THE KINETICS OF BIODEGRADATION OF TRANS 4-METHYL-1-CYCLOHEXANE CARBOXYLIC ACID

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By

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ABSTRACT

This thesis presents the study of biodegradation factors of a candidate naphthenic acid compound, the trans isomer of 4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA). Low molecular weight components of naphthenic acids such as trans-4MCHCA are known to be toxic in aquatic environments and there is a need to better understand the factors controlling the kinetics of their biodegradation. In this study, a relatively low molecular weight naphthenic acid compound and a microbial culture developed in our laboratory (primarily *Alcaligenes paradoxus* and *Pseudomonas aeruginosa*) were used to study the biodegradation of this candidate naphthenic acid. The purpose of the research was to evaluate the kinetic parameters and model the biodegradation of this compound in three bioreactor systems: batch reactors, a continuously stirred tank reactor and immobilized cell reactors.

In batch reactors, the maximum specific growth rate ($0.52\pm0.04\ d^{-1}$) of the consortium at $23^{o}C$ and neutral pH was not highly variable over various initial substrate concentrations (50 to 750 $mg\cdot l^{-1}$). Batch experiments indicated that biodegradation can be achieved at low temperatures; however, the biodegradation rate at $4^{o}C$ was only 22% of that at room temperature ($23^{o}C$). Biodegradation at various pH values indicated a maximum specific growth rate of $1.69\pm0.40\ d^{-1}$ and yield ($0.41\pm0.06\ mg\cdot mg^{-1}$) at a pH of 10.

Study of the candidate substrate using a continuously stirred tank reactor and the microbial culture developed in the batch experimentations revealed that the kinetics of the candidate naphthenic acid are best described by the Monod expression with a maximum specific growth rate of $1.74\pm0.004\ d^{-1}$ and a half saturation constant of $363\pm17\ mg\cdot l^{-1}$. The continuously stirred tank reactor achieved a maximum reaction rate of 230 $mg\cdot(l\cdot d)^{-1}$ at a residence time of 1.6 d (39 h).

Two high porosity immobilized cell reactors operating continuously over three months were found to consume trans-4MCHCA at a rate almost two orders of magnitude

higher than a continuously stirred tank reactor. The immobilized cell systems attained a maximum reaction rate of 22,000 $mg\cdot(l\cdot d)^{-1}$ at a residence time of 16 minutes. High porosity immobilized cell reactors were shown to effectively remove a single naphthenic acid substrate in continuously fed operation to dilution rates of 90 d^{-1}. A plug flow model best represented the degradation in the immobilized cell systems and was demonstrated to be a useful tool for studying the effects of parameter variation and prediction of reactor performance. This work highlights the potential of augmented bioremediation systems for the degradation of naphthenic acids.

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Dedication

To my husband David for your love, eternal optimism and support.

To my children Matthew, Adam and Mikhaila for your patience.

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NOMENCLATURE AND ABBREVIATIONS

Nomenclature

A – cross-sectional area of the reactor (cm^2)

A_b – cross-sectional area of biofilm (cm^2)

A_p – cross-sectional area of packing fibre (cm^2)

D – dilution rate (d^{-1})

$\mathcal{D}$ – axial dispersion coefficient ($cm^2 \cdot s^{-1}$)

EC50 – effect concentration (% v/v)

F – liquid flow rate of the substrate medium ($ml \cdot h^{-1}$)

IC50 – concentration producing 50% reduction in light (% v/v)

IC20 – concentration producing 20% reduction in light (% v/v)

k_e – rate of endogenous decay (d^{-1})

K_i– Haldane inhibition constant ($mg \cdot l^{-1}$)

K_s – Monod saturation constant ($mg \cdot l^{-1}$)

K_{sx}– Contois saturation constant (mg substrate·mg biomass^{-1})

L – length of reactor (cm)

LC50 – lethal concentration (% v/v)

LD50 – lethal dose (% v/v)

Pe – Peclet number (unitless)

r_A – the maximum biodegradation rate ($mg \cdot (l \cdot d)^{-1}$)

S – substrate concentration ($mg \cdot l^{-1}$)

SA_p = packing surface area (cm^2)

S_i – initial substrate feed concentration ($mg \cdot l^{-1}$)

S_{ss} – substrate concentration at steady state ($mg \cdot l^{-1}$)

Sz – steady state substrate concentration at any point z in the direction of liquid flow ($mg \cdot l^{-1}$)

t – thickness of the packing (cm)

V – reactor volume (ml)

V_r – volumetric reaction rate ($mg \cdot (l \cdot d)^{-1}$)

V_w – working volume of reactor (ml)

V_{wbiof} – volume of wet biofilm (ml)

w – width of stainless steel packing fibre (cm)

W_b – dry weight of biomass in biofilm (mg)

W_p – weight of clean stainless steel packing (mg)

X_l – biomass concentration in the free liquid effluent ($mg \cdot l^{-1}$)

X_{biof} – biomass concentration in the biofilm ($mg \cdot l^{-1}$)

X_T – total biomass concentration in the reactor ($mg \cdot l^{-1}$)

Y – biomass yield coefficient (mg biomass·mg substrate^{-1})

z – axial distance in direction of flow (cm)

Abbreviations

2DCHAA – 2,2-dicyclohexylacetic acid

APCI – atmospheric pressure chemical ionization

CFU – colony forming unit

CI – chemical ionization

CSTR – continuously stirred tank reactor

EI – electron ionization

ESI – electrospray ionization

ESI-MS – electrospray ionization – mass spectrometry

FAB – fast atom bombardment

FID – flame ionization detection

FT-IR – Fourier transform infrared

GC – gas chromatography

ICR – immobilized cell reactor

LSI – liquid secondary ion

MPN – most probable number

MS – mass spectrometry

OD – optical density

PAH – polycyclic aromatic hydrocarbon

PC – plate count

QTOF-MS – quadrupole time of flight mass spectrometry

RO – reverse osmosis

rpm – revolutions per minute

SE – standard error

SEM – scanning electron microscopy

SPE – solid phase extraction

SSE – sum of the squares of the error

TIC – total ion current

trans-4MCHCA – 4 methyl-1-cyclohexane carboxylic acid (trans isomer)

UV – ultraviolet

VOC – volatile organic compound

Greek Symbols

α – coefficient for effective biomass concentration in biofilm (unitless)

δ – biofilm thickness (mm)

δ_{eff} – effective biofilm thickness (mm)

η – porosity of packing bed (unitless)

ϕ_L – liquid hold up in the reactor (unitless)

ρ_{ss} – density of stainless steel ($mg \cdot cm^{-3}$)

μ – specific growth rate (d^{-1})

μ_g – gross specific growth rate (d^{-1})

μ_m – maximum specific growth rate (d^{-1})

μ_{net} – net specific growth rate (d^{-1})

θ_i – residence time (d)

CHAPTER 1
INTRODUCTION

1.1 Background

1.1.1 Naphthenic Acids

Naphthenic acids (NAs) are a complex mixture of organic acid surfactant compounds which naturally occur in crude oils. NAs encompass a large range of alkyl-substituted acyclic and cyclic carboxylic acids with the general chemical formula $C_nH_{2n+z}O_2$, where (n) indicates the carbon number and *Z* specifies the number of hydrogen atoms that are lost as the structure becomes more compact. There are many different classes of carboxylic acids present in crude oils (Meredith et al., 2000; Lo et al., 2003). NAs are commercially extracted from various fractions of petroleum. The most common products are metal naphthenates which are used in the wood-preservation industry. NAs are also prepared for many other uses including: fuel additives, paint driers, lubricants and in the manufacturing of tires (Deineko et al., 1994; Brient et al., 1995; Holowenko et al., 2001; Clemente et al., 2003a).

NAs are present in oil sands in Canada which comprise the second largest proven global crude reserve after reserves in Saudi Arabia. With regard to the petroleum industry, their corrosive nature which results from their acidity is detrimental to the refinery processes and equipment (Quagraine et al., 2005b; Headley et al., 2007). NAs are released from the bitumen during the alkaline hot water extraction process. The NAs are concentrated during the extraction processes and are retained in the tailings pond surface waters (Holowenko et al., 2002). NAs have been implicated as some of the most toxic substances in oil sands tailings and have been identified as priority substances impacting the aquatic environments (Headley et al., 2002a).

The majority of Canadian crude oil reserves are found in Alberta's oil sands located in the Athabasca Basin in northeastern Alberta. In 2004, Alberta Energy reported that production of crude oil from these reserves had reached 1.1 million barrels per day (Department of Energy, Government of Alberta, 2007). Approximately, 0.1 to 0.2 m^3 of tailings pond water is accumulated for each tonne of oil sands processed. In 2003, it was estimated that approximately 4×10^8 m^3 of tailings comprised of a slurry of sand, clay, water and unrecovered bitumen in a stable aqueous suspension are retained in the Athabasca region and that the total volume was expected to increase to over 1 billion m^3 as soon as 2020 (Lo et al., 2004; Quagraine et al., 2005b).

