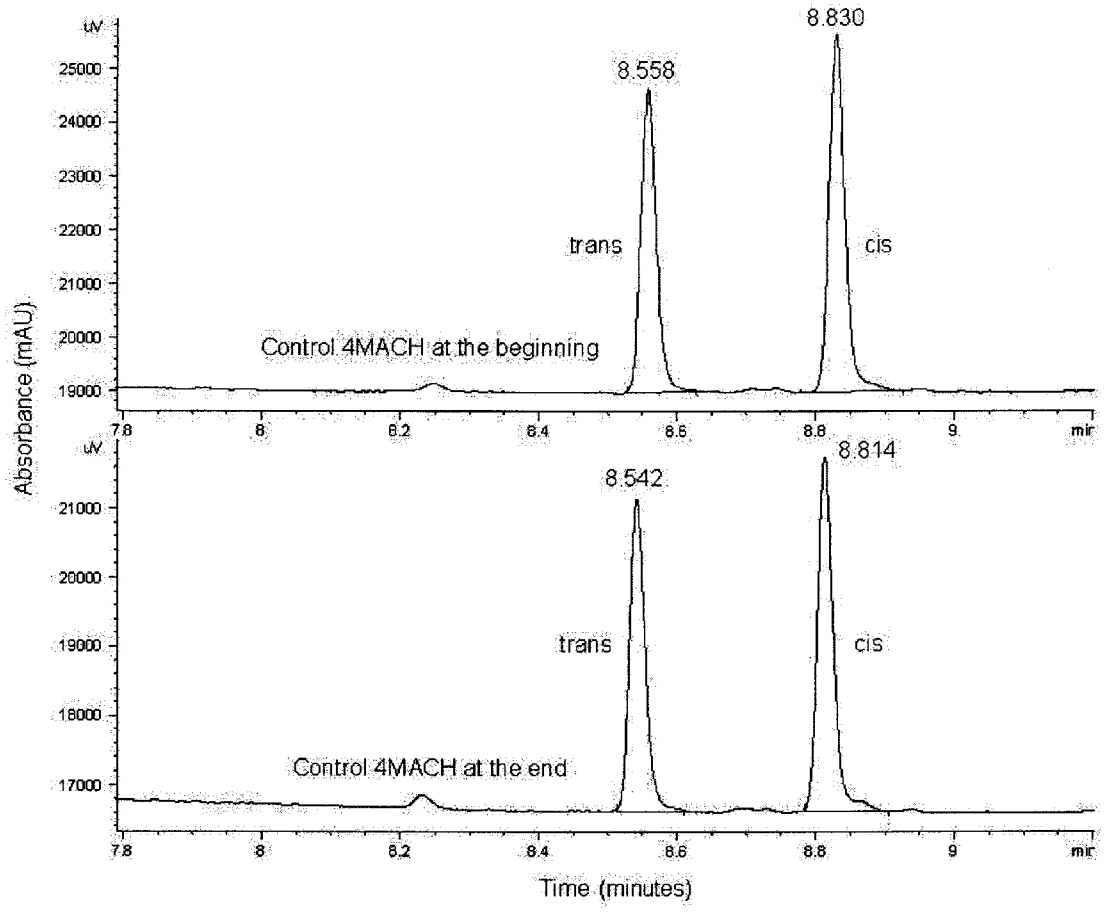


Figure 4.3 Chromatograph of control 4MACH peaks at the beginning and the end of the first experiment.

4.2.3 Kinetic Constant Calculations

The concentration of NAs was measured daily in the first experiment and three times per week in the last three experiments. The NAs data collected for each experiment is presented in Appendix A. The natural log of NAs concentration relative to the initial NAs concentration in each bioreactor was calculated and plotted versus time.

Plots of relative NAs concentration versus time on semi log axes confirmed that the biodegradation which occurred during the experiments followed first-order kinetics. This is demonstrated by the example linear plots illustrated in Figure 4.4 and the corresponding R^2. For example, the first-order rate constants (K) from the biodegradation of *trans*- 4MACH (0.0025 hour^{-1}) and *trans*- 4MCCH (0.0028 hour^{-1}) were calculated by using linear regression (Figure 4.4).

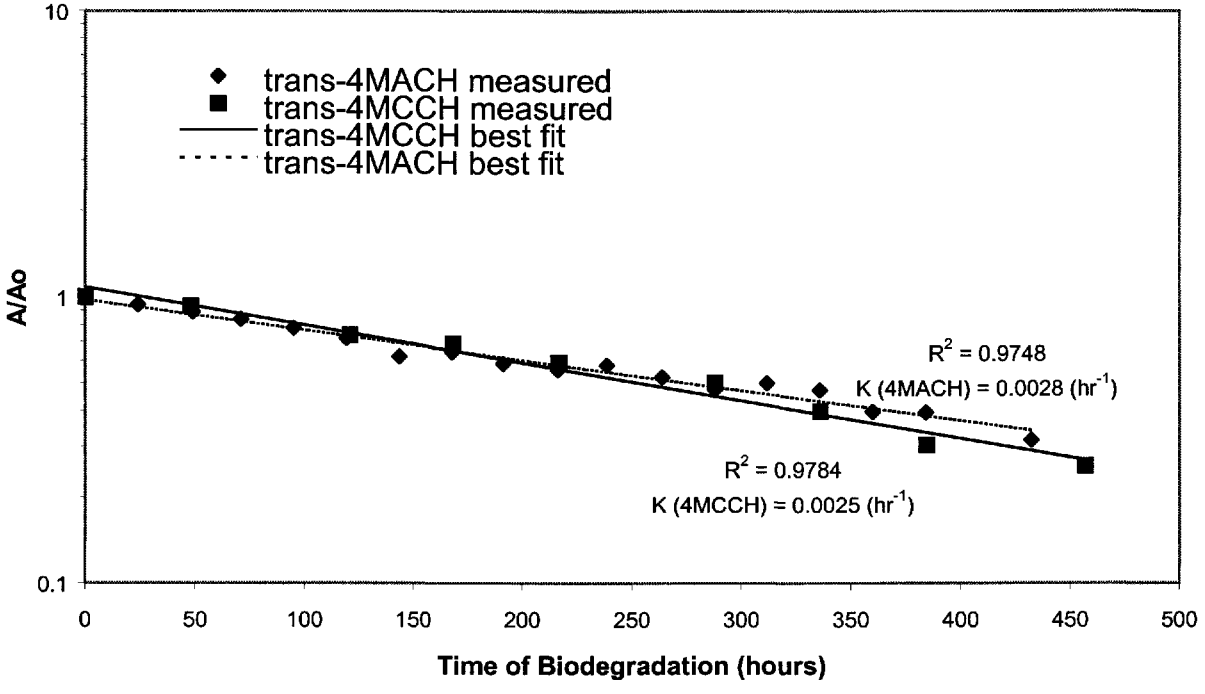


Figure 4.4 Biodegradation kinetics of 9mg/l of *trans*- 4MCCH and *trans*- 4MACH at room temperature (20°C) from non-amended Athabasca River water (pH 8.7).

The values of the first-order rate constant from each biodegradation test water for each quality condition and the corresponding coefficient of determination (R^2) are given in Table 4.2. The first order kinetic model provided an excellent fit to the experimental results for the *trans*- isomers (demonstrated by R^2 values near unity), while results from the *cis*- isomers were rather uncertain (R^2 values significantly less than unity).

Table 4.2 Rate constant (K) and coefficients of determination of predominant geometric isomers of NAs biodegradation in Athabasca River water.

Sample Description	Average K ($hour^{-1}$) and R^2 values at each experiment			
	trans- isomers		*cis*- isomers	
	K ($hour^{-1}$)	R^2	K ($hour^{-1}$)	R^2
Experiment 1 4MACH (9mg/l)				
Non-amended level				
Raw River Water*	0.0025	0.97	0.0007	0.40
Amended level				
5mg/l HA added*	0.0027	0.96	0.0005	0.61
pH to 7	0.0025	0.97	0.0004	0.51
pH to7;5mg/l HA*	0.0030	0.97	0.0008	0.63
Experiment 2 4MACH (7.5mg/l)				
pH 3.5	N/A	N/A	N/A	N/A
Temp. at 30°C	0.0079	0.96	0.0018	0.98
Temp. at 10°C	0.0006	0.97	0.0002	0.32

Table 4.2 continued

	trans- isomers		*cis*- isomers	
	K ($Hour^{-1}$)	R^2	K ($hour^{-1}$)	R^2
Experiment 3 4MCCH (9mg/l)				
Non-amended level				
Raw River Water*	0.0028	0.96	0.0006	0.71
Amended level				
pH to 6*	0.0014	0.94	0.0006	0.84
Temp. at 30°C	0.0060	0.97	0.0012	0.94
Temp. at 10°C	0.0008	0.97	0.0005	0.76
Experiment 4 3MCCH (12mg/l)				
Temp. at 30°C	0.0021	0.99	0.0016	0.92

*Athabasca river water at room temperature (20±2°C)

The average half-life values were calculated using the K values presented in Table 4.2. The half-life calculation procedure was described in section 3.4. A comparison of calculated half-life values and half-life ratios for the geometric isomers of 4MACH, 4MCCH and 3MCCH are presented in Table 4.3.

Table 4.3 Calculated half-lives and half-life ratios of geometric isomers of 4MACH, 4MCCH and 3MCCH biodegradation in Athabasca River Water.

Sample Description	Average $t_{1/2}$ (days) and *trans*-$t_{1/2}$/*cis*-$t_{1/2}$ values at each experiment.		
	trans- isomers $t_{1/2}$ (days)	*cis*- isomers $t_{1/2}$ (days)	*trans*- $t_{1/2}$ / *cis*- $t_{1/2}$
Experiment 1 4MACH (9mg/l)			
Non-amended level			
Raw River Water*	11.6±0.3	41.3±0.1	0.28
Amended level			
5mg/l HA added*	10.7±0.4	57.8±0.1	0.19
PH to 7	11.6±0.4	72.2±0.1	0.16
pH to7;5mg/l HA*	9.6±0.4	36.1±0.1	0.27
Experiment 2 4MACH (7.5mg/l)			
pH 3	N/A	N/A	N/A
Temp. at 30°C	3.7±0.9	16.0±0.3	0.23
Temp. at 10°C	48.1±0.0	144.38±0.0	0.33

Table 4.3 continued

	trans- isomers $t_{1/2}$ (days)	*cis*- isomers $t_{1/2}$ (days)	*trans*- $t_{1/2}$ / *cis*- $t_{1/2}$
Experiment 3 4MCCH (9mg/l)			
Non-amended level			
Raw River Water*	10.1±0.5	48.1±0.1	0.21
Amended level			
pH to 6*	20.6±0.2	48.1±0.1	0.42
Temp. at 30°C	4.8±0.9	24.1±0.2	0.20
Temp. at 10°C	41.3±0.1	57.8±0.1	0.71
Experiment 4 3MCCH (12mg/l)			
Temp. at 30°C	13.8±0.26	18.1±0.2	0.70
			Avg. = 0.34

*Athabasca River Water at room temperature (20±2°C)

4.3 Effects of Geometric Isomerism

The average half-life values listed in Table 4.3 show that the *trans*- isomers were biodegraded more quickly than *cis*- isomers in all cases. Therefore, the *cis*- isomers appear to be more persistent than *trans*- isomers. In general, there is approximately a 1 to 3 ratio of *trans*- isomers over *cis*- isomers half-lives for each water quality condition. This value is obtained from the average ratio (*trans*- $t_{1/2}$/ *cis*- $t_{1/2}$) in Table 4.3.

In the first experiment, *cis-* isomers had a large range of half-life values (see Table 4.3). For example, the largest half-life value of *cis-* 4MACH amended river water sample at pH 7 in the first experiment was 72.2±0.1 days, while the lower half-life for *cis-* 4MACH at pH 7 plus 5mg/l of HA was 36.1±0.1 days. In contrast the half-life values of the *trans-* isomers of 4MACH were much more consistent. For example, the average half-lives of *trans-* 4MACH in non-amended river water samples at room temperature (20±2°C) and in amended to a pH level of 7 in the first experiment were 11.6±0.3 and 11.6±0.4 days, respectively.

Based on the experimental data, the *trans-* isomer biodegraded faster than the *cis-* isomer. An example is shown in Figure 4.5 where the measured K values for *trans-* and *cis-* 4MACH in non-amended Athabasca river water (pH 8.7, temperature 20±2°C) were 0.0025 and 0.0007 $hour^{-1}$ respectively. The corresponding half-lives were 11.6±0.3 days and 41.3±0.1 days, respectively.