The toxicity of the liquid wastes from the tailings pond water has been largely attributed to the salinity and presence of organic compounds, collectively referred to as NAs (Hsu et al., 2000; Headley et al., 2002a; Lo et al., 2003; Quagraine et al., 2005a; Bataineh et al., 2006; Headley et al., 2007). Other concerns with the tailings pond water include the presence of clay particulates as well as other acidic fractions present in the bitumen, including phenol and cresols. In Canada, environmental regulations require the tailings to be retained on-site to prevent release into the environment due to concerns about their toxicity. The toxicity of the liquid wastes from the tailings ponds has largely been attributed to NAs having both acute and chronic toxicity to aquatic organisms (Quagraine et al., 2005b). NAs occur as a complex mixture of compounds in the environment and there has not been conclusive identification of the specific compounds which are most toxic and/or corrosive. Toxicity does not necessarily correlate directly to concentration of NAs (McMartin, 2003).

The petroleum industry intended to reduce the toxicity of the oil sands tailings waste, in part, by natural biodegradation. However, the volumetric capacity of the existing tailings ponds where natural biodegradation occurs is not sufficient to cope with the wastewater production increases predicted by the petroleum industry. As a result the net accumulation of tailings already encompasses more than 5,000 hectares (Peachey,

2005). In all cases, the widespread presence of NAs and their potential impact on the environment needs to be better understood.

1.1.2 Current Research

There is currently no ambient marine water or surface water quality guidelines for NAs in Canada or the United States (Quagraine et al. 2005b); however, on-going research is being conducted pertaining to their mobility, toxicity and biodegradability. In addition to the need to better understand the fate and mobility of these compounds, one of the most prominent hurdles has been the analytical tools for detection and quantification of NAs in various media. The complexity of the NA mixtures continues to pose a major challenge in the development of a suitable analytical method for the detection and quantification of these compounds (Bataineh et al., 2006). There is currently no method that identifies or quantifies individual acids within the mixture (Clemente and Fedorak, 2005). Furthermore, full separation and identification of individual structures of compounds have not been achieved to date (Scott et al., 2005).

Repeated attempts to extensively biodegrade NAs from Athabasca oil sands tailings have been unsuccessful using laboratory cultures of tailings pond water bacteria (Scott et al., 2005). Only a few studies have included biodegradation of individual compounds of commercially available NAs (Tanapat, 2001). Some studies on individual NAs have reported the impact of molecular structure on biodegradation and adsorption on soils. Additional studies have evaluated commercial preparations of NA mixtures as well as available individual NA compounds (Herman et al., 1994; Lai et al., 1996; Holowenko et al., 2001; Clemente and Fedorak, 2005). Research conducted at Syncrude revealed that under aerobic conditions, NAs would undergo microbial biodegradation and a reduction in relative toxicity can be achieved but the influential factors are not well characterized (Leung et al., 2003). Laboratory studies of weathered light crude exposed to microbial degradation observed an increase in the concentrations of cyclic carboxylic acids with (n) greater than 20 showing that biodegradation of the lower carbon numbers was prevalent. These results concurred with previous studies (Holowenko et al., 2001).

The study of combinations of single compounds and NA mixtures and commercially prepared mixtures has for the most part been conducted for purposes of toxicity evaluation. In doing so, the kinetics of biodegradation have not been the focus of the research but rather the effects of biodegradation on toxicity. Herman et al. (1994) studied a candidate NA compound with a single ringed structure similar to that used in this research together with a mixture of organic acids extracted from tailings. At the time of Herman's research, the hurdle to evaluation of biodegradation was the analytical capabilities. This study concluded that microbial activity was able to reduce the organic carbon by 20%. Based on a toxicity evaluation by Microtox™, a complete absence of detectable toxicity following biodegradation of the naphthenic acids was reported although no reduction in any gas chromatographic peaks were observed using the derivatization method of gas chromatography with flame ionization detection. Subsequently, Lai et al. (1996) studied the use of phosphate as an enhancement nutrient in the biodegradation of tailing pond water. Lai et al. used a simple compound (linear Z=0 with 2 rings) in various ages of tailings pond water. Lai et al. reported a link between biodegradation and toxicity reduction. The 2-ringed compound showed no decrease in toxicity before and after the degradation study. The straight chained compound degraded in the fresh tailings pond water and lower temperatures reduced the degradation rates (Lai et al., 1996). Clemente et al. (2005) reviewed the research to date and reported that a surrogate or model compounds added to naphthenic acid extract from tailings pond water showed no decrease in toxicity by Microtox™ evaluation. Often in this research, due to the analytical limitations, biodegradation was observed in terms of microbial activity and substrate utilization was not monitored. Biokinetic information was not reported in these studies. A significant observation in these studies is the sensitivity of different toxicity evaluation methods. On the basis of the existing literature, a need emerged for better understanding of the factors controlling the kinetics of NA biodegradation and the results of toxicities. These gaps in the research formed the basis for this study.

1.1.3 Research Approach

As with many complex environmental concerns such as those presented by the polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons and ester phthalates,

scientists have addressed the complexity by means of segregation and/or simplification of the environment and control of the variables. This approach can be carried out by several means such as identifying indicator compounds or representative model compounds, using prepared simple mixtures of model compounds or initially starting with a single, simple structured compound with physical properties representative of those naturally occurring. The latter approach often is conducted with a safe and cost effective candidate compound that can be easily acquired and studied in a laboratory setting prior to scale up or observation in the real environment.

In the case of environmental concerns related to hydrocarbon contamination, this approach has been taken by many federal organizations including Health Canada, Environment Canada as well as with the provincial regulatory agencies. An example of this approach includes toxicological evaluation and remediation studies based on critical contaminants of concern such as benzene, toluene, ethyl benzene and xylenes to represent the light petroleum hydrocarbons (Canadian Council of Ministers of the Environment, 2006). Similarly, benzo(a)pyrene is often used as an indicator of PAH contamination when assessing the toxicity and hazards associated with petroleum contaminated sites. A similar approach has been used with the assessment of human health risks by ester phthalates. For the evaluation of the biodegradation process of NAs, this investigation has also used this approach with the evaluation of a single NA compound, trans 4-methyl-1-cyclohexane carboxylic acid. Oil sands acid extracts are known to contain over 5000 components covering a range of molecular weights and ring structures (Headley and McMartin, 2004). The rationale for the selection of this compound is described in detail in Section 1.5.1. The insights gained by the study of a single compound may not necessarily be directly applicable to other components in such a complex mixture nor should the results be extrapolated blindly. However, the use of a single compound as an initial kinetic assessment tool is of significant use in future kinetic studies of other single components and ultimately mixtures.

1.2 Biodegradation

Interest in the microbial biodegradation of pollutants has intensified in recent years as scientists and engineers strive to find sustainable ways to cleanup contaminated environments. Biodegradation as a treatment method is non-destructive, cost- and treatment-effective and often a logistically favourable cleanup technology. The field of biodegradation of contaminated soil, groundwater, surface water and air is expanding rapidly. Currently, a significant portion of environmental research is focused on contaminants in soil, wastewater and groundwater resulting from the processing of crude oil products. Biotechnologies for treatment of contaminated sites can be classified as in-situ or ex-situ. Bioremediation is widely recognized and utilized in the remediation of petroleum contaminated sites in Canada. Research focused on biodegradation of the NAs is being undertaken in several areas. Together with characterization of the NAs, research efforts are beginning to be targeted at the biodegradation process naturally occurring in the tailings pond and its relationship with time (Holowenko et al., 2002). Preliminary analyses of the biodegradation rate for two specific NA isomers in Athabasca River water have been carried out (Tanapat, 2001). Tanapat's research demonstrated that chemical structure has a significant effect upon biodegradation rates for the isomers studied.

Research to date has revealed some success with the biodegradation of commercial grade NA preparations (Clemente, et al. 2004); however, this observed biodegradation does not correlate with biodegradation of the oil sands NAs. Published research indicates the potential for microbiological degradation of the tailings NAs as indicated by the presence of microbial communities (Herman et al., 1994; Lai et al., 1996; Meredith et al., 2000). These studies indicate that biodegradation in unamended wastewater will decrease the acute toxicity. NAs have been observed to be recalcitrant in aquatic environments. Hence, the observed rates of biodegradation do not meet with the wastewater production increases resulting in the increase in tailings retained at the mining sites. As a result, alternative or enhanced biodegradation techniques are required. Enhanced biodegradation of other persistent environmental contaminants associated with the petroleum industry has been demonstrated with the use of ex-situ bioremediation techniques in well designed bioreactors (Purwaningsih, 2002; Riess et al., 2005).

Differences in the chemical composition of oil sands tailings extract and commercially prepared mixtures of NAs may provide an explanation for the greater resistance of oil sands tailings to microbial attack. The rate of biodegradation may be affected by the chemical structure of the NAs and other oil sands acids present in the contaminated tailings as compared with that of the commercial mixtures. Given the diversity of NA compounds, changes in the chemical structure during biodegradation are related to the toxicity of the mixture. Although NAs are known to be persistent biomarkers used in identification of oil source maturation, little is established regarding their relative degradation pathways and kinetics in aquatic environments (Headley and McMartin, 2004).

1.3 Bioreactors

Due to varying and adverse environmental conditions, often in-situ biodegradation is a very slow process and can not be effectively controlled. Where environmental conditions are not amenable to in-situ treatment of contamination, ex-situ processes are considered. Treatment of contaminated soil and liquid streams in controlled bioreactors has proven to be an effective approach in reducing the concentration of contaminants. The design of bioreactors has often been used to enhance the biodegradation rates of environmental contaminants in wastewaters (Quail and Hill, 1991). In similar cases involving environmentally persistent contaminants related to petroleum operations such as PAHs and volatile organic compounds (VOCs), biodegradation has been studied with candidate compounds such as phenol, naphthalene, methyl napthalenes, pyrene, toluene and ethanol (Quail and Hill, 1991; Nikakhtari and Hill, 2006; Yu et al., 2006; Clarke et al., 2007). These cases have also evaluated the biodegradation kinetics with the use of various types of bioreactors such as the continuously stirred tank reactor (CSTR), roller bioreactors, batch reactors, packed bed reactors, and fluidized bed reactors. In recent years, biodegradation research has shown that superior performance in bioreactors supporting high biomass hold-ups such as packed bed reactors, fluidized bed reactors and other types of immobilized cell reactors

(ICRs) can be achieved. High biomass hold-ups are achieved by immobilization of the cells on the surfaces of a suitable carrier matrix (Quail, 1990).

In the case of recalcitrant contaminants, biodegradation limitations need to be identified and characterized so that the degradation can be stimulated either by supplementing the contaminated waste waters or by constructing bioreactors (Folsom et al., 1990). Characterization of the degradation kinetics is a necessity for the design of the large scale systems.

In this study, a single naphthenic acid compound and a microbial culture developed in our laboratory were used to study the biodegradation of a candidate NA compound and to evaluate the kinetics of the process in several bioreactor configurations. These systems were studied under aerobic conditions. The fundamentals of each of these bioreactor systems are well known and described in previous studies (Nauman, 2001; Shuler and Kargi, 2002; Gadekar et al., 2006). Details of the theoretical modelling of each bioreactor are described in detail in the relevant chapters.

1.4 Objectives

As a result of the complexity of the mixture, the current analytical delays required in the quantification of the mixture and the relevance of a commercial mixture as a surrogate for the tailings pond NAs (Scott et al. 2005; Armstrong, S.A. 2008), the research activities conducted within the scope of this project include the study of the biodegradation factors of a single naphthenic acid compound, the associated kinetic parameters and modelling the treatment of this compound in three bioreactor systems: batch reactors, a continuously stirred tank reactor and immobilized cell reactors.

1.5 Biodegradation Experiments

1.5.1 Preliminary Experimental Work

Within the first year of this study, biodegradation of a commercially available NA mixture (Fluka Standard) was studied. While biodegradation of the mixture was apparent,

quantitative assessment of the kinetics was difficult to achieve due to uncertainty in measurement of the Fluka NA concentrations as a result of interference from the media. As well, intermediate sample preparation steps such as solid phase extraction (SPE) presented a significant time delay. This time delay was not conducive to attaining thorough kinetic data for microbial growth.

The purpose of this preliminary work was to quantify the biodegradation of NAs and study the effects of bioreactor design on the extent of biodegradation with the use of a commercially available NA standard and ultimately NAs extracted from the tailings pond water. However, as a result of the complexity of the mixture, the ongoing criticisms of using a commercial grade NA mixture as a surrogate, the research activities were revised to study the kinetics of biodegradation of a candidate NA and verification of the associated kinetic parameters.

Several NA compounds were evaluated to find a suitable individual NA compound for kinetic studies. Low molecular weight components in oil sands naphthenic acids, such as 4-methyl-1-cyclohexane carboxylic acid (4MCHCA) are known to be biodegradable in aquatic environments. The trans-isomer of 4MCHCA (referred to herein as trans-4MCHCA) was thus investigated in this study, to elucidate factors controlling the kinetics of biodegradation using batch cultures. The evaluated factors included concentration of the substrate, pH and temperature. An earlier research study indicated that the trans-isomer would be more amenable to biodegradation than the cis-isomer (Headley et al., 2002b). The chemical formula for trans-4MCHCA is $CH_3C_6H_{10}CO_2H$ with a formula weight of 142.2 $g \cdot mol^{-1}$. It has a single ring structure fitting the formula of NAs where the *Z* series equals -2 (CAS Number 13064-83-0). Figure 1.1 represents the molecular structure of trans-4MCHCA.

During the experimentation with individual NA compounds a suitable analytical tool was developed in our laboratories to provide timely quantification of biodegradation of the candidate compound. As well at this time, a suitable means of biomass

quantification was developed. Details of the biomass calibration curve for the consortium used in this study are presented in Appendix A.

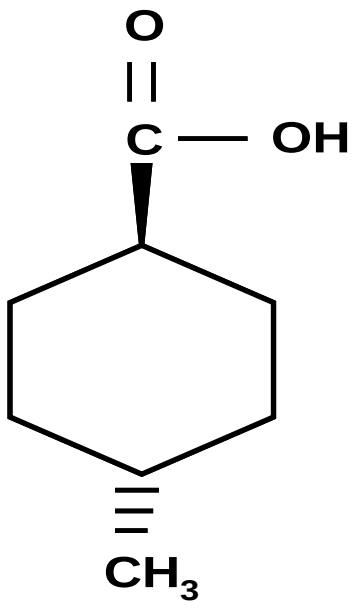

Figure 1.1 Molecular structure of trans 4-methyl-cyclohexane carboxylic acid (Sigma-Aldrich).

1.5.2 Batch Experimentation

Initially, microbial growth was monitored for several commercially available candidate NA compounds in 250 ml shake flasks each containing 100 ml of medium, 100 $mg \cdot l^{-1}$ of substrate in solution and 10% inoculating culture (v/v). Upon development of a microbial consortium capable of degradation of the substrate at varying concentrations, experiments were conducted in batch reactors. The characteristics of a batch reactor are that neither inflow or out flow of reactants or biomass occurs during the experiment. Cells are cultured in the reactor with an initial volume that is not altered by further nutrient addition or removal.

Batch experiments for this study were carried out in 500 ml shake flasks each containing 150 ml of media with varying substrate concentrations (50, 100, 250, 500 and 750 and 800 $mg \cdot l^{-1}$). The range of concentrations evaluated, which are significantly higher than the concentrations observed currently in tailing pond waters (20 to 120 mg

NAs·l^{-1} (Clemente et al., 2005)) allow for determination of kinetic principles and limiting conditions for the selected substrate. Vigorous mixing was achieved by a magnetic stirrer (200 rpm). Shake flasks were maintained at room temperature (23±2^{o}C) with a pH of 7.

Based on the results of the initial substrate concentration experiments, the analytical method developed for the study and available funding resources, two initial substrate concentrations were selected for the remaining experiments (250 mg·l^{-1} and 500 mg·l^{-1}).

Upon completion of the room temperature experiments, additional batch experiments were carried out in controlled environments where the temperature was adjusted to: 4, 8, 12, 16, 23, 30, 37, 40 and 42^{o}C. For these temperature studies, the initial substrate concentration remained constant at 500 mg·l^{-1} and similarly adjusted to a neutral pH. Samples were collected at intervals and tested for optical density and substrate concentration.

Batch experiments were also carried-out to assess the effects of initial pH (5.5, 7.0, 8.5, 10.0, 11.0, and 13.0) at room temperature (23^{o}C) on the activity of the bacterial culture and biodegradation of trans-4MCHCA. The initial substrate concentrations for these experiments were 500 mg·l^{-1}. Data obtained from the batch experiments were evaluated to determine specific growth rates and yield for the various environmental conditions.

1.5.3 Continuously Stirred Tank Reactor

Previous studies on biodegradation indicate that the stirred tank reactors are an effective type of bioreactor for the evaluation of biodegradation of soluble contaminants (Purwaningsih, 2002). A continuous flow bioreactor was used to determine the kinetics at a steady state in the well mixed system. The CSTR is suitable for aerobic, continuous flow, well mixed biodegradation studies. The reactor is utilized in its simplest form as a chemostat reactor. A working volume of 550 ml was used to minimize the volume of stock solution required.

Initially the bioreactor was operated in batch mode. Once complete substrate removal was achieved, the bioreactor was operated in continuous mode. Feed stock solution was pumped into the reactor at an initial flow rate of 1.0 $ml \cdot h^{-1}$ and incrementally increased to a maximum flow rate of 22 $ml \cdot h^{-1}$ (overall 12 flow rates were tested). Substrate and biomass concentrations were monitored on a daily basis. The pH was adjusted daily to maintain the reactor at approximately 7. Each flow rate was maintained until steady state conditions were attained by observation of residual substrate concentration and the biomass concentration by means of optical density. Steady state conditions were maintained for a minimum of three residence times or three days. During steady state, the biomass conditions were monitored by most probable number and plate count. The flow rate was then increased stepwise until cell wash-out was observed. After the maximum experimental residual substrate concentration was observed, the CSTR reactor flow rate was decreased to 10 $ml \cdot h^{-1}$ again in order to asses the reproducibility of the results. The reactor was also operated prior to shut down in batch mode to provide biomass for the development of the biofilms used in the ICRs described in Section 1.5.4 below. Data obtained by the CSTR experimentation provided the basis for determining a theoretical kinetic model that was best fit to provide experimental parameters.