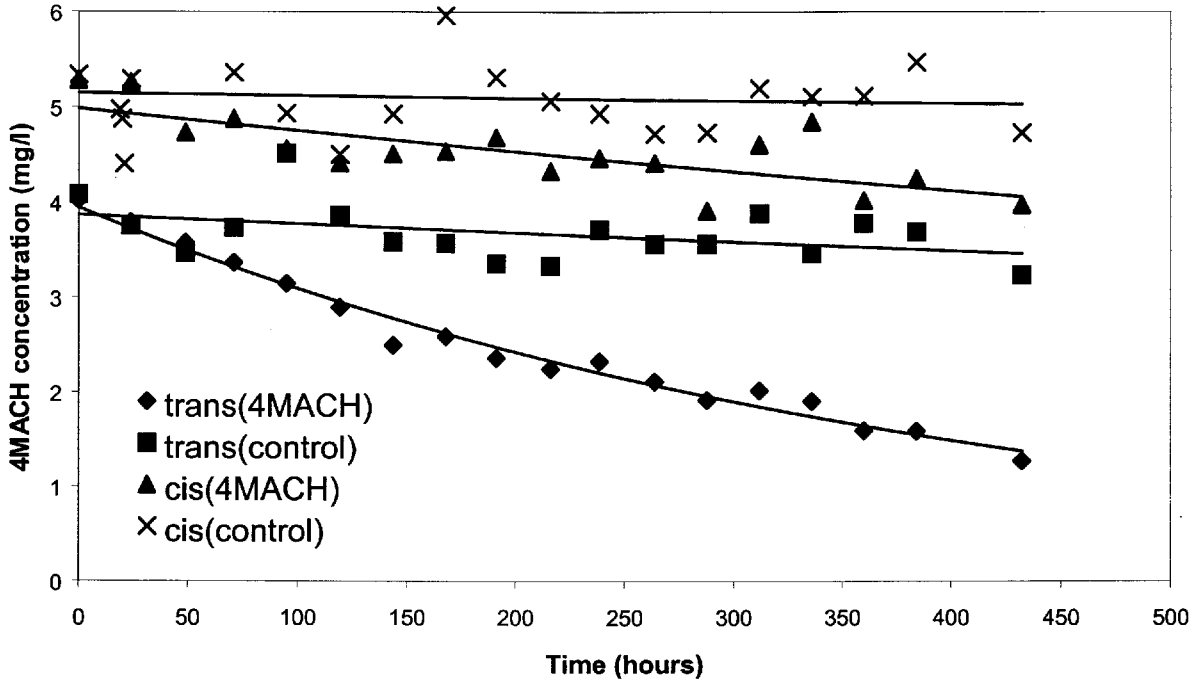


Figure 4.5 Biodegradation of 9mg/l of *cis*- and *trans*- isomers of 4MACH for non-amended Athabasca River Water (pH 8.9) (20±2°C).

As summarized in Table 4.3, there is a general trend for half-lives. The *trans*-isomers appear to degrade more rapidly than the corresponding *cis*- isomers. The *trans*- isomers are thus more bioavailable in natural waters than the *cis*- geometric isomers. The difference in bioavailability is rationalized by considering the intramolecular hydrogen bonding which can occur for only the *cis*- isomer (see Figure 4.6). It is proposed that the closed structures of the *cis*- isomers are more stable to microbial degradation than the more open geometry of the *trans*-isomers. Moreover, the *cis*- isomer requires additional energy to break the intramolecular hydrogen bonding, while *trans*- isomers require less energy. This proposed reason supports a slow degradation of *cis*- isomer in comparison to the *trans*- isomer.

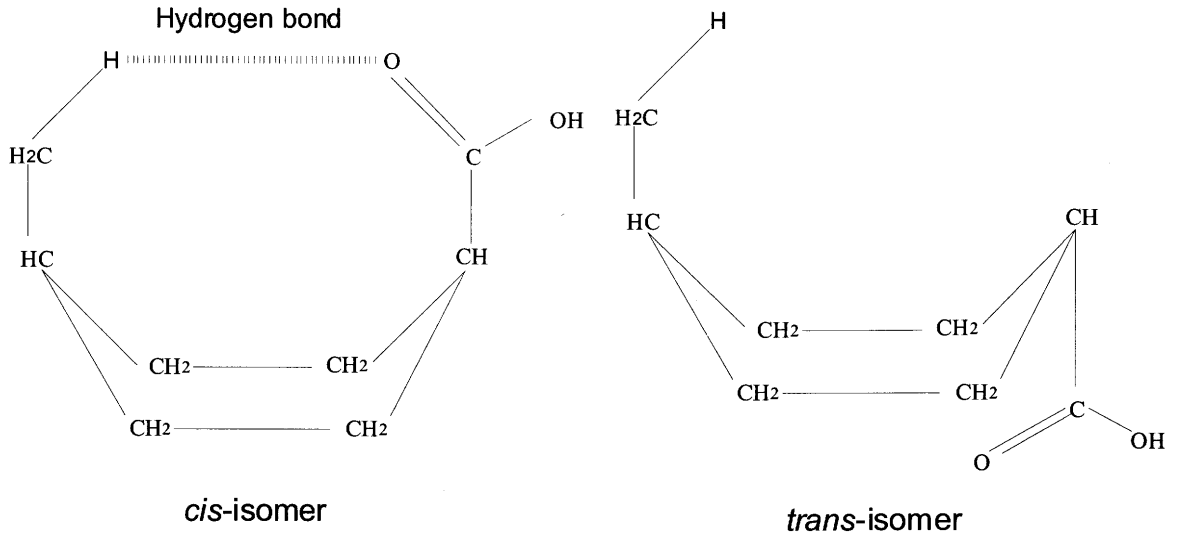


Figure 4.6 Intramolecular hydrogen bonding occurring in *cis*- isomers.

According to Table 4.2, there is limited scatter of results for the *trans*- isomer, while *cis*- isomers have greater scatter and variability of results for different NAs and amendment conditions. As well, this trend is evident in the control data (see Figure 4.5). This scatter in the *cis*- data likely influences the R^2 results since the magnitude of the scatter in the controls is similar to the magnitude of the change in concentrations at the end of the experiment.

4.4 The Effects of NAs Concentration and Species

Various NAs species and concentrations were used in the experiments as prescribed in Table 3.1. This allowed the relative effect of NAs species and concentration upon biodegradability to be investigated.

Structural differences pertaining to whether the carboxylic acid was directly attached to the cyclohexane ring, or attached via a $-CH_2$ group, do not appear to have a significant effect on the measured rate constants in the *trans*- isomer. For

example, consider the biodegradation of *trans*- 4MCCH (9mg/l) and *trans*- 4MACH (9mg/l) for non-amended raw river water, shown in Figure 4.4. The two NAs in question have the same substituted hexane ring, and differ only by the presence or absence of a $-CH_2$ entity attached to the terminal carboxylic acid. Under the experimental conditions, this structural difference of *trans*- isomers appears to have only a minor effect on the rate of biodegradation. The measured K values for *trans*- 4MACH and *trans*- 4MCCH at room temperature (20±2°C) were 0.0025 and 0.0028 $hour^{-1}$ respectively, while the average half-lives were 11.6±0.3 days and 10.1±0.5 days (Figure 4.7). In contrast there is a significant difference in the rate of biodegradation of *cis*- isomers. The measured K values for *cis*- 4MACH and *cis*- 4MCCH at room temperature (20±2°C) were 0.0007 and 0.0005 $hour^{-1}$ respectively, while the average half-lives were 41.3±0.1 days and 48.1±0.1 days (Figure 4.7).

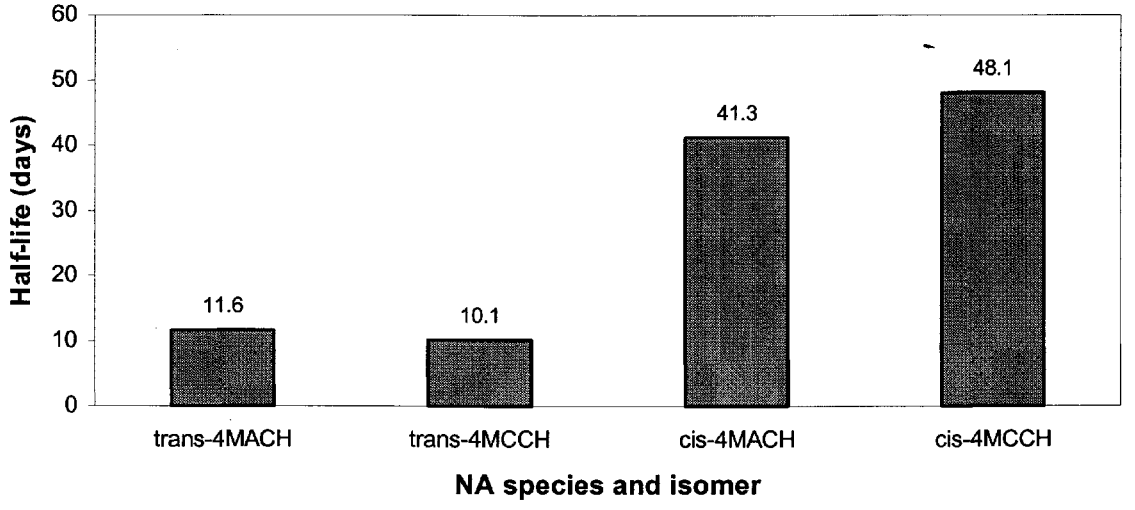


Figure 4.7 Comparison of *trans*- 4MACH, *trans*- 4MCCH, *cis*- 4MACH, and *cis*- 4MCCH half-life values under non-amended raw river water in the first and third experiment, respectively.

There is a significant difference in the rate of degradation of the 4MCCH and 3MCCH structural isomers (Figure 2.5). Under similar experimental conditions

(non-amended, temperature 30±1°C), the more open structure of the *trans*-4MCCH isomer permitted it to degrade more rapidly than the *trans*- 3MCCH isomer, with half-lives of 4.8±0.9 and 13.8±0.3 days respectively. Interestingly, this order is reversed for the corresponding *cis*- isomers, where the measured half-lives for *cis*- 4MCCH and *cis*- 3MCCH were 24.1±0.2 days and 18.1±0.2 days respectively (Figure 4.8). This reversal in the half-lives relative to the corresponding *trans*- isomers may reflect in part the closed stable conformations of the *cis*- isomers, arising from intramolecular hydrogen bonding.

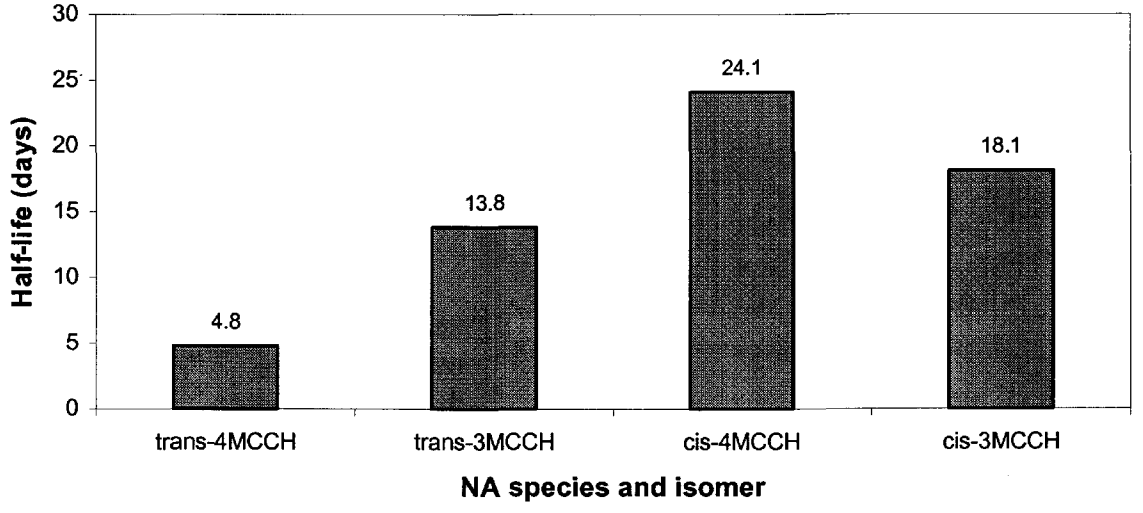


Figure 4.8 Comparison of *trans*- 4MCCH, *trans*- 3MCCH, *cis*- 4MCCH, and *cis*-3MCCH half-life values under non-amended temperature 30°C in the third and fourth experiment, respectively.

4.5 Water Quality Effects

4.5.1 Humic Acid Amendments

The aim of adding HA from the base condition is to provide an alternate carbon and energy source for the bacteria. The effect of humic acid (HA) amendment

was monitored in the first experiment only. The original dissolved organic carbon concentration was enhanced by adding 5 mg/l of HA.

The rate of biodegradation of *trans-* 4MACH in the amended sample (10.7±0.4 days) did not significantly differ from the non-amended sample (11.6±0.3 days) as shown in Figure 4.9. On the other hand, a significant effect on the rate of biodegradation appeared in the *cis-* isomers. The measured half-life of *cis-* 4MACH in the non-amended sample was 41.3±0.1 days, whereas, the half-life of *cis-* 4MACH in the amended sample was 57.7±0.1 days. The effect of the *cis-* isomer on the rate of biodegradation may be influenced due to the greater scatter and variability of results. Therefore the *trans-* isomer result in HA amended sample is more reliable.

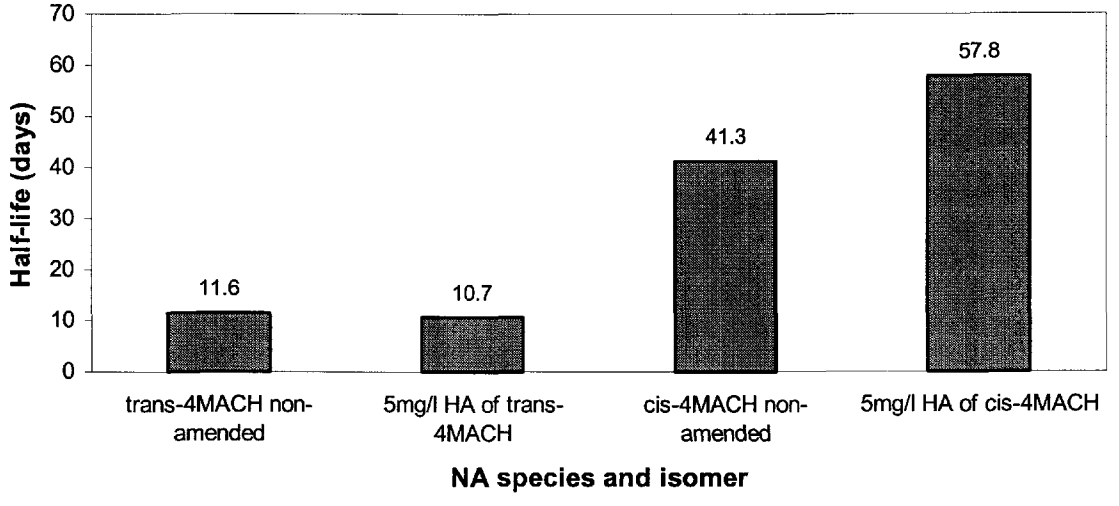


Figure 4.9 Comparison of *trans-* and *cis-* 4MACH biodegradation in the non-amendment and HA amendment in the first experiment.

Lai *et al.* (1996) expected to find an increasing rate of biodegradation of NAs for DOC in the range of 3-10 mg/l. However, Lai et al. (1996) found no significant effect of DOC on observed rate of NA biodegradation. The experimental results of this investigation support the obervation of Lai *et al.* (1996).

4.5.2 pH

The pH levels were measured at the start and end of each experiment. pH in the bioreactor experiments did not change by more than ±0.5. Changes of less than ±0.1 were observed in the river water control samples. From this, it can be concluded that there was no significant change in pH due to physical process or chemical reactions in the bioreactors.

Table 4.4 shows the results of pH analyses conducted for each experiment. The pH was monitored closely throughout each experiment.

Table 4.4 Results of pH analyses in the first four experiments at various pH levels.

Sample Description	Start	End
9mg/l 4MACH (Experiment 1)		
Non-amended	8.7	8.5
5mg/l HA added	8.7	8.3
pH to 7	7.0	6.5
pH to 7; 5mg/l HA	7.1	6.8
7.5mg/l 4MCCH (Experiment 2)		
pH to 3.5	3.5	3.3
Temp. at 10°C	8.6	8.7
Temp. at 30°C	8.7	8.4
9mg/l 4MCCH (Experiment 3)		
non-amended	8.7	8.3
pH to 6	6.0	5.9
Temp. at 30°C	8.8	8.7
Temp. at 10°C	8.7	8.5
12mg/l 3MCCH (Experiment 4)		
Temp. at 30°C	8.8	8.5

Under all conditions, there were slight decreases of pH in the bioreactor water samples. The release of carbon dioxide during NAs mineralization may have caused the pH to decline slightly as the experiments progressed (Oh and Tuovien, 1994; Greer *et al.,* 1990; McMartin, 2000).

In the first experiment at neutral pH (pH 7) did not appear to have a significant effect on the rate of biodegradation of *trans*- 4MACH (11.6±0.4 days) in comparison to the *trans*- 4MACH non-amended sample (11.6±0.3 days). On the other hand, in the third experiment at pH 6 *trans*- 4MCCH (20.6±0.2 days) appeared to have a significant effect on the rate of NAs biodegradation in comparison to the *trans*- 4MCCH non-amended sample (10.1±0.5 days). It is concluded that *trans*- 4MCCH at pH 6 may be outside the preferred or optimum range (pH 7 to pH 8.7) and probably inhibits the growth of microbial populations. This comparison is shown in Figure 4.10.

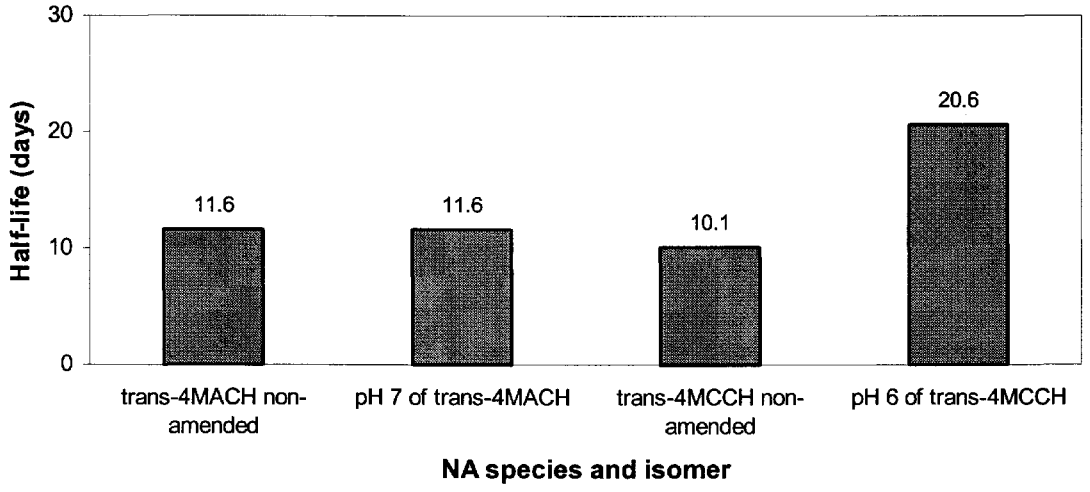


Figure 4.10 Comparison of *trans*- 4MACH (pH 7) and *trans*- 4MCCH (pH 6) biodegradation results under non-amended (raw river water) and pH amendments.

The effect of pH on the rate of NA biodegradation was reversed for the corresponding *cis-* isomers (Figure 4.11), where the measured half-life for *cis-* 4MCCH at pH 6 and *cis-* 4MCCH non-amended samples were 48.1±0.1 and 48.1±0.1 days, respectively. The measured half-life values of *cis-* 4MACH at pH 7 and *cis-* 4MACH non-amended samples were 72.2±0.1 days and 41.3±0.5 days, respectively.

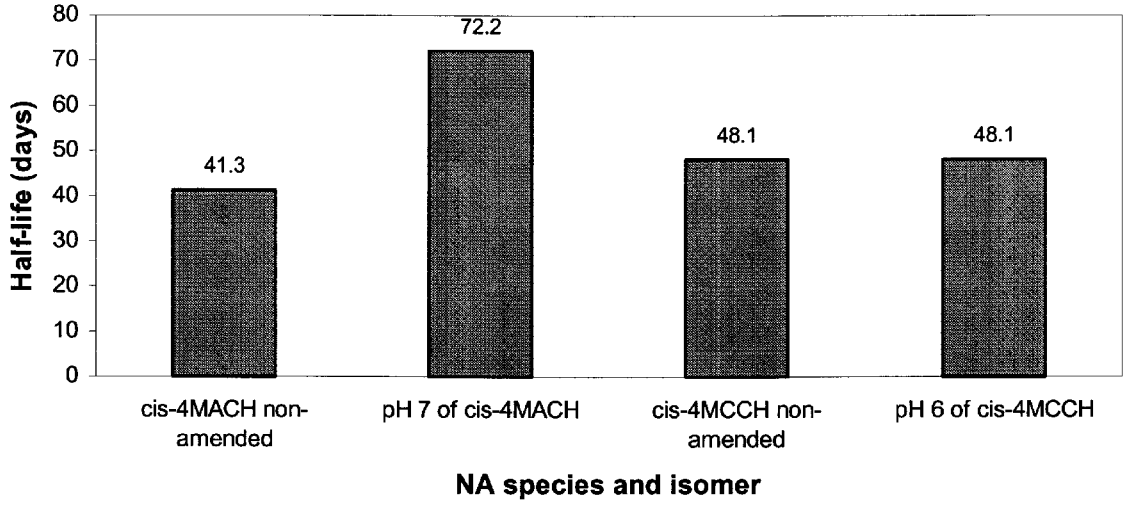


Figure 4.11 Comparison of *cis-* 4MACH (pH 7) and *cis-* 4MCCH (pH 6) biodegradation results under non-amended (raw river water) and pH amendments.

Lai *et al.* (1996) stated that there was no significant effect of pH on biodegradation rate of NAs in the range of pH 7.3-8.5. This statement is supported the results for the *trans-* isomers in this investigation.

In the second experiment the biodegradation rate of 4MACH was negligible. For this particular experiment, the microbial populations were reduced to zero as they were not able to acclimatize to an extreme pH value. The pH was maintained at approximately 3.5 throughout the experiment as shown in Table 4.3. Microbial

analysis proved that no microbial communities were living in the highly acidic bioreactors.

4.5.3 Humic Acid and pH Amendments in Combination

NAs biodegradation in reactors where pH and DOC were both adjusted was investigated in the first experiment. The combination of HA amendment plus pH 7 seemed to slightly increase the rate of *trans*- 4MACH (9.6±0.4 days) biodegradation in comparison to the non-amended *trans*- 4MACH (11.6±0.3 days) as shown in Figure 4.12. The same combination slightly increased the rate of biodegradation of *cis*- isomer in comparison to the non-amended *cis*- 4MACH. The measured half-life of *cis*- 4MACH in the non-amended sample was 41.3±0.1 days, while the half-life of *cis*- 4MACH in the combination of pH amendment with 5 mg/l of HA was 36.1±0.1 days.

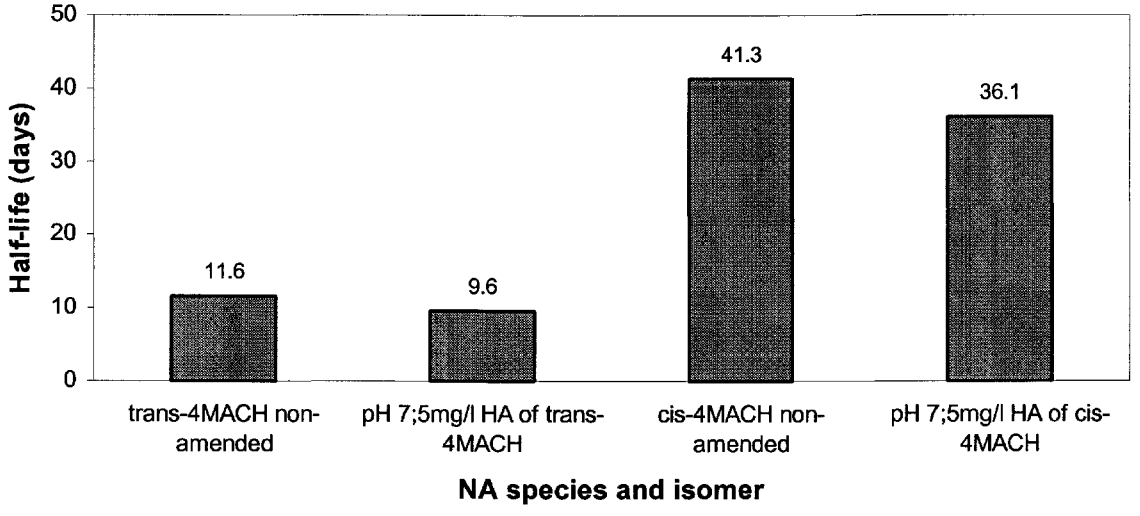


Figure 4.12 Comparison of *trans*- and *cis*- 4MACH biodegradation in the combination of pH amendment with 5 mg/l of HA and non-amended levels in the first experiment.