1.5.4 Immobilized Cell Reactors

Three ICRs were used to evaluate the suitability of a biofilm for biodegradation of trans-4MCHCA and the performance of this type of reactor in comparison with the freely suspended cell system. Additionally, the kinetic parameters determined using the kinetic data generated in the CSTR were used to model the performance of the ICRs. The benefit of modelling the bioreactor is to predict accurately the experimental trend in steady state biodegradation operations. This mathematical model can be used to evaluate limitations in the system and determine the relative sensitivity of the variable parameters. This tool could serve to aid in the future evaluation of bioreactors and potentially other NA compounds.

The biofilm was developed by trickling partially degraded effluent from the CSTR over 35 g of stainless steel packing material for a two month period. At this time, the packing was removed and divided into three sections for three ICRs operated in submerged mode with forced air. The steel packing material had a porosity of 99% in each ICR. Two ICRs were operated at two initial substrate concentrations (500 $mg \cdot l^{-1}$ and 250 $mg \cdot l^{-1}$) of trans-4MCHCA for approximately three months. Each reactor was studied concurrently at increasing flow rates to determine the substrate removal rate at various steady state conditions. The flow rates were increased step-wise to a maximum of 100 $ml \cdot h^{-1}$ in order to find the maximum volumetric reaction rate. Steady state points were determined in a manner similar to that described in Section 1.5.3.

During the entire study, the third ICR served to assess the reproducibility of the results at selected flow rates. When the reactor was not running as a duplicate system, it was used for complete biodegradation of the effluents from the CSTR and the other two ICRs for the purpose of safe disposal.

During operation of the ICRs, microbial identification was carried out in order to identify the dominant microbial species capable of degradation of the candidate NA at the designated substrate concentrations. Throughout experimentation, the reactors were sampled daily for residual substrate concentration and freely suspended biomass in the liquid collected from the bottom of the reactor. On a daily basis the pH and air flow rate were monitored and adjusted as required to maintain neutral and aerobic conditions in the reactor. At steady state conditions, which were achieved typically every 5 to 7 days in the absence of any operational interruptions, the biomass concentration was also assessed by most probable number method and the diversity of the consortium was monitored by plate counts.

Upon disassembly of the ICRs, the reactors were drained for 1 hour to determine the volume of liquid. Following the measurement of the liquid volume the packing material with biofilm was carefully removed from the bioreactor and weighed to obtain the total weight of the dry biomass in each reactor. One reactor was retained for a

subsequent industrial related experiment described in Section 1.5.5 and then dismantled for determination of the dry biomass weight.

1.5.5 Tailing NA Extract Experiment in an ICR

Subsequent to completion of the ICR experiments, a two week preliminary study was carried out to utilize the established biofilms for degradation of oil sands tailings NA extract. This experiment was conducted at an investigatory concentration of 100 $mg \cdot l^{-1}$ and a flow rate of 5 $ml \cdot h^{-1}$ in order to collect samples which were analyzed with currently accepted methods for quantitatively evaluating NAs in aqueous solutions. The results of this work are presented in Appendix B.

1.6 Toxicity Reduction Evaluation

The purpose of toxicity tests in this biokinetic study was to identify any reduction in toxicity of the bioreactor effluent. The *Artemia salina* test (Toussaint et al., 1995) was used as a rapid screening toxicity test similar to that used for previous studies of bioreactor effluents (Tarighian et al., 2001; Riess et al., 2005). At the time that degradation was established and the biokinetic studies were underway, toxicity tests were conducted for the various reactors to evaluate the effects of treatment on the toxicity of effluent from each reactor. The *A. salina* test was used for each reactor to give an indication of toxicity reduction in comparison with other bioreactor performance. However, consideration was given to a test organism representative of a freshwater ecosystem within the scope of the project. *Daphnia magna* was selected as the fresh water test organism. Selected *D. magna* tests were conducted in a regulated toxicity testing facility. The endpoint of this test was the LC50 which in addition to providing information of the toxicity of the candidate compound (which has not been studied to date) it also provided a quantitative toxicity reduction evaluation of reactor performance. A complete study of the toxicity of the candidate NA compound was outside the scope of the biokinetic study.

In accordance with current regulatory guidelines in Alberta (Department of Energy, Government of Alberta, 2007) for the disposal of oil related waste, another commonly conducted rapid screening test, Microtox™, was undertaken. The purpose of this test was to conduct a toxicity screening test with a quantitative endpoint for comparison with both the reactor performance and the LC50 of a freshwater organism as well as comparison with the *A. salina* which is also a marine organism. In a study comparing *D. magna*, trout and Microtox™, the IC20 most closely compared with trout toxicity (10% v/v vs. LD50 of 7% v/v). The *D. magna* test was most sensitive with an LD50 of 2% (v/v) in tailings water (Clemente and Fedorak, 2005).

The toxicity tests conducted for the evaluation of the biological support medium, the candidate NA influent and bioreactor effluents were selected in such a manner to efficiently obtain information on the toxicity reduction of the bioreactor on the candidate NA compound and to act as another means of quantitatively comparing reactor efficiencies.

The steps of the *A. salina* toxicity test were as follows (Toussaint et al., 1995). Brine shrimp eggs purchased from a local pet store were added to 100 ml of reverse osmosis (RO) water containing 25 $g \cdot l^{-1}$ NaCl. This flask was kept at room temperature and was aerated for approximately 36 hours. After 36 hours, 200 µl of the brine shrimp solution were added to two rows of a 96 well micro array plate (2 rows containing 8 wells per row) using an eight channel multi-pipettor. An equivalent volume of the evaluated effluent was added to each well in a similar manner. Controls were used with the medium, the *A. salina* support medium and the contaminant solution at the concentration of the input feed to the bioreactor. All motile shrimp were counted using a 7X magnification Optivisor™ headset. Shrimp visibly moving during observation were said to be alive. Those not moving were said to be dead and were not counted. The test was performed over five hours with a single concentration of the effluent.

Daphnia magna and Microtox™ tests were conducted by a certified environmental analytical company (ALS Laboratory Group Environmental Division).

Microtox™ carried out by ALS Laboratories was conducted in Grand Prairie, Alberta in accordance with Directive 050 of the Alberta Energy and Utility Board Appendix 4, (1996). Microtox™ was carried out within 48 hours of shipping. *D. magna* carried out in Winnipeg, Manitoba was specified in accordance with Environment Canada protocol (2000). *D. magna* tests were conducted over 48 hours within 24 hours of shipping.

1.7 Statistical Analyses

Statistical analyses of experimental data are reported throughout this thesis in terms of mean values ± one standard deviation. With assumption of a normal distribution, this interval has a 68.26% confidence interval. In biological studies, it is common to use this level of confidence (Nikakhtari, 2005a). In selected cases, the 95% confidence interval has been reported or plotted to illustrate more conservative limits in variance. Linear and non-linear regression analyses were conducted within Excel™ software by methods described in Brent (1977) and Kennedy and Neville (1964).

1.8 Layout of Thesis

This thesis has been prepared in manuscript style with 4 chapters forming the sequential progression of this study. Initially, a suitable analytical tool was developed to permit quantitative observation of the biodegradation of an individual NA compound and is presented in Chapter 2. Following the development of a timely analytical method, batch experimentation was carried out to assess the influence of temperature, pH and initial concentration on the biodegradation of the selected NA compound. The experimental portion of this component of the study is presented in Chapter 3 together with determination of kinetic information such as specific growth rates and yield. During experimentation in the batch system, the microbial consortium capable of biodegrading the NA under varying environmental conditions was developed. With the viable microbial consortium, experimental determination of the kinetic parameters of the NA compound by means of the CSTR was studied and reported in Chapter 4 together with the experimental results from the ICRs. Evaluation of biodegradation with ICRs is compared with the results of the CSTR. Chapter 5 presents the modelling of the

performance of the ICR using the kinetic information obtained in Chapter 4. Chapter 6 presents overall discussion of the results and recommendations for future research. References have been compiled for the entire thesis at the end of this document.

CHAPTER 2
QUANTIFICATION OF TRANS 4-METHYL-1-CYCOHEXANE CARBOXYLIC ACID IN WATER BY GAS CHROMATOGRAPHY-FLAME IONIZATION DETECTION

Contribution of PhD Candidate

The analytical method presented herein has been solely designed to facilitate the quantification of the candidate naphthenic acid (NA) compound studied. The GC-FID method was modified for the purposes of this study by Janice Paslawski with technical support and guidance from Dr. G.A. Hill, R. Blondin and K.M. Peru. All written text was prepared by Janice Paslawski with editorial guidance from Dr. M. Nemati, Dr. G.A. Hill and Dr. J.V. Headley.