Under the experimental conditions (Table 4.3), the combination of the amendments of pH and DOC levels at the initial *trans*- 4MACH concentration of 9 mg/l (9.6±0.4 days) appeared to slightly accelerate the biodegradation process in comparison to the DOC amendment alone (10.7±0.4 days), while the combination amendment for *cis*- 4MACH (36.1±0.1 days) significantly accelerated the biodegradation process in comparison to the DOC amendment alone (57.8±0.1 days).

4.5.4 Temperature

Lai *et al.* (1996) stated that low temperature (5°C) greatly reduces the biodegradation rate, while the rate increases at a higher temperature level (10°C). This means that decreasing temperature slows the rate of biodegradation, while increasing temperature accelerates the rate of biodegradation.

The effect of temperature was investigated in the last three experiments as prescribed in Table 3.1. The rate of biodegradation observed at 10°C was significantly slower than those observed at room temperature (20°C) and at 30°C (see Table 4.1). A temperature of 10°C was found to decrease the rate of NAs biodegradation in all cases. In contrast, 30°C appeared to dramatically accelerate the biodegradative process.

The effect of temperature on the half-life of the NAs is shown Figure 4.13. This effect likely reflects the corresponding changes in microbial activity and is particularly evident for the temperature change from 10 to 20°C compared to the corresponding change from 20 to 30°C.

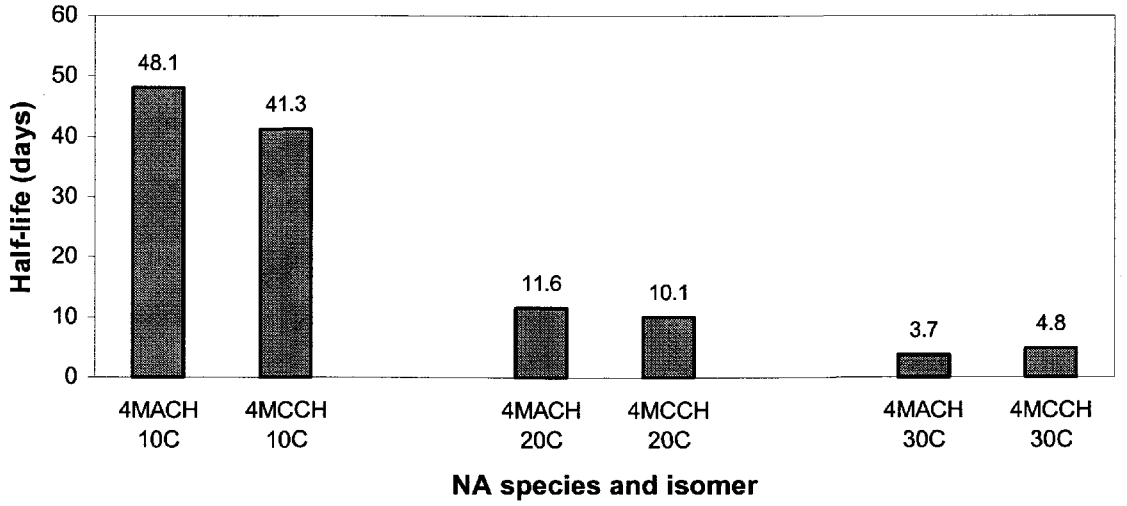


Figure 4.13 Comparison of the half-lives of *trans*- 4MACH and *trans*- 4MCCH results for non-amended river water at 10, 20 and 30°C.

For non-amended river water, there was greater than a ten-fold increase in the value of half-life (from 3.7 to 48.1 days) for the biodegradation of *trans*- 4MACH for an increase in temperature from 10°C and 30°C. This trend was observed for all NAs investigated, with values of half-life at 30°C falling in the range of 3.7 to 4.8 days, compared to the larger values of 41.3 and 48.1 days measured at 10°C.

Figure 4.14 shows a comparison of representative *cis*- isomers biodegradation results in non-amended river water for various temperature conditions.

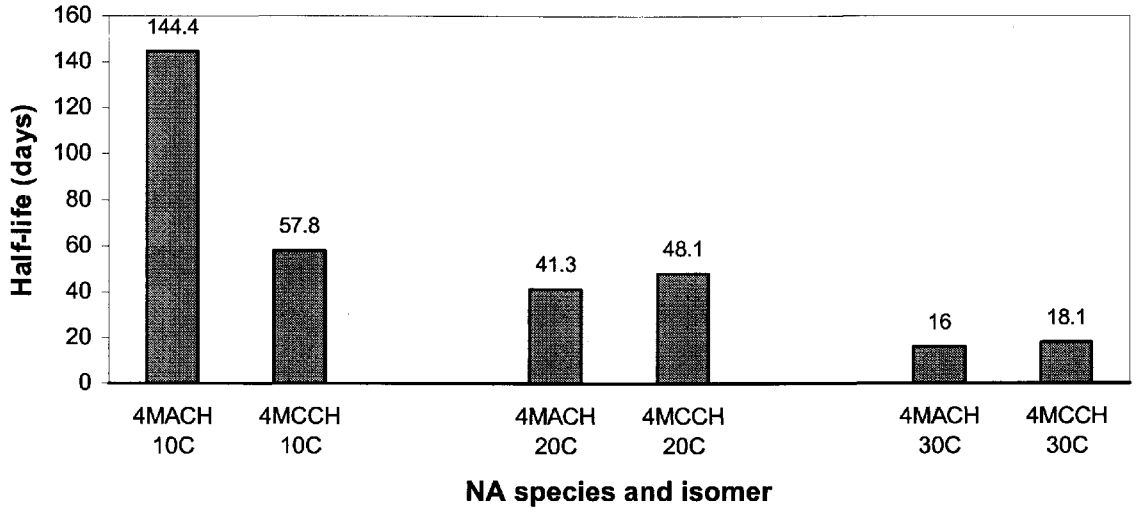


Figure 4.14 Comparison of half-lives of *cis*- 4MACH and *cis*- 4MCCH for non-amended (raw river water) at 10, 20 and 30°C.

For non-amended water, there was approximately a nine-fold decrease in the value of half-life (from 144.4 to16.0 days) for the biodegradation of *cis*- 4MACH for corresponding increase in temperature from 10°C and 30°C. Values of half-life at 30°C fell in the range 16.0 and 18.1 days, compared to the corresponding lower values of 57.8 and 144.4 days measured at 10°C.

As expected, NAs will therefore be more persistent at lower temperatures (Lai *et al.*, 1996) and this in turn, may have a profound significance on the prevalence in the northern oil sands environment.

4.5.4.1 Effect of Temperature on Reaction Rate Constant

Increasing temperature increases the chemical reaction rate and the reaction rate constant. This relationship can be described by the Arrhenius equation given in section 2.4.1. Figure 4.15 shows a comparison of experimental results for *trans*-

4MACH plotted according the Arrhenius relationship compared to predicted results using Equation 2.7 (for average θ over the range of 10° to 30°C).

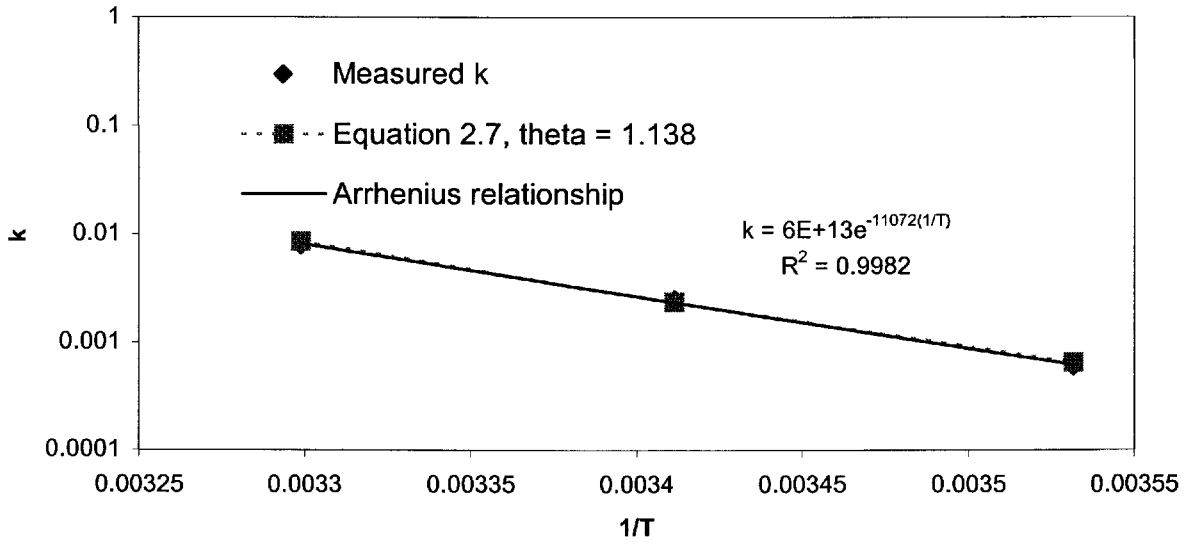


Figure 4.15 Comparison of Arrhenius and theta plots for the *trans*- 4MACH in non-amended water at 10 to 30 °C.

Figure 4.16 shows a comparison of measured k plotted according to the Arrhenius relationship and predicted k using the theta formation for the *cis*- 4MACH non-amended water at 10 to 30°C.

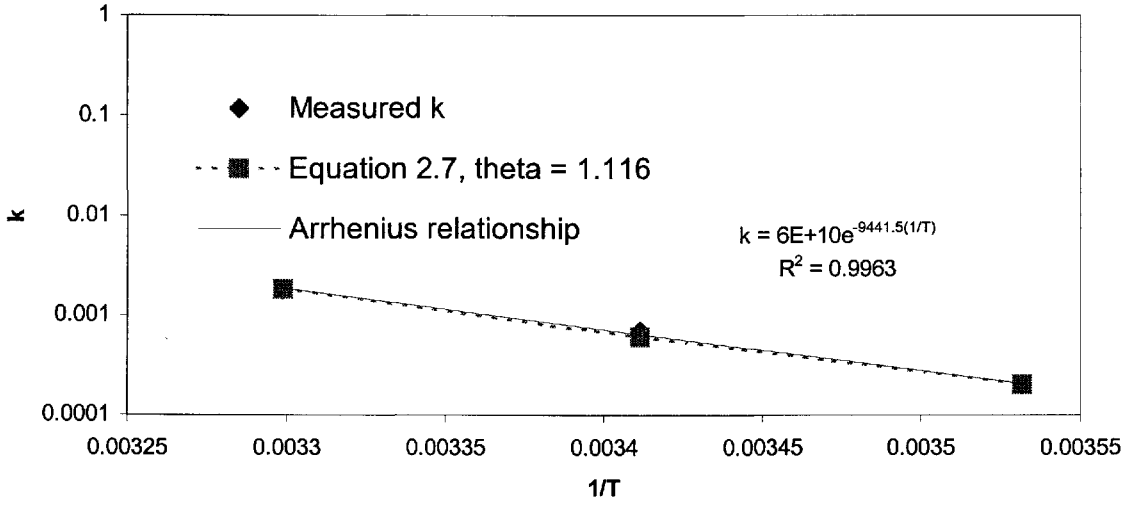


Figure 4.16 Comparison of Arrhenius and theta plots for the *cis*- 4MACH non-amended water at 10 to 30 °C.

Figure 4.17 shows a comparison of measured k plotted according to the Arrhenius relationship and predicted k using the theta formation for the *trans*- 4MCCH non-amended water at 10 to 30°C.

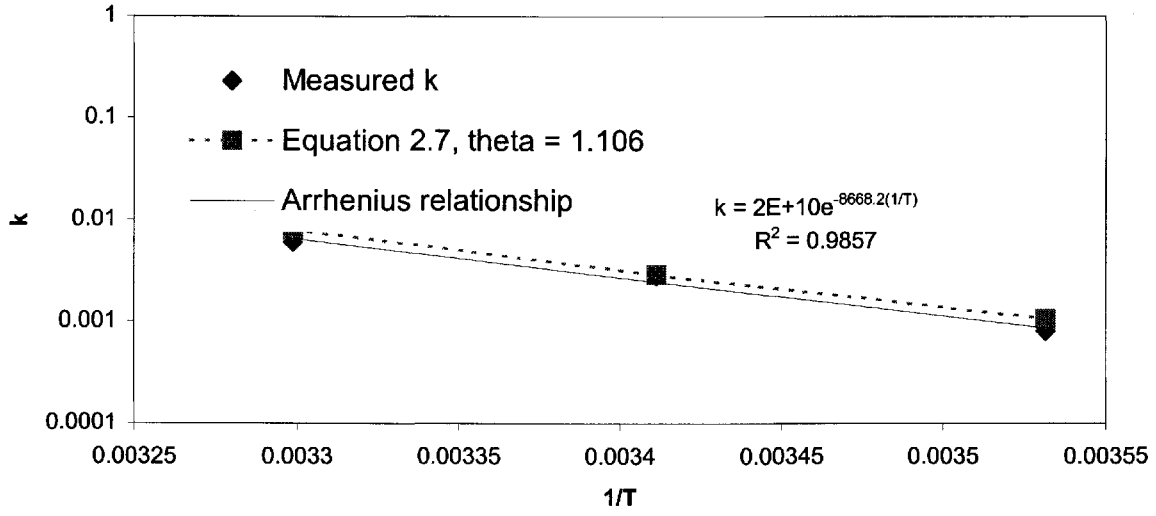


Figure 4.17 Comparison of Arrhenius and theta plots for the *trans*- 4MCCH non-amended water at 10 to 30 °C.

Figure 4.18 shows a comparison of measured k plotted according to the Arrhenius relationship and predicted k using the theta formation for the *cis*- 4MCCH non-amended water at 10 to 30°C.

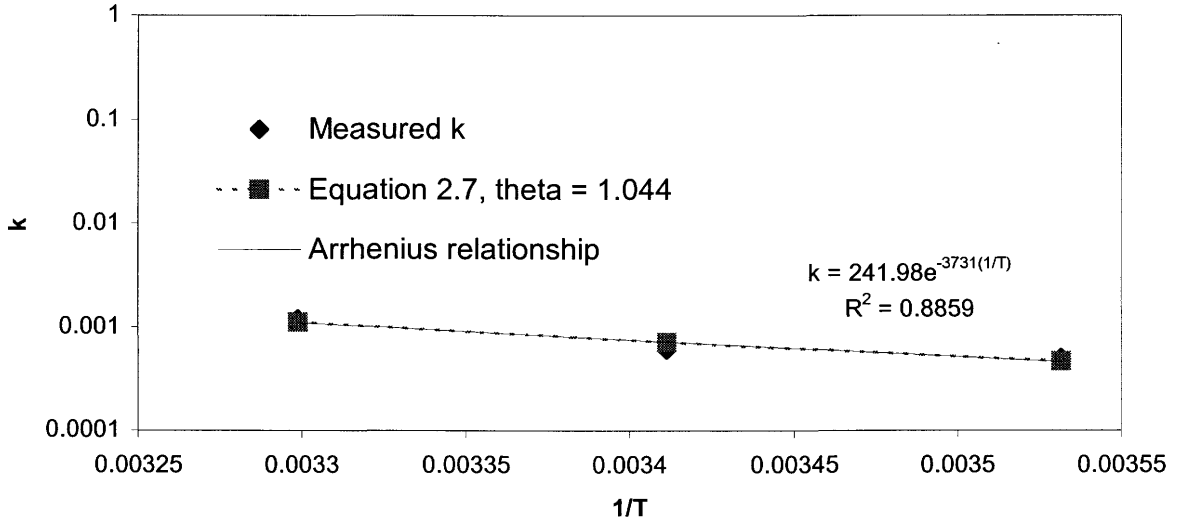


Figure 4.18 Comparison of Arrhenius and theta plots for the *cis*- 4MCCH non-amended water at 10 to 30 °C.

Based on the Arrhenius the plots shown in Figure 4.15 to Figure 4.18, there was no significant difference in prediction of reaction rate constants based upon the Arrhenius equation or the theta formulation.

A summary of the activation energy (Ea), pre-exponential factor (A) and theta (θ, temperature coefficient) values of 4MACH and 4MCCH geometric isomers is given in Table 4.5. The measured Ea values for *trans*- and *cis*- 4MACH non-amended water at 10 to 30°C were 22.0 and 18.76 kcal/mole, respectively. The corresponding pre-exponential factors (A) were 6.0×10^{13} and 6.0×10^{10} liter/mole, respectively. The average θ value was 1.127. In the 4MCCH investigation, the measured Ea values for *trans*- and *cis*- isomers were 17.23 and 7.41 kcal/mole,

respectively. The corresponding pre-exponential factors (A) were 2.0×10^{10} and 2.42×10^{2}, average θ value was 1.075.

Table 4.5 Results of activation energy (Ea), pre-exponential factor (A) and θ (temperature coefficient) values of 4MACH and 4MCCH geometric isomers.

	Ea (kcal/mole)	A (liter/mole)	θ
trans- 4MACH	22.0	6.0×10^{13}	1.138
cis- 4MACH	18.76	6.0×10^{10}	1.116
trans- 4MCCH	17.23	2.0×10^{10}	1.106
cis- 4MCCH	7.41	2.42×10^{2}	1.044

4.7 Engineering Considerations

A number of toxic effects of oil sands process wastewater have been traced to the presence of NAs (CONRAD, 1998). Biodegradation is an important mechanism for removing these chemicals from process wastewater and from natural ecosystems. Biodegradation decreases the concentration of NAs by transforming them into innocuous organic compounds, carbon dioxide and water.

Studies of biodegradation kinetic behavior are essential to the practice of environmental engineering. Biodegradation is an inexpensive and widely used method for removing organic compounds from wastewater and is the primary mechanism responsible for their destruction in natural waters. Data on biodegradation kinetics and factors affecting the rates of biodegradation are required for design and operation of biological treatment systems, for modeling the fate of organic chemicals in surface and groundwater systems and for establishing limits on the discharge of organic compounds to the environment.

Reaction rate coefficients (k) are required for the design of lagoon treatment systems. For example, the solution to the governing mass balance equation for a single cell, completely mixed lagoon for steady state flow conditions is (Sawyer *et al.*, 1994):

$$S/So = 1/ (1+ k (V/Q)) \quad (4.1)$$

where S and So are the effluent and influent NAs concentration (mg/l), respectively, and V and Q are volume of lagoon (Mlitres) and wastewater flowrate (Mlitres/d), respectively. The required detention time in the lagoon (V/Q) can be determined given an initial NA concentration, the desired effluent NA concentration and an estimated k based upon the results of this study. After the detention time has been determined, the required volume of lagoon can be calculated by multiplying the wastewater flowrate by the required retention time in the lagoon. Similar analysis can be conducted for completely mixed reactors in series, or for flow reactors based upon their governing mass balance equations and an estimated k value

In addition, rate coefficients (k) and mass balance analysis can be used to predict the concentration of NAs in a river downstream of an effluent discharge point. For example, for steady state conditions the governing mass balance relationship in the two-dimensional mixing zone downstream of an effluent discharge point is given by the following equation (Putz *et al.*, 1984):

$$hu(\partial S/\partial x) = \partial/\partial z\ (h\varepsilon_z(\partial S/\partial z)) - hkS \quad (4.2)$$

where u is the depth averaged velocity (m/s) in the x longitudinal (or downstream) direction (m), z is the transverse (or cross stream) direction (m), ε_z is the transverse mixing coefficient (m^2/s), h is the depth of flow (m), and S is the depth

averaged concentration of NAs (mg/L). Estimated rate coefficient (k) values (expressed in units of 1/sec) can be used in analytical or numerical solutions to equation 4.2 in order to predict concentrations of NAs at various positions in the river downstream of an outfall.

The results of this study show that primary factors, such as temperature, pH and DOC affect the biodegradation rate of NAs. Relatively high temperatures were employed in this research to study the rate of biodegradation. As a result, temperature showed a pronounced effect upon the rate of biodegradation compared to pH and DOC amendments. In addition, biodegradation rate constants in the laboratory are generally more rapid than those found in the field because conditions in the laboratory are optimized for microbes. Therefore, caution must be exercised in attempting to predict biodegradation rates in the field. The relationship developed using the coefficient θ can be used as a guide for predicting the rate constant (k) of geometric isomers of NAs in Athabasca River at different temperatures in the range of 10 to 30°C.

Under natural water quality conditions there are several other factors affecting the biodegradation rate of NAs. For example, salinity, dissolved oxygen, redox potential, nutrient availability, sediment structure and sunlight. Other important factors include the sorption of organic compounds to sediment matter, toxicity thresholds of complex organic chemical mixtures to the microbial community, the structure of the microbial community present and the degree of acclimatization of the microbial community to the organic compounds (U.S. EPA, 1982). Estimates of the attenuation rate of NAs in field situations must consider the cumulative effect of all these factors.

Previous studies of the chemical structure of NAs have revealed that the alkyl-substitution of the cycloalkane ring may affect the biodegradation rate as well as

the number of cycloalkane rings (CONRAD, 1998). These factors are difficult to investigate because representative pure compounds are not commercially available (CONRAD, 1998). This investigation of three representative NAs has shown that the *trans*-isomers degraded more rapidly than the respective *cis*-isomer. Therefore, it is important to consider the effects of structural isomers when assessing NAs aquatic toxicity, particularly since NAs are complex mixtures of hundreds of compounds, comprised of a number of geometric isomers.

5. CONCLUSIONS

The following conclusions can be made based upon the results of this investigation:

1. Athabasca River water contains microorganisms well adapted to mineralizing NAs. Heterotrophic populations in the river water were able to utilize the NAs as a carbon source.

2. Changes in temperature had a significant effect on the rate of biodegradation. For example, for non-amended raw river water, there was greater than a ten-fold increase in the value of K (from 0.0006 to 0.0079 $hours^{-1}$) for the biodegradation of *trans*- 4MACH at temperature 10°C and 30°C. NAs will be more persistent at lower temperature in Athabasca River water than those found in higher temperature. Temperature effects conform to the Arrhenius relationship and the applicable θ values for adjustment of rates are 1.138, 1.116, 1.106 and 1.044 for *trans*- 4MACH, *cis*- 4MACH, *trans*- 4MCCH and *cis*- 4MCCH, respectively, over the range of 10 to 30°C.

3. DOC amendments (less than 5mg/l humid acid) had insignificant effect on the rate of biodegradation.

4. pH had little effect upon the rate of biodegradation provided the bacteria populations were not compromised. In the range of pH 8.7-7.0 the effects were generally insignificant. At pH 6, 4MCCH was more slowly biodegraded than at the ambient pH of approximately 8.7. It is concluded that 4MCCH at pH 6 may be getting outside of the preferred or optimum pH range and inhibits the growth of microbial populations. In the case of extreme pH amendment (pH 3.5) in the second experiment, the microbial population was reduced to zero in the raw

Athabasca river water as they were not able to acclimatize to the extreme pH value.

5. For the NAs investigated, the *trans*- isomers degraded more rapidly than the respective *cis*- isomers in all cases. The closed structures of the *cis*- isomers are more stable to microbial degradation than the more open geometry of the *trans*-isomers. The *trans*- isomers are thus more bioavailable in natural water than the *cis* geometric isomers. It is proposed this difference in bioavailability is the result of intramolecular hydrogen bonding which can occur for only the *cis*- isomers.

6. Intramolecular hydrogen bonding provides a rationale for the relative differences observed in biodegradation rate for the geometric isomers. Subtle differences in structures of geometric isomers can thus have a significant effect on their bioavailability and related toxicity in natural waters. Therefore, careful attention must be given to structural isomers when assessing aquatic toxicity of NAs, particularly since NAs are generally a complex mixture of hundreds of compounds, comprising a number of geometric isomers. The bioavailability of individual NA compounds within these mixtures cannot be assessed using average rate constants or half-lives measured for NA mixtures. Information regarding the composition of the NA mixture and specific rate constants for each of the compounds is required.

7. The structural differences of side chains of NAs appeared to have only a minor effect on the rate of biodegradation. For example, the measured K values for *trans*- 4MACH and *trans*- 4MCCH at room temperature (20°C) were 0.0025 and 0.0028 $hour^{-1}$.

8. Under water quality and environmental condition similar to this study, it is generally expected that *cis*- isomer will be more persistent and will be biodegraded less than the *trans* geometric isomers.

6. RECOMMENDATIONS

NAs are a complex mixture of hundreds of compounds. Therefore, additional commercially available pure compounds should be tested in the laboratory to assess the influence of different structures, such as the number of cycloalkane rings or the alkyl-substitution on the cycloalkane ring, upon NA biodegradation.

The results of this investigation demonstrated that temperature has a significant effect upon NA biodegradation rate. Therefore, further laboratory investigations should be conducted at temperatures lower than 10°C that simulate winter conditions in the Athabasca River.

In general, there are numerous other factors affecting microbial growth and metabolism. These include salinity, redox potential, nutrient availability, sediment structure and sunlight. Therefore, the influence of these other important factors should be investigated with a series of laboratory tests. In addition, the biodegradation effectiveness of microbial populations taken from other surface water sites subjected to NAs input should be investigated.

The results of the laboratory test programs recommended above should be compared to field studies of NA biodegradation. For example the reduction of NAs concentration that occurs in a treatment lagoon system or in a river system should be investigated. Samples taken from the lagoon system, and at various locations within a river system would allow quantification of the reduction in NA concentration compared to input conditions. The governing mass balance equations for these field situations combined with measured hydraulic and geometric parameters could be used to calculate k values that occur under field conditions. These calculated k values could then be compared to the results of laboratory tests for similar environmental conditions. The field studies should

extend over several years in order to fully investigate seasonal effects such as temperature changes on biodegradation rates.