Contribution of this Chapter to the Overall Study

This chapter describes a simple analytical method (gas chromatography flame ionization detection (GC-FID)) for the analysis of a candidate naphthenic acid compound, specifically 4-methyl-1-cyclohexane carboxylic acid (trans isomer). This new method for direct analyses of a single NA compound in media used to support microbial growth has been successfully used in this study for the purposes of evaluating the biokinetics of the degradation of a the NA compound. The sensitivity of the method was well suited for the monitoring and degradation of the NA compound and facilitated manipulation of the bioreactor operating conditions for evaluation of kinetics.

Keywords: naphthenic acids; gas chromatography flame ionization detection; biodegradation

2.1 Introduction

Environmental assessment of naphthenic acids (NAs) is currently limited by the analytical methods available for characterization of the principle toxic components in complex oil sands mixtures. There is a need to further develop routine and rugged methods for the characterization of oil sands NAs present in natural waters (Headley et al., 2007). Quantitation has been originally achieved using Fourier transform infrared (FT-IR) spectroscopy (Hao et al., 2005 references therein). Concurrently, qualitative methods have been carried out with gas chromatography-mass spectrometry (GC-MS) (Lo et al., 2003; Hao et al., 2005; Headley et al., 2007). Some of the current methods for detecting NAs in water require complex preparation procedures including liquid-liquid extraction and solid-phase extraction (SPE) (Bataineh et al., 2006; Merlin et al., 2007).

A variety of mass spectrometric techniques have been used for the study of the NAs within a given sample, including GC-MS, electron ionization (EI), liquid secondary ion mass spectrometry (LSI-MS), fast atom bombardment (FAB), chemical ionization (CI), atmospheric pressure chemical ionization (APCI) and recently electrospray ionization (ESI) and quantitative quadrupole time of flight-MS (QTOF-MS) (Barrow et al., 2003; Barrow et al., 2004; Bataineh et al., 2006). All of the above methods have unique strengths and limitations.

Applications of ESI-MS, however, have emerged as the technique most widely used for studies of environmental samples (Headley et al., 2007). One of the limitations of the ESI-MS procedure is that quantification can be prone to matrix effects arising from the presence of salts and co-eluting components in complex oil sands mixtures. For example, this is particularly evident for quantitative analyses of biological samples. The ionization resulting from the component of media in which the NAs are being identified often interferes significantly with the quantitation of the NAs. While there are approaches to help eliminate such concerns, using for example, prior clean up of samples or internal standards, there is much research still needed to further develop accurate analytical methods for naphthenic acids in environmental samples.

NAs are difficult to analyze directly by conventional gas chromatography techniques because of their polarity and thermal instability of the carboxylate functional group. To circumvent this problem, NAs are commonly derivatized prior to analysis using GC-MS. However, with careful choice of instrumental conditions, GC-FID (flame ionization detection) can be utilized for direct analysis of the candidate naphthenic acid in water and biological media.

Research in soil, plant, groundwater and surface water environmental areas require the quantitative determination of NAs in both natural and synthetic waters such as ground water and surface waters as well as laboratory media used for the support of microbial activity. Examples of these areas of research include the evaluation of the adsorption characteristics of NAs in synthetic groundwater (Janfada et al., 2006), the uptake of NAs in plants (Armstrong et al., 2008) and the biodegradation of NAs in waste waters undertaken in this study (Paslawski et al., 2008).

The area of biodegradation of NAs is not clearly understood (Bataineh et al., 2006). Biokinetics is a field which requires timely analyses of substrate and biomass concentrations in order to evaluate microbial communities and their metabolic function. As an initial step in the understanding of the biokinetic of NAs, candidate compounds of NAs have been studied (Headley et al., 2002b; Bataineh et al., 2006). For the purposes of determining the biokinetics of degradation of the candidate naphthenic acid, direct analysis was desirable in order to minimize losses through extraction or derivatization steps.

In the previously mentioned studies, the media in which the NAs required quantitation included an array of nutrients and trace minerals. In order to address this background interference, SPE is undertaken as a preparatory step prior to quantitative analyses via ESI-MS. SPE requires a significant amount of handling time which delays access to the results. Additionally, the requirement of pH adjustments and the additional SPE step can compromise the nature of the sample (Janfada et al., 2006). For

quantification, FID has the main attraction since it is widely available in most analytical laboratories. Although not as ideally suited for characterization of NA mixtures as ESI-MS, FID has additional features of a wide linear range, uniform response and simple calibration procedure, owing to the fact that the detector response of hydrocarbons is minimally influenced by the chemical nature of the compound (Scott et al., 2005). In addition, the robustness of the FID (with appropriate injector and column conditions) to water makes it particularly useful for the direct detection of pollutants in natural water samples (Skoog et al., 1998), thus eliminating time consuming extraction procedures. For example, direct GC-FID analysis of samples has been reported for research of the biokinetics of polycyclic aromatic hydrocarbons in water (Riess et al., 2005; Nikakhtari and Hill, 2006; Yu et al., 2006).

2.2 Experimental

2.2.1 Chemical

For this study, the trans-isomer of 4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA) was selected as a candidate NA compound due to its availability and potential for biodegradation. The molecular formula for this compound is $CH_3C_6H_{10}CO_2H$ (CAS Number 13064-83-0) with a molecular weight of 142.20 Daltons. The candidate NA compound used in the study was purchased from Sigma-Aldrich (98% purity). Trans-4MCHCA is in a dry state and was prepared for the purposes of the biodegradation study in solution with a medium consisting of various reagent grade chemicals and trace minerals required to support the growth and activity of the bacteria used for the purpose of biodegradation. This medium composition was selected on the basis of previous studies (Hill, 1974). The composition of the pH neutral (7) medium is presented in Table 2.1.

Prior to investigation, a stock solution of 1,000 $mg \cdot l^{-1}$ trans-4MCHCA (which was determined to be the solubility limit in medium) was prepared in a 100 ml volumetric shake flask. Complete dissolution of 1,000 $mg \cdot l^{-1}$ of trans-4MCHCA required

approximately 24 hours of vigorous mixing on a magnetic stir plate at room temperature (23±2^{o}C).

Table 2.1 Modified McKinney's medium

Macronutrient Composition

Constituent	Nutrient Formula	Concentration ($mg \cdot l^{-1}$)	Nutrient Name
A	KH_2PO_4	840	Potassium phosphate monobasic
B	K_2HPO_4	750	Potassium phosphate dibasic
C	$(NH_4)_2HPO_4$	474	Ammonium phosphate dibasic
D	NaCl	60	Sodium chloride
E	$CaCl_2$	60	Calcium chloride
F	$MgSO_4 \cdot 7H_2O$	60	Magnesium sulphate heptahydrate
G	$Fe(NH_4)_2SO_4 \cdot 6H_2O$	20	Ferrous ammonium sulphate 6 hydrate

Trace Mineral Medium Micronutrient Composition		1 $ml \cdot l^{-1}$	Per litre of macronutrient medium

Constituent	Name	Concentration ($mg \cdot l^{-1}$)	Nutrient Name
A	H_3BO_3	600	Boric acid
B	$CoCl_2$	400	Cobalt chloride
C	$ZnSO_4 \cdot 7H_2O$	200	Zinc sulfate
D	$MnCl_2$	60	Manganese chloride
E	$Na_2MoO_4 \cdot 2H_2O$	60	Sodium molybdate
F	$NiCl_2$	40	Nickel chloride
G	$CuCl_2$	20	Copper chloride

2.2.2 Equipment

GC-FID analyses of trans-4MCHCA were performed with a dedicated Hewlett Packard (HP) GC-FID (5890 HP Series II with Chemstation operating software). The GC was operated with helium as the carrier and detector makeup gas (1.0 $ml \cdot minute^{-1}$ and 29 $ml \cdot minute^{-1}$, respectively), hydrogen and medical grade (breathing) air were used as

the combustion gas on the FID. The inlet was operated in splitless mode (purge valve on at 1.00 minutes). Good separation was achieved using a HP-INNOWAX Polyethylene Glycol (19091N-113) column manufactured by Agilent J&W Scientific and distributed by Agilent Technologies. The column specifications were as follows:

Length: 30 m
Inside Diameter: 0.32 mm
Film thickness: 0.25 μm

Operating conditions were as follows:

Inlet temperature: 220^{o}C
Column oven temperature: 225^{o}C
Detector temperature: 245^{o}C
Column oven temperature program: 90^{o}C for 1.10 minutes, ramped to 225^{o}C at 25^{o}C·minute^{-1} and held for 2.40 minutes.
Sample injection volume: 0.5 μL

The noted operating conditions resulted in a retention time of 7.6±0.3 minutes. The run time per injection was 8.9 minutes.

Calibration was conducted with seven standard solutions prepared using the stock solution described previously. The concentration of trans-4MCHCA in the standard solution ranged between 25 mg·l^{-1} and 1,000 mg·l^{-1} (25, 50, 100, 250, 500, 750 and 1,000 mg·l^{-1}). Each standard solution was injected 3 times from the minimum to the maximum concentration. Between each standard, 3 injections of Millipore water were run to prevent the possible accumulation of the substrate which may contribute to carry-over during analysis of samples. Using the standard solutions a linear calibration curve was observed with a regression coefficient of 0.9995.