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APPENDIX A: RESULTS of GC-FID ANALYSIS

Table A.1 non-amended flask results (1st experiment).

Time (hours)	*trans*- isomer			*cis*- isomer		
	4MACH (mg/l); pH 8.7			4MACH (mg/l); pH 8.7		
0	3.88	3.92	3.33	5.02	5.17	5.37
23	3.83	3.92	3.96	4.91	5.32	5.16
46.3	3.82	3.54	3.70	5.11	5.03	5.03
70.6	3.97	3.72	3.36	5.17	4.81	5.74
94.6	3.61	3.82	3.92	4.93	5.24	5.26
119.6	3.68	3.56	3.46	4.92	4.75	4.70
141.6	3.17	3.49	3.43	4.57	4.95	4.80
165.6	3.35	3.01	3.06	5.01	4.63	4.48
189.9	3.01	2.78	2.88	4.63	4.39	4.42
214.2	2.54	2.51	2.42	4.33	4.83	4.17
238.5	2.68	2.50	2.56	4.66	4.54	4.52
261.8	2.41	2.13	2.52	4.34	4.25	4.50
286.8	2.65	1.98	2.18	4.84	4.13	4.52
309.1	2.55	2.13	2.30	4.81	4.07	4.85
334.4	2.43	1.94	1.96	4.87	4.36	4.46
358.4	2.29	1.72	1.72	4.72	3.96	3.85
382.4	2.31	1.83	1.89	5.01	4.45	4.75
406.4	1.74	1.95	2.01	4.61	4.53	4.15
430.4	1.74	1.74	1.29	3.74	4.77	4.26
454.7	1.75	1.53	1.48	4.32	4.01	4.48
502.7	1.27	1.29	1.23	3.74	3.82	4.12

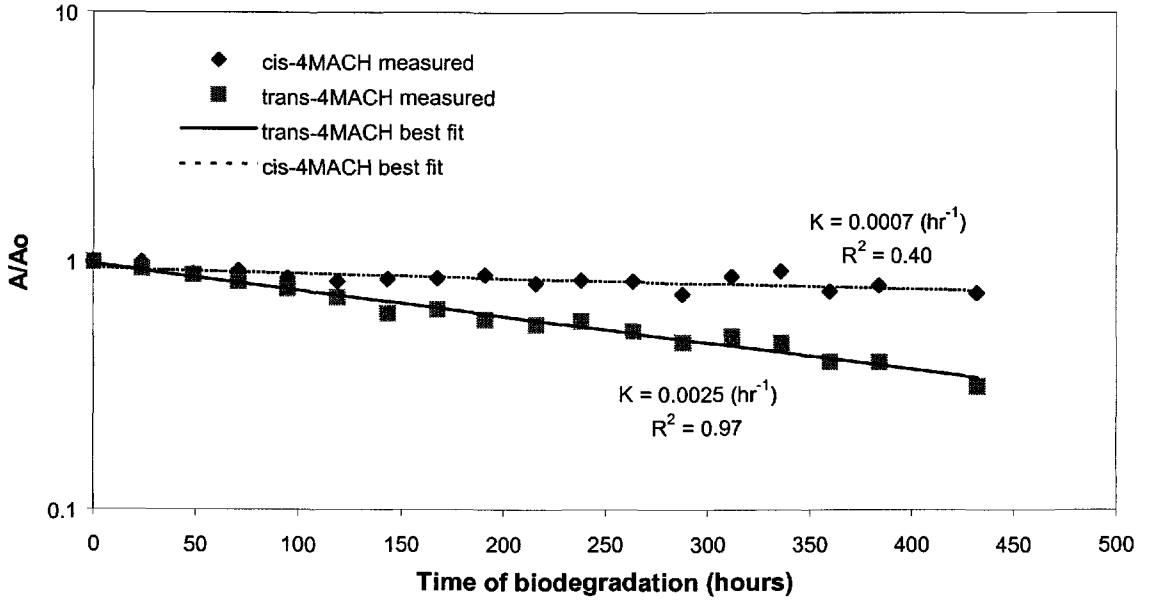

Figure A.1 Biodegradation kinetics of non-amended water at room temperature of *trans*- and *cis*- 4MACH in the first experiment.

Table A.2 Results from the flasks amended to pH 7 (1st experiment).

Time (hours)	*trans*- isomer			*cis*- isomer		
	4MACH (mg/l); pH 7			4MACH (mg/l); pH 7		
0	5.35	4.33	5.11	6.29	5.18	6.07
23	3.85	3.80	3.95	4.61	4.55	4.75
46.3	3.62	3.99	3.80	4.78	5.07	4.85
70.6	3.76	4.00	3.97	4.51	4.73	4.72
94.6	3.40	3.43	3.76	4.30	4.19	4.64
119.6	3.27	3.32	3.32	4.10	4.11	4.19
141.6	3.29	3.38	3.31	4.37	4.41	4.39
165.6	2.99	2.93	3.09	4.37	4.03	4.35
189.9	2.63	3.10	3.06	3.91	4.40	4.41
214.2	2.55	2.78	2.73	4.60	4.36	4.36
238.5	2.58	2.73	2.49	4.65	4.23	3.96
261.8	2.19	2.44	2.38	4.16	4.23	3.99
286.8	1.95	2.55	2.25	4.19	4.58	2.24
309.1	1.83	2.12	2.31	4.16	4.27	3.96
334.4	1.63	2.23	1.83	3.87	4.43	3.71
358.4	1.39	1.83	1.78	3.42	3.63	3.64
382.4	1.38	2.09	1.93	3.73	4.32	4.39
406.4	1.10	1.95	1.85	3.60	4.44	4.37
430.4	0.82	1.68	1.98	3.02	4.29	5.02
454.7	0.78	1.45	1.52	3.38	3.89	3.88
502.7	0.52	1.08	1.26	2.86	3.55	3.67

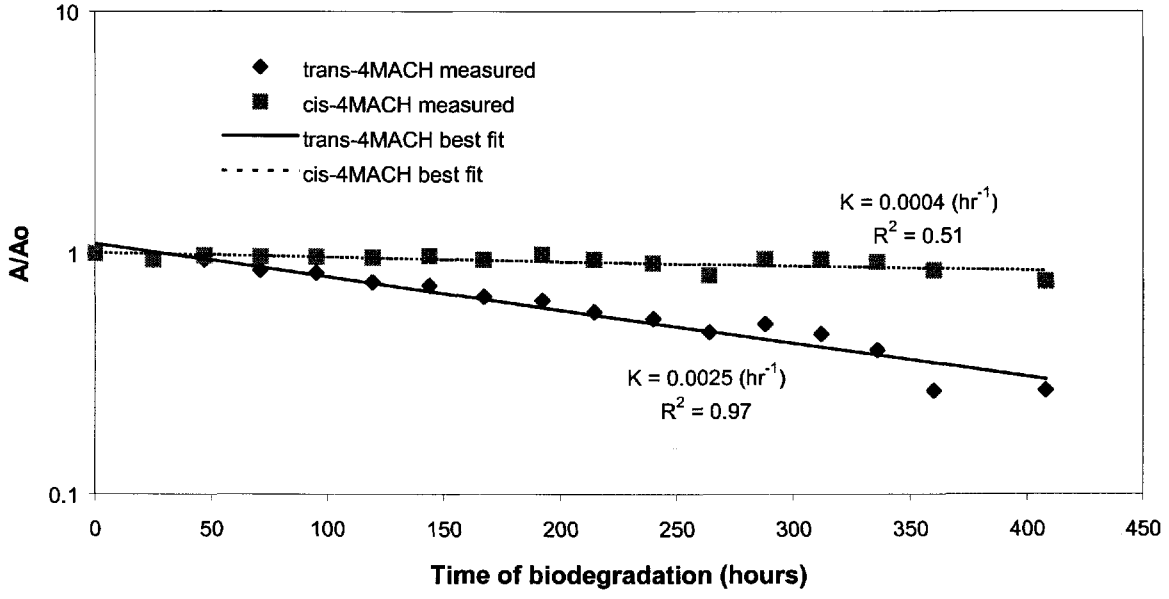

Figure A.2 Biodegradation kinetics of amended pH 7 at room temperature of *trans*- and *cis*- 4MACH in the first experiment.

Table A.3 Results from the flasks amended with 5 mg/l of HA and non-amended pH (1st experiement).

Time (hours)	*trans*- isomer			*cis*- isomer		
	4MACH (mg/l);pH 8.7 plus 5mg/l of HA			4MACH (mg/l);pH 8.7 plus 5mg/l of HA		
0	4.22	4.02	4.20	4.03	3.85	3.98
23	3.82	4.63	4.19	3.68	4.08	4.02
46.3	3.76	4.03	4.31	3.71	4.17	4.21
70.6	4.45	3.90	4.15	4.18	3.69	3.90
94.6	3.72	3.80	3.38	3.68	3.77	3.31
119.6	3.35	3.67	3.63	3.44	3.80	3.73
141.6	2.69	3.19	3.43	3.09	3.51	3.89
165.6	2.71	3.04	2.88	4.00	3.48	3.94
189.9	2.35	2.87	2.54	3.81	3.75	3.60
214.2	1.99	2.85	2.11	3.71	3.89	3.61
238.5	2.01	2.93	2.21	3.81	3.62	4.02
261.8	1.91	2.56	2.12	3.80	3.66	4.00
286.8	1.93	2.23	1.95	3.57	3.63	4.04
309.1	1.67	2.13	1.75	3.66	3.96	3.75
334.4	1.51	1.51	1.39	3.81	3.74	3.48
358.4	1.52	1.17	1.33	3.61	3.38	3.45
382.4	1.54	1.17	1.48	3.60	3.35	3.46
406.4	1.40	0.81	1.13	3.55	2.90	3.09
430.4	1.19	0.65	0.97	3.17	2.71	3.12
454.7	1.06	1.52	0.81	2.99	3.88	3.11
502.7	0.86	0.47	0.47	2.96	2.76	2.67

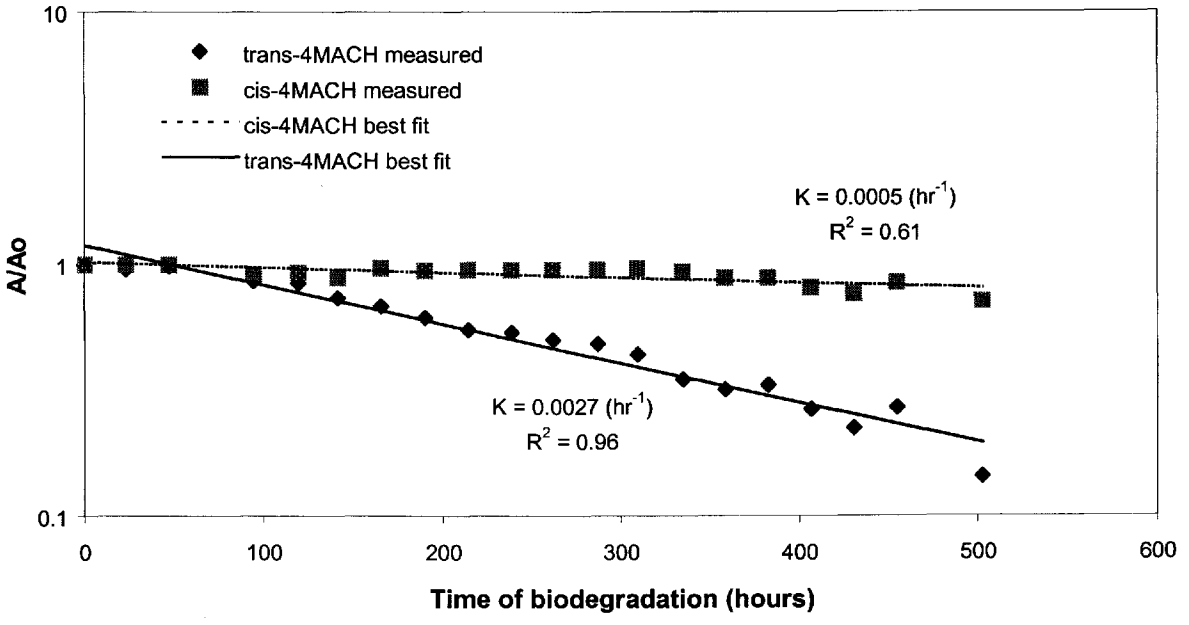

Figure A.3 Biodegradation kinetics of amended DOC with 5mg/l of HA at room temperature of *trans*- and *cis*- 4MACH in the first experiment.

Table A.4 Results from the flasks amended with 5 mg/l of HA and pH 7 (1st experiment).