The retention time of the trans-4MCHCA on the column was 7.6±0.3 minutes. Determination of the area within the substrate peak was carried out by integrating the

area under the peak occurring between 7.3 minutes and 7.9 minutes. This range allows for any slight shifts in the retention time over the course of the inlet liner which may arise from use between replacement and/or cleaning. Column replacement or maintenance was not required during the course of this study (15 months).

A 5 μL syringe was used for sample injection and the injection volume was 0.5 μL. This injection volume was found to be optimum for sensitivity and facilitated direct analyses of the candidate compound in a water based medium without extinguishing the flame. In order to minimize the accumulation of the medium salts on the plunger of the syringe, between analyses Millipore water was injected after analyses of a maximum of 5 samples (3 injections per sample). Typically, corrosion of the syringe causing the plunger to bind and malfunction does not occur during continuous operation but only during the period when the GC-FID is not operated. In order to effectively prolong the use of the syringe, the syringe was removed between runs and cleaned subsequent to use. The cleaning of the syringe was best achieved by rinsing or soaking in Millipore water and intermittently cleaned by vacuuming Millipore water through the syringe. Once the plunger moved freely through the syringe, the syringe was stored until subsequent use. Typically, with proper care, a syringe could be used up to 4 months (approximately 2,000 injections).

The glass inlet liner (Supelco Splitless Injection Sleeve with a Double Taper Part No. 2-0485, 05) was sealed with a Supelco Thermo-O-Ring™ 6.35 mm outer diameter (Part No. 21003-U) which provided a leak free seal between the inlet liner and the injection port body. With numerous injections, the liner tends to accumulate residual salts from the medium which in turn will attract the trans-4MCHCA during analyses. Build up of analyte on the inlet can be monitored by two means. First, the concentration measured on a Millipore blank analyzed between substrate samples would normally report background levels (2 to 5 $mg \cdot l^{-1}$) at the end of 3 injections. This cleaning procedure would record a higher concentration on the third injection (up to 10 $mg \cdot l^{-1}$) indicating that the inlet and column could not be cleaned with 3 injections of the Millipore blank and cleaning or replacement of the inlet would be required. A second indicator used as a

means of identifying analyte and mineral retention on the inlet was based on the reported concentration for the first injection of Millipore water subsequent to the substrate sample injections exceeding 10 $mg \cdot l^{-1}$. This observation would indicate that a substantial amount of analyte was being retained on the inlet liner from the previous batch of samples. The third injections of Millipore water were used as the basis for determining the background noise for the method.

In order to minimize the uncertainty in the quantitation of the substrate concentration and the amount of residual accumulation on the liner, the liner should be cleaned or replaced after approximately 500 injections. When the variation in the three injections of a given sample exceeds 10 $mg \cdot l^{-1}$ or the accumulation of analyte on a Millipore water blank series of injections following the sample analyses exceeds 10 $mg \cdot l^{-1}$, the liner and septum should be changed.

Furthermore, in order to minimize the accumulation of the medium salts on the syringe and inlet liner, the samples are diluted to within the expected range of 60 $mg \cdot l^{-1}$ and 200 $mg \cdot l^{-1}$ in order to prolong the life of the column, inlet liner and the syringe. In the event that the sample concentration is unknown, samples are analyzed in the order of expected increasing concentration. The column and syringe are injected with Millipore water after a maximum of five samples. The accumulation of analyte on the first and last (for the purposes of this study, three injections of each sample were used) Millipore water injections are used to determine the reliability of the unknown sample concentration. Typically, the accumulation of analyte on the column and liner that would be carried over onto the first Millipore blank injection will not exceed 10 $mg \cdot l^{-1}$. This carryover effect will be cleared by the third injection to an average background noise residual concentration equivalent to 3.56 ± 0.91 $mg \cdot l^{-1}$. In the instance that a sample with an unexpectedly high concentration is injected through the column (for example in excess of 200 $mg \cdot l^{-1}$) the samples may be re-analyzed with Millipore water injections between each sample to ensure the reliability of the analyses.

Verification of the applicability of this method to other candidate NA compounds, specifically those candidate compounds with a more complex molecular structure, was considered with a preliminary evaluation of a second candidate compound. The compound used for verification of the method was 2,2-dicyclohexylacetic acid (2DCHAA) ($C_{14}H_{24}O_2$) which represents Z= -4, a two ringed candidate NA. 2DCHAA (CAS No. 52034-92-1, 99% purity) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.3 Results and Discussion

A new method for direct analyses of a candidate NA compound in media used to support microbial growth has been successfully used in this study for the purposes of evaluating the biokinetics of the degradation of the candidate NA compound. The following section presents results of the application of the GC-FID method for the biodegradation study of trans-4MCHCA by a microbial consortium.

Using the standard solutions, a linear calibration curve was developed (regression coefficient of 0.9995). Figure 2.1 presents the linear calibration of trans-4MCHCA standards prepared in the support medium up to the solubility limit of 1,000 $mg \cdot l^{-1}$. As illustrated in Figures 2.2 through 2.6, the method was well suited to monitoring the growth and utilization of trans-4MCHCA by bacteria in a biological medium. Figure 2.2 shows a typical GC-FID chromatograph for a 100 $mg \cdot l^{-1}$ trans-4MCHCA standard eluting at approximately 7.6 minutes. The candidate compound was readily detected and resolved from components eluting in the biological medium.

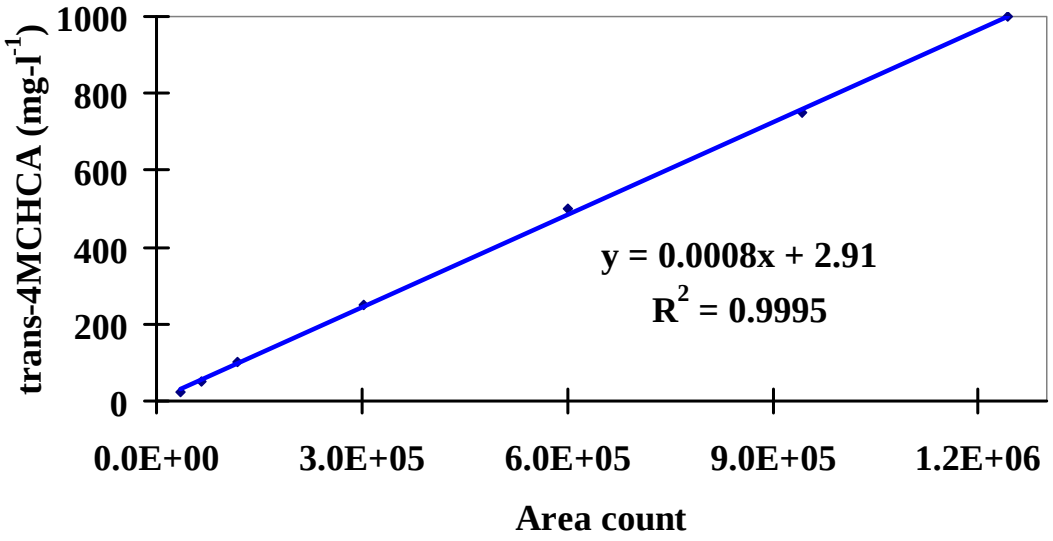

Figure 2.1 Calibration curve trans-4MCHCA.

The average standard deviation of 3 injections of unknown samples collected over seven months of a continuous bioreactor study (308 samples) was 3.64 $mg \cdot l^{-1}$ with a standard deviation of 0.48 $mg \cdot l^{-1}$. The corresponding background noise observed for the 3rd injection of Millipore water blank analyzed between samples was 3.56 $mg \cdot l^{-1}$ with a standard deviation of 1.67 $mg \cdot l^{-1}$. The method detection limit as calculated by the maximum noise observed plus three standard deviations of the observed noise was 11.2 $mg \cdot l^{-1}$. The sensitivity of this method was thus well suited for the monitoring of degradation of the NA compound. The method facilitated efficient manipulation of the bioreactor operating conditions for evaluation of kinetics. For illustrative purposes, an example of a batch kinetic experiment conducted with an initial substrate concentration of 100 $mg \cdot l^{-1}$ is presented in Figure 2.3. The degradation was monitored by GC-FID analysis of the samples at a minimum frequency of 24 hours throughout the course of the experiment with increased sampling frequency (6 hours to 12 hours) during the exponential growth phase where the maximum substrate utilization coincided.