Time (hours)	*trans*- isomer			*cis*- isomer		
	4MACH (mg/l); pH 7 plus 5mg/l of HA			4MACH (mg/l); pH 7 plus 5mg/l of HA		
0	4.17	4.09	4.24	4.98	5.08	4.46
23	3.83	3.72	4.26	4.56	4.59	5.36
46.3	3.50	3.57	3.46	4.40	4.45	4.26
70.6	3.90	3.82	3.87	4.64	4.56	4.59
94.6	3.42	3.48	3.50	4.36	4.35	4.34
119.6	3.37	3.37	3.67	4.45	4.29	4.53
141.6	3.39	3.11	3.33	4.66	4.17	4.20
165.6	2.84	2.74	2.96	4.20	3.93	3.78
189.9	2.32	2.83	2.87	4.14	4.61	3.63
214.2	2.05	2.22	2.64	4.26	3.56	3.72
238.5	1.89	2.41	2.94	4.31	4.25	4.08
261.8	1.87	2.18	2.83	4.71	3.99	4.23
286.8	1.43	2.11	2.03	4.09	4.07	3.15
309.1	1.27	2.13	2.46	4.03	4.03	3.90
334.4	0.97	1.65	2.26	3.62	3.29	3.81
358.4	0.81	1.85	2.31	3.37	3.96	4.06
382.4	0.90	2.04	2.46	3.69	3.38	4.71
406.4	0.65	1.94	2.02	3.24	3.34	3.96
430.4	0.52	1.48	2.61	3.09	3.40	3.15
454.7	0.47	1.65	1.90	3.24	2.92	3.94
502.7	0	1.38	1.36	2.64	3.38	3.12

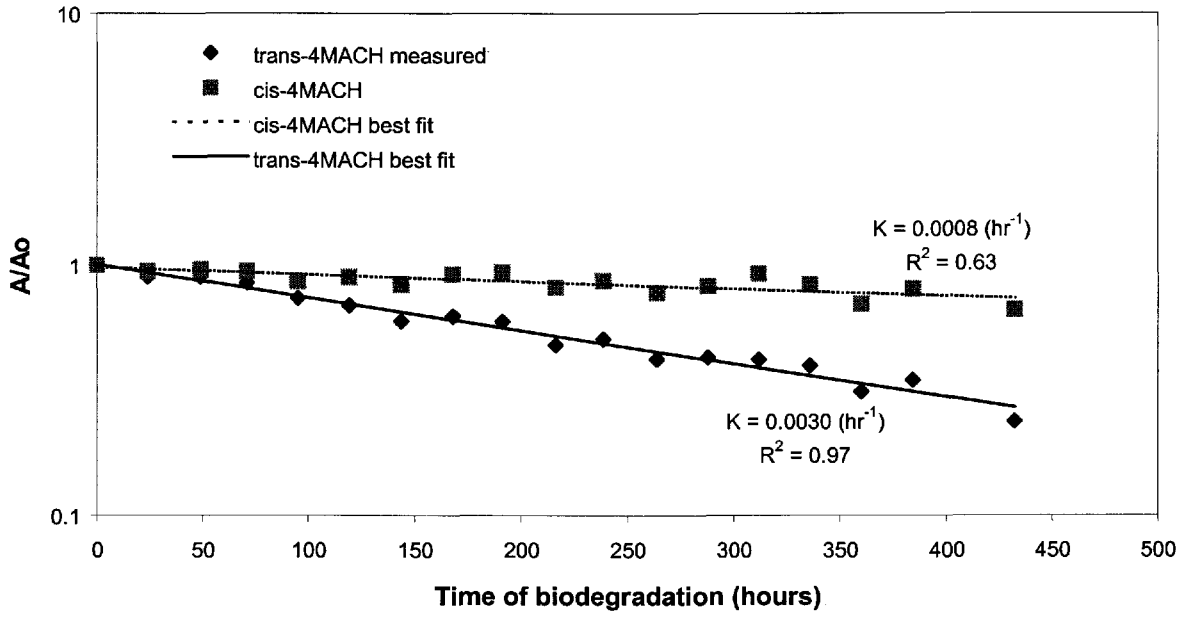

Figure A.4 Biodegradation kinetics of amended DOC with 5mg/l of HA plus pH 7 at room temperature of *trans-* and *cis-* 4MACH in the first experiment.

Table A.5 Results from the flasks amended to pH 3.5 (2nd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MACH (mg/l); pH 3.5		4MACH (mg/l); pH 3.5	
0	3.30	3.49	2.96	3.16
73	3.06	3.33	2.88	3.10
121	3.09	3.18	2.85	2.90
168	3.33	3.26	3.14	3.03
241	3.58	3.24	3.26	3.05
289	3.50	3.21	3.20	3.06
337	3.23	3.33	3.09	3.07
409.3	N/A	N/A	N/A	N/A
456.6	3.41	3.41	3.31	3.31

Table A.6 Results from the flasks amended temperature to 30°C (2nd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MACH (mg/l); pH 8.7 at 30°C		4MACH (mg/l); pH 8.7 at 30°C	
0	3.25	3.42	3.77	3.93
73	2.90	2.95	3.41	3.75
121	2.76	1.97	3.45	3.10
168	2.13	1.14	3.60	3.23
241	1.08	0	3.07	1.92
289	0.52	0	2.84	1.76
337	0	0	2.51	1.64
409.3	0	0	1.95	1.56
456.6	0	0	1.93	1.45

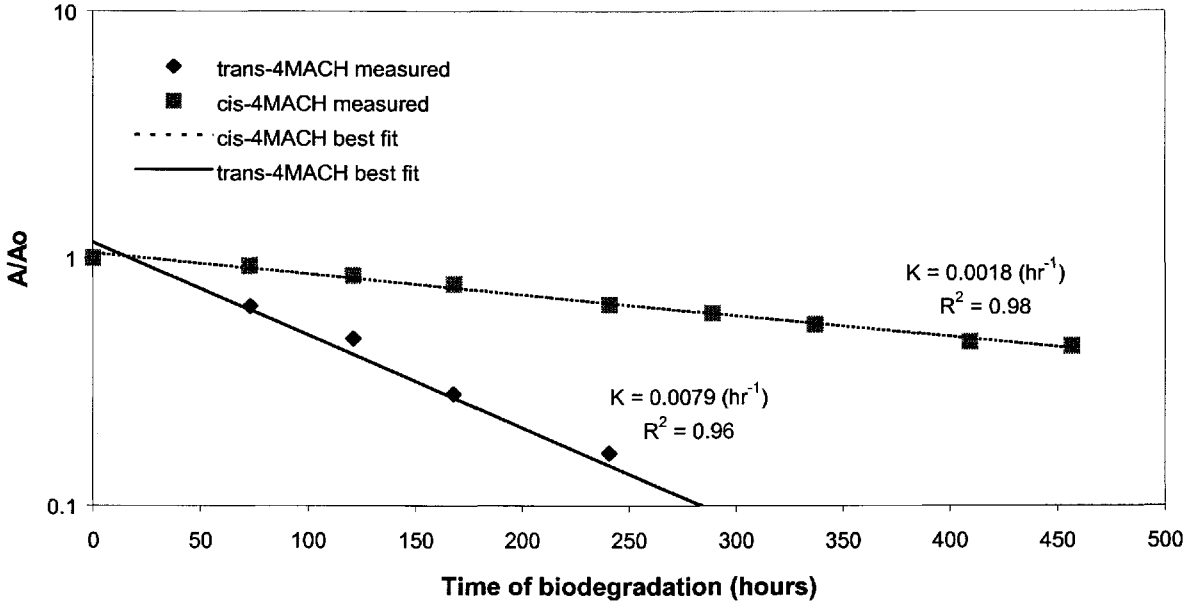

Figure A.5 Biodegradation kinetics of non-amended water at 30°C temperature of *trans*- and *cis*- 4MACH in the second experiment.

Table A.7 Results from the flasks amended temperature to 10°C (2nd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MACH (mg/l); pH 8.7 at 10°C		4MACH (mg/l); pH 8.7 at 10°C	
0	3.50	3.36	3.77	3.66
73	3.36	3.17	3.69	3.47
121	2.96	3.09	3.38	3.51
168	2.98	2.91	3.62	4.46
241	2.92	2.84	3.75	3.43
289	2.57	2.74	3.45	3.29
337	2.63	2.87	3.37	3.41
409.3	2.42	2.90	3.19	3.19
456.6	2.52	2.77	3.34	3.21

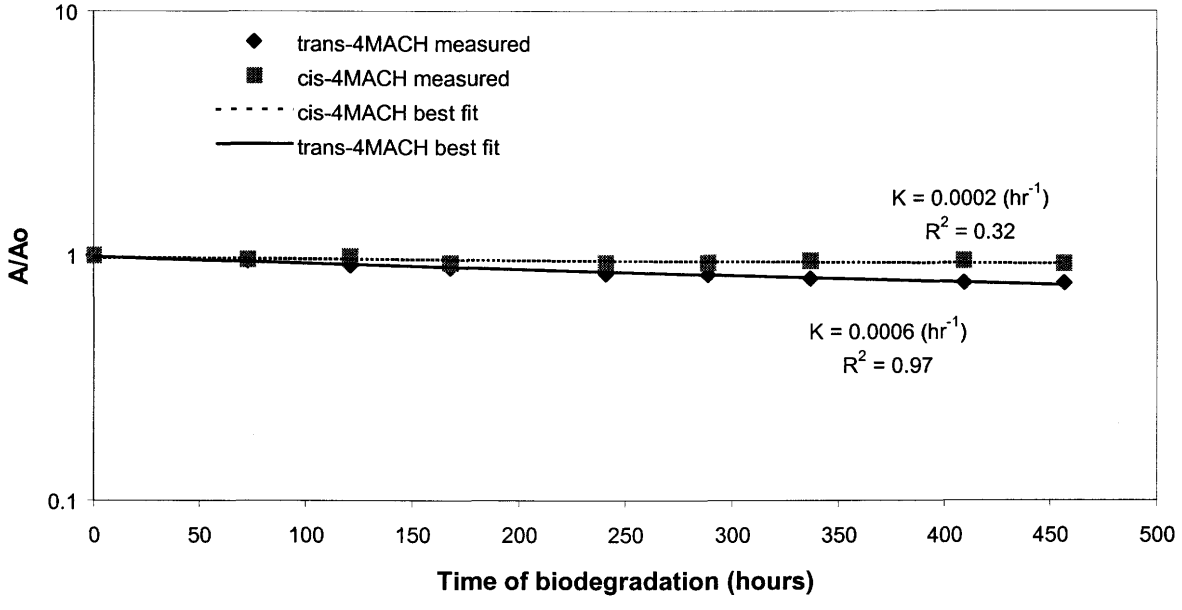

Figure A.6 Biodegradation kinetics of non-amended water at 10°C temperature of *trans*- and *cis*- 4MACH in the second experiment.

Table A.8 Results from the non-amended water samples (3rd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MCCH (mg/l); pH 8.7		4MCCH (mg/l); pH 8.7	
0	1.90	1.99	5.03	6.01
48	1.81	1.83	5.85	5.81
121	1.42	1.31	4.77	5.41
168.3	1.36	1.26	4.48	5.14
216.6	1.02	1.29	4.40	5.61
287.9	1.16	0.80	4.49	4.14
336.2	0.80	0.75	4.51	4.11
384.5	0.48	0.59	4.08	3.92
456.8	0.50	0.50	3.43	3.43
504.8	0	0	3.38	3.21

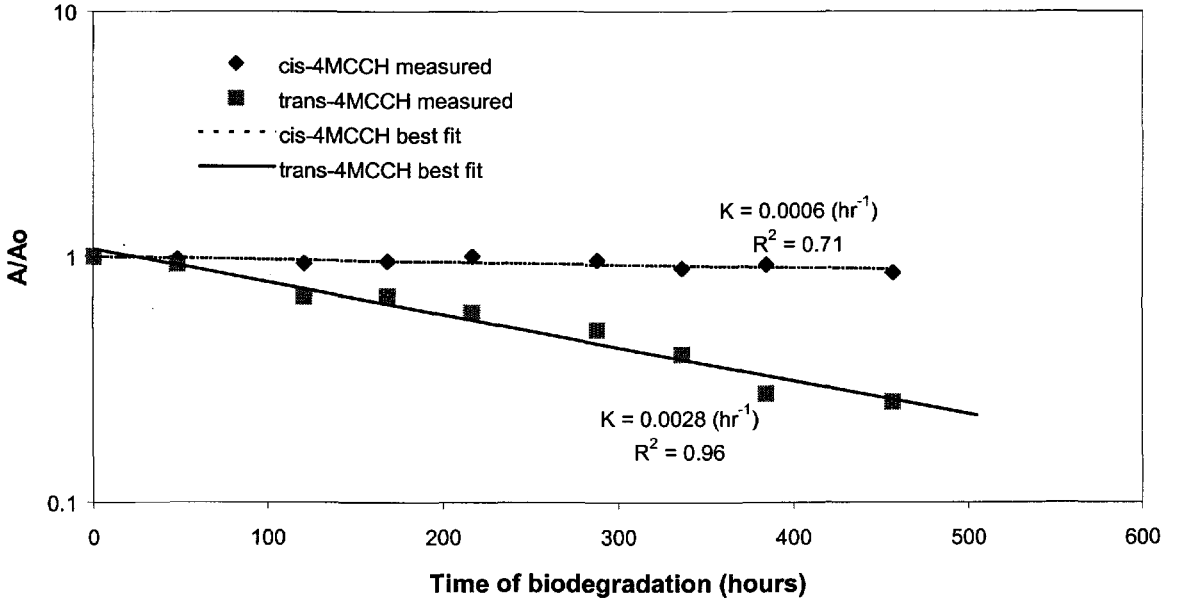

Figure A.7 Biodegradation kinetics of non-amended water at room temperature of *trans*- and *cis*- 4MCCH in the third experiment.