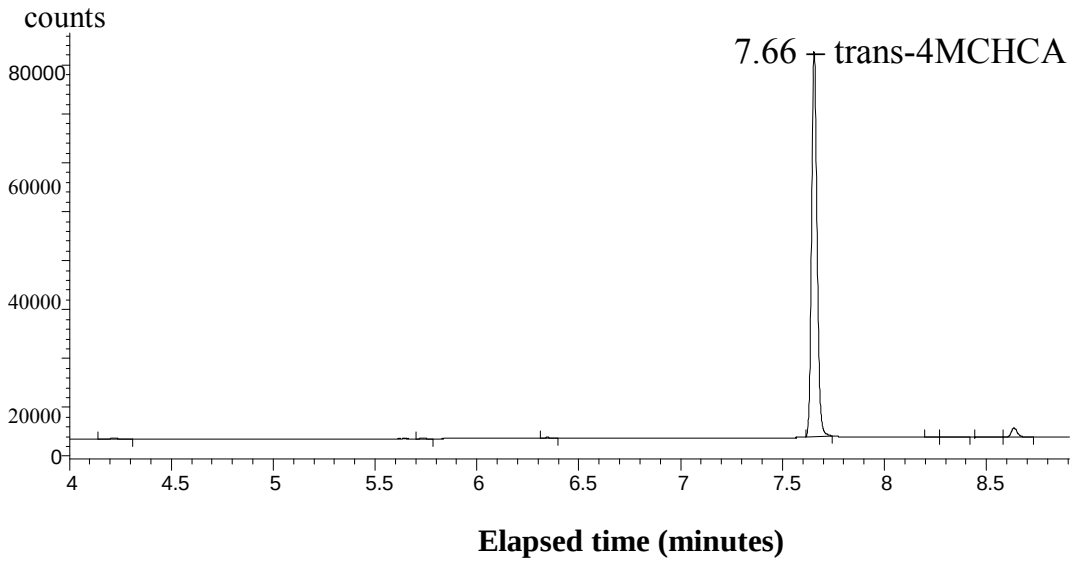

Figure 2.2 Chromatogram of 100 $mg \cdot l^{-1}$ analyte standard.

The sensitivity of the method was also sufficient to monitor the subsequent biodegradation in a bioreactor, as illustrated in Figure 2.4, showing the chromatogram of the resultant effluent. For comparison, to assess the contribution to carry over that must be monitored, a chromatogram of Millipore water injected after analyses of the analyte is presented in Figure 2.5. Similarly, for comparative purposes, a chromatograph of sterile medium in the absence of the analyte is presented in Figure 2.6. The maximum background noise for the chromatographs of the sterile medium observed (2.77 ± 0.17 $mg \cdot l^{-1}$) was less than the average background noise for Millipore blank injections (3.56 ± 1.67 $mg \cdot l^{-1}$) supporting the usefulness of this method.

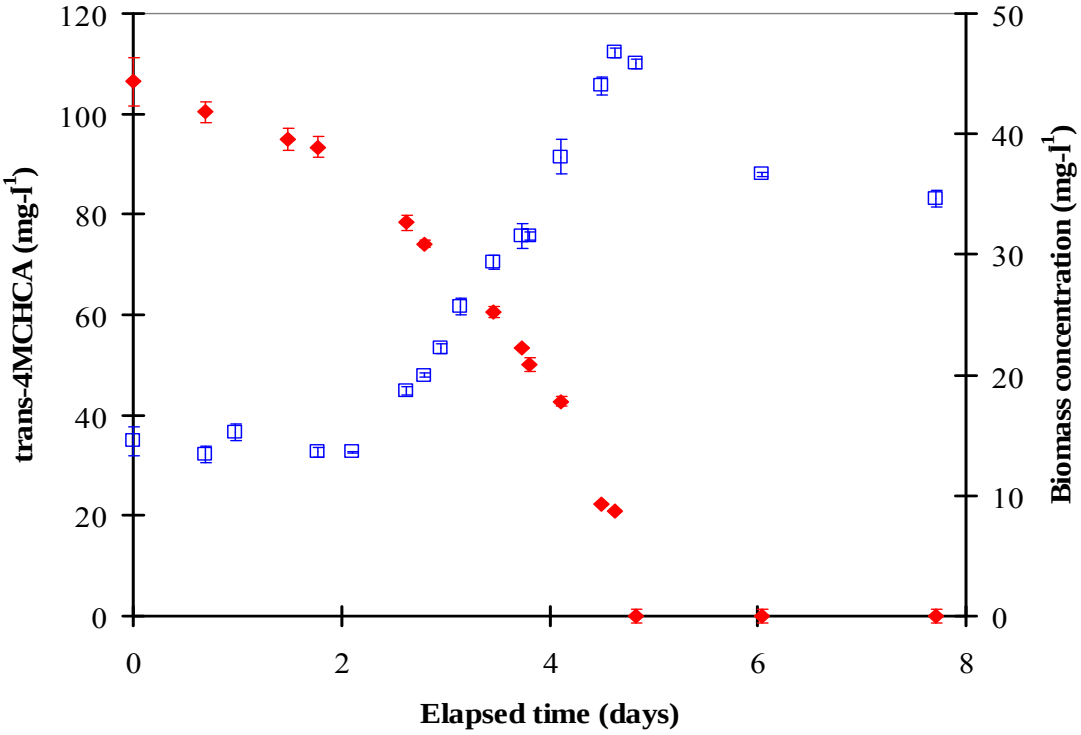

Figure 2.3 Batch experimental degradation of trans-4MCHCA with time (initial substrate concentration of 100 mg·l^{-1}). ♦represents concentration of trans-4MCHCA; □ represents biomass concentration; error bars represent 95% confidence intervals.

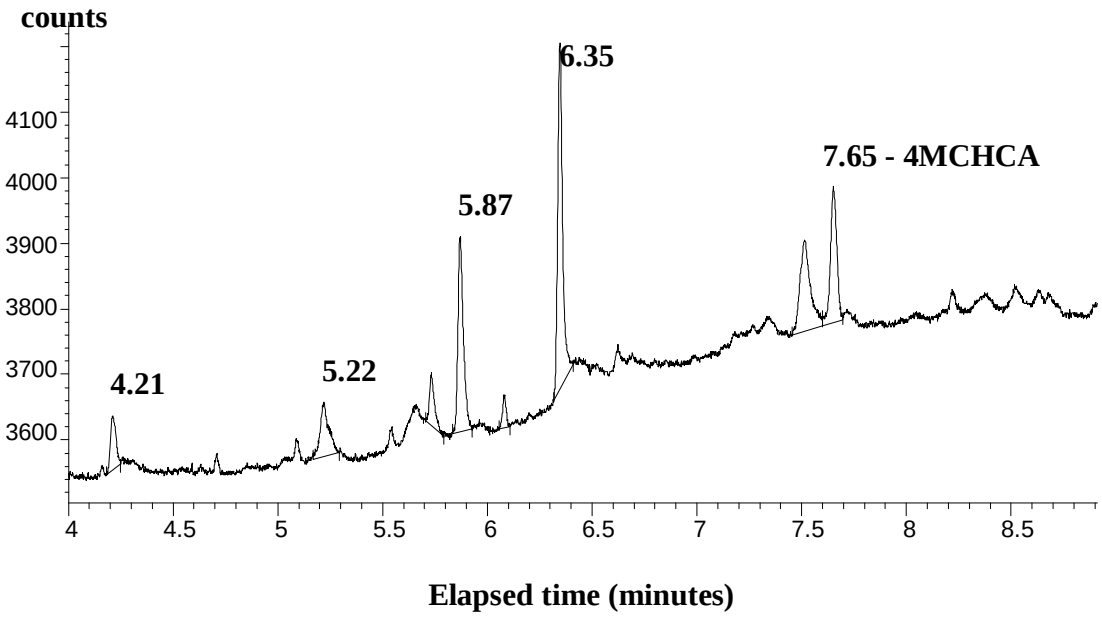

Figure 2.4 Chromatogram of a fully degraded trans-4MCHCA effluent sample.

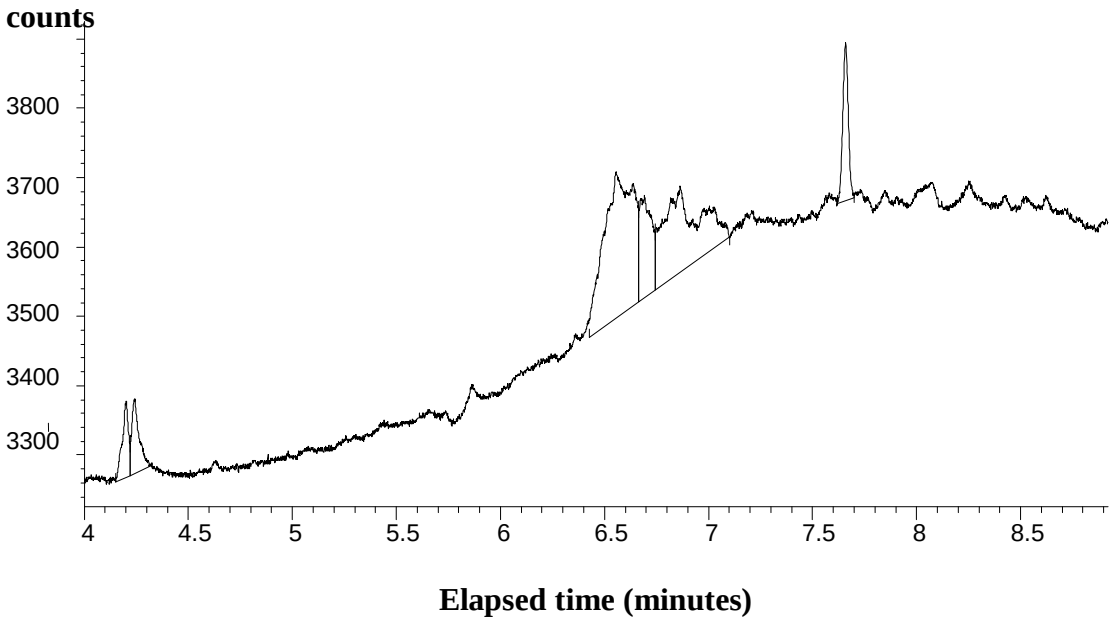

Figure 2.5 Chromatogram of Millipore water 3rd injection following analyses of unspecified trans-4MCHCA samples.

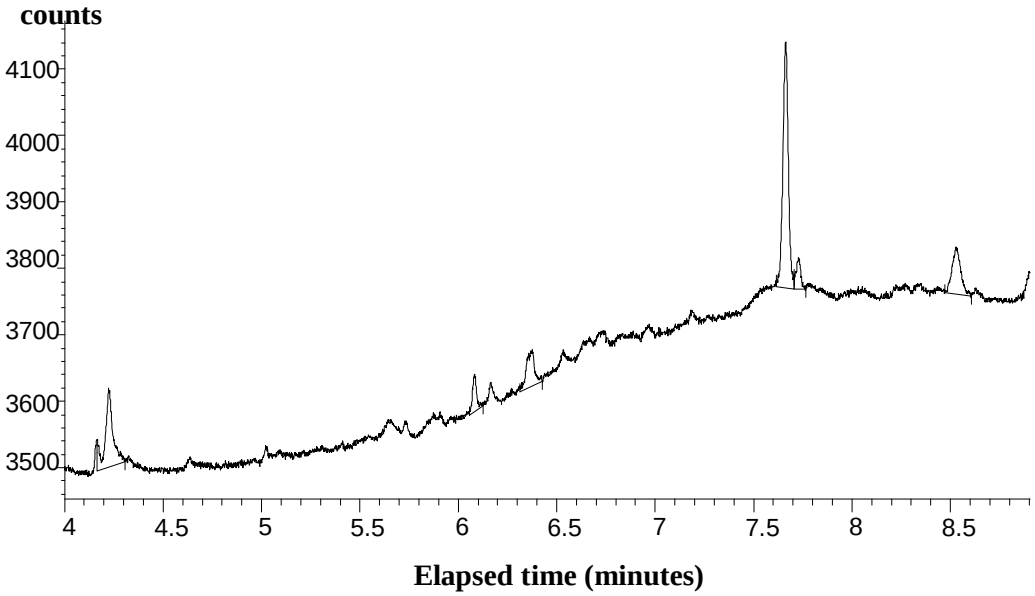

Figure 2.6 Chromatogram of medium sample.

These data were reproducible over an 18 month period in which the method was observed to be robust, reliable with little or no downtime, providing attention was given to preventative maintenance procedures described in the experimental section.

The primary advantage of the GC-FID method was the direct injection of the candidate naphthenic acid compound in the medium used to support the biological activity. Elimination of complex extraction methods or derivatization of compounds allows for timely monitoring of the biological activity. Other advantages of this method include consistency in the calibration of the candidate compound as well as repeatability between injections of an unknown concentration as well as repeatability and stability of the prepared standards. In order to consistently monitor the quantitative concentration of the compound, a dedicated GC-FID and a single candidate compound was used. Due to the general availability and low maintenance requirement of the GC-FID in environmental research settings, this requirement did not pose a significant disadvantage.

With dedicated instrumentation and column, the liner needed to be replaced or cleaned approximately every 3 months. One of the primary disadvantages of analyses of NA compounds in a medium containing minerals nutrients is the adhesion of the salts to the analytical apparatus (i.e. syringe, column inlet and liner). This is a common problem with analyses of most substrates in biosystems. The cleaning of the syringe following each analytical batch run prolonged the life of the syringe significantly. As with most analyses by GC-FID regular inlet and liner maintenance (i.e. replacement of the liner and/or cleaning) was required to obtain reproducible analytical results. A further concern with the analyses of biosystems pertains to the continued biodegradation of the substrate after the time of sampling. This factor was minimized in this study by microfiltration of the samples to remove the micro-organisms, and either immediate analyses or storing the samples at -17^{o}C prior to analyses. Analysis was typically carried out for all stored samples within 2 to 3 days. In spite of these precautions, a certain amount of on-going degradation may occur during the time required to analyze a given batch of samples. The GC-FID method is thus advantageous in this regard relative to other methods that require significantly more sample handling and analytical time.

The GC-FID method can be adapted for quantifying other naphthenic acids. For example, a second candidate compound was considered at the calibration level to verify the applicability of the GC-FID method for other NAs. 2DCHAA, another dry state candidate compound was prepared in medium for calibration with the GC-FID settings and column used for the analyses of trans-4MCHCA presented herein. The calibration was observed to be linear as illustrated in Figure 2.7, with a correlation of 0.9897. This calibration was only used for preliminary analyses with GC-FID. The limitation observed during a preliminary evaluation of 2DCHAA included persistence of the compound in the inlet indicated by the presence of the peak on the chromatographic profile after several blank injections which may be attributed to its higher molecular weight and more complex ring structure. This limitation could possibly be eliminated by dilution of the sample, but was not pursued further in the current investigation. Detailed biodegradation experiments for this compound were not carried out during the course of this study.

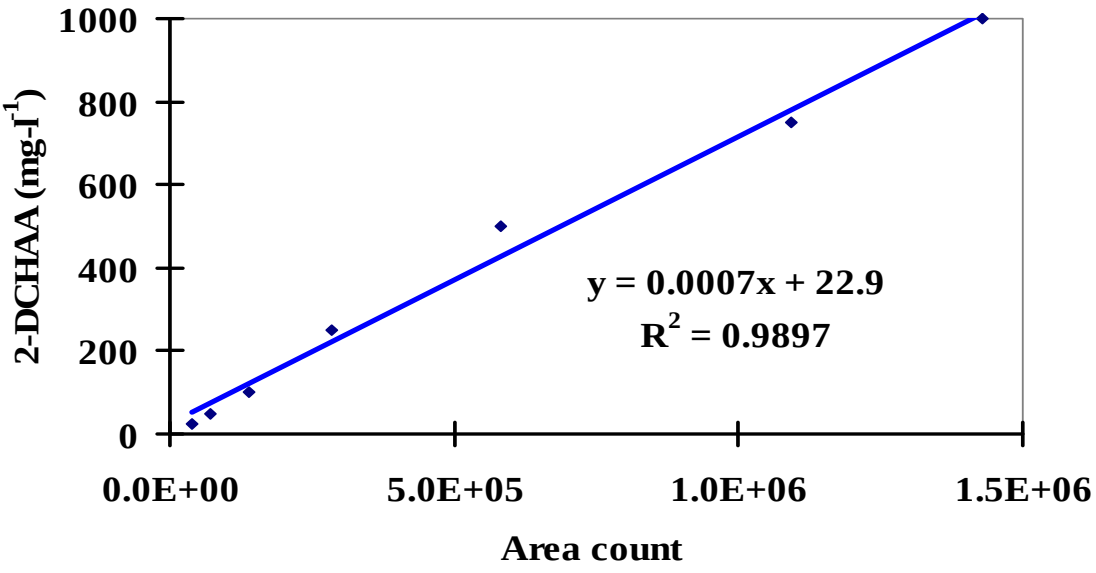

Figure 2.7 Calibration curve 2DCHAA.

2.4 Conclusions

A new method for direct analyses of a candidate NA compound in media used to support microbial growth has been successfully developed in this study for the purposes of evaluating the biokinetics of the degradation of the candidate NA compound. The sensitivity of the method was well suited for the monitoring and degradation of the NA compound and facilitated manipulation of the bioreactor operating conditions for evaluation of kinetics.

CHAPTER 3
BIODEGRADATION KINETICS OF TRANS 4-METHYL-1-CYCLOHEXANE CARBOXYLIC ACID IN BATCH REACTORS

A similar version of this chapter has been submitted to the journal Biodegradation in February 2008 and accepted subject to revisions in April 2008.

Contribution of the PhD Candidate

Experimental design was developed for the purposes of this study by Janice Paslawski with technical input and guidance from Dr. M. Nemati, Dr. G.A. Hill and Dr. J.V. Headley. All written text was prepared by Janice Paslawski with editorial guidance from Dr. M. Nemati, Dr. G.A. Hill and Dr. J.V. Headley.

Contribution of this Chapter to the Overall Study

This chapter presents the basis for the biokinetic experimentation of trans-4MCHCA studied throughout the course of this research. A viable consortium was developed capable of biodegradation of trans-4MCHCA in batch reactors in various environmental conditions. The kinetics of biodegradation in a batch system was studied under various initial substrate concentrations, temperatures and pH. The analytical technique for quantification of the substrate concentration, described in Chapter 2, was developed concurrently with the development of a viable microbial consortium. This consortium and batch kinetic results form the basis for the remainder of this study in involving the evaluation of biodegradation in two reactor configurations.

Key words: biodegradation, kinetics, naphthenic acids

Chapter 3

3.1 Introduction

Canada ranks the second largest in terms of global proven crude oil reserves (15% of world reserves