Table A.9 Results from the flasks amended pH to 6 (3rd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MCCH (mg/l); pH 6		4MCCH (mg/l); pH 6	
0	1.99	1.89	5.40	5.12
48	2.01	1.65	5.67	4.47
121	1.64	1.08	4.90	5.08
168.3	2.11	1.56	4.42	5.11
216.6	1.54	1.34	4.78	4.70
287.9	1.52	1.25	N/A	N/A
336.2	1.23	0.99	4.35	3.84
384.5	1.38	0.87	4.58	3.50
456.8	1.03	1.19	4.05	4.63
504.8	1.25	0.85	3.89	4.44

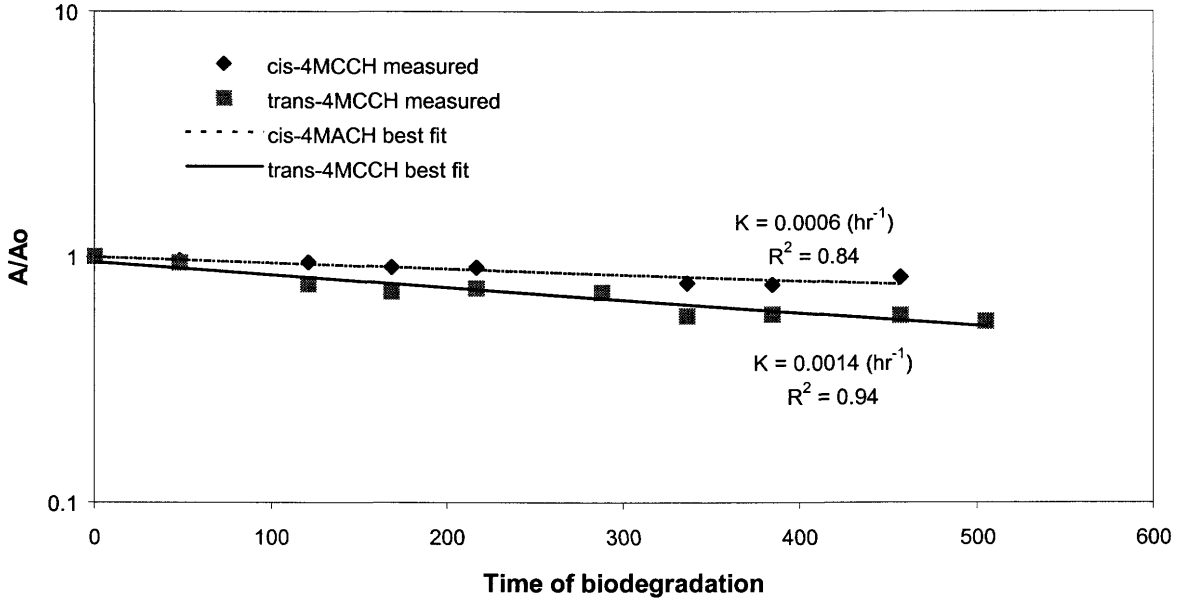

Figure A.8 Biodegradation kinetics of amended pH 6 at room temperature of *trans*- and *cis*- 4MCCH in the third experiment.

Table A.10 Results from the flasks amended temperature to 30°C (3rd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MCCH (mg/l); pH 8.7 at 30°C		4MCCH (mg/l); pH 8.7 at 30°C	
0	1.28	1.66	5.42	5.41
48	0.70	1.54	4.80	5.69
121	0.47	1.17	4.92	5.52
168.3	0.36	0.80	4.46	4.66
216.6	0.26	0.56	4.02	4.27
287.9	0	0.25	3.85	3.41
336.2	0	0	3.52	3.49
384.5	0	0	3.21	3.18
456.8	0	0	2.68	3.69
504.8	0	0	2.23	3.30

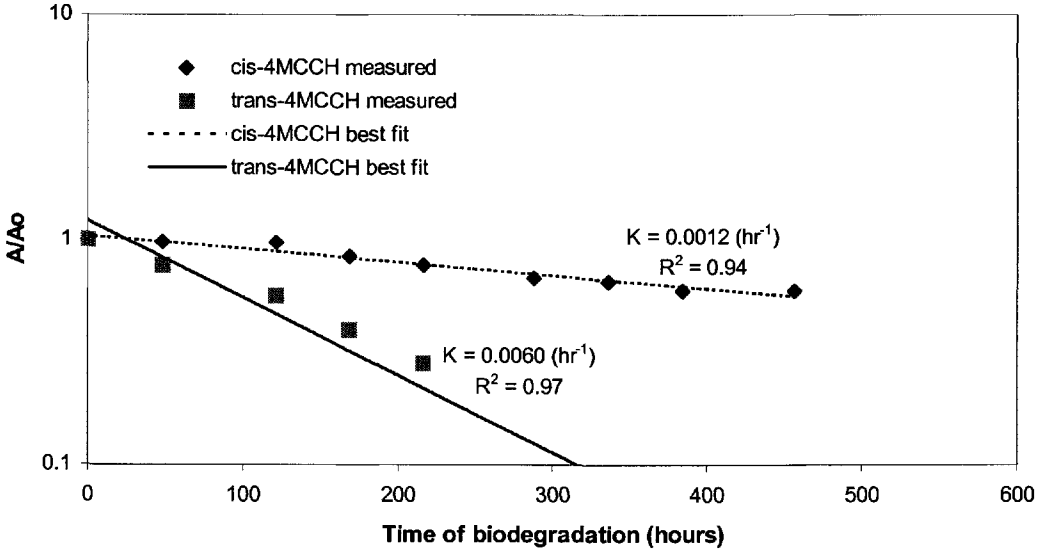

Figure A.9 Biodegradation kinetics of non-amended water at 30°C temperature of *trans*- and *cis*- 4MCCH in the third experiment.

Table A.11 Results from the flasks amended temperature to 10°C (3rd experiment).

Time (hours)	*trans*- isomer		*cis*- isomer	
	4MCCH (mg/l); pH 8.7 at 10°C		4MCCH (mg/l); pH 8.7 at 10°C	
0	2.33	2.31	6.19	5.66
48	2.15	2.19	5.69	5.59
121	2.02	2.07	5.46	5.54
168.3	2.05	1.86	5.60	5.21
216.6	1.95	1.91	5.48	5.64
287.9	1.70	1.87	4.97	5.67
336.2	2.06	1.45	4.64	4.64
384.5	1.64	1.54	5.02	5.31
456.8	1.43	1.16	5.05	4.17
504.8	1.34	1.13	4.89	4.12

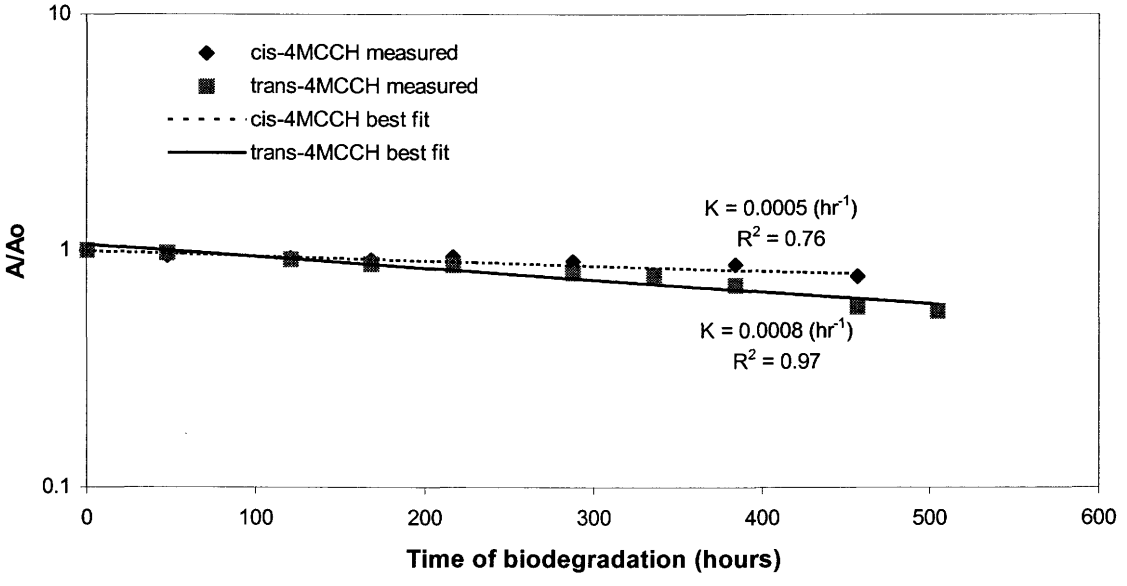

Figure A.10 Biodegradation kinetics of non-amended water at 10°C temperature of *trans*- and *cis*- 4MCCH in the third experiment.

Table A.12 Results from the flasks amended temperature to 30°C (4th experiment).

Time	*trans*- isomer			*cis*- isomer		
(hours)	3MCCH (mg/l); pH 8.7 at 30°C			3MCCH (mg/l); pH 8.7 at 30°C		
0	8.10	8.51	8.98	3.68	3.83	4.09
47	6.57	8.11	7.69	3.00	3.69	3.54
94.3	7.09	8.00	5.80	3.26	3.74	2.66
191.6	5.44	5.46	6.00	2.60	2.53	2.95
263.9	4.58	4.99	5.21	2.33	2.49	2.74
336.9	4.32	4.89	3.87	2.26	2.96	2.08

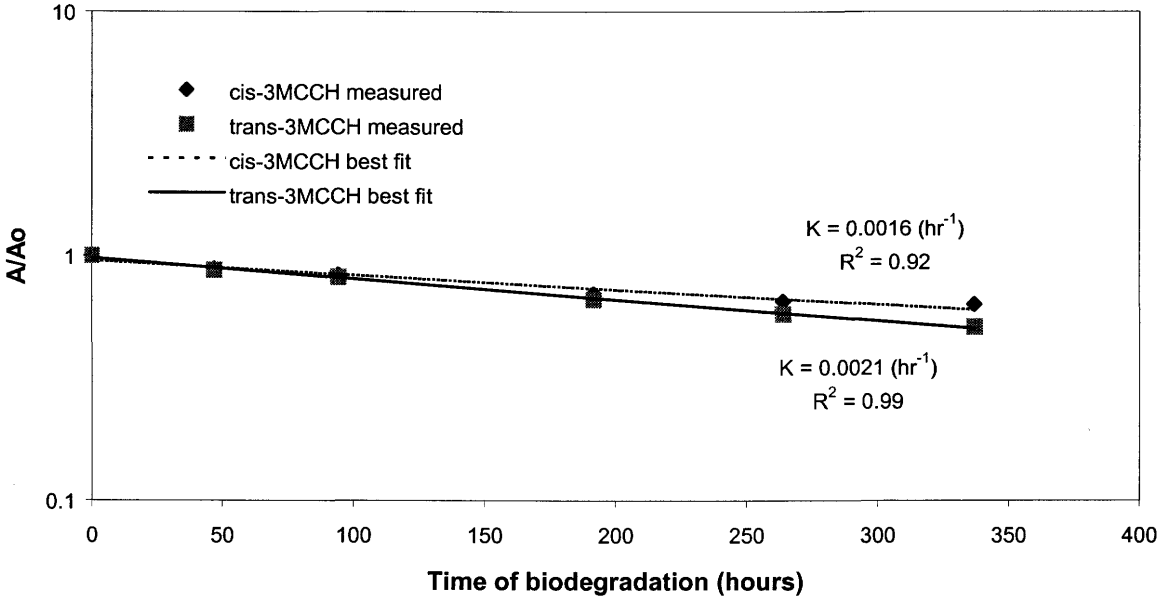

Figure A.11 Biodegradation kinetics of non-amended water at 30°C temperature of *trans*- and *cis*- 3MCCH in the fourth experiment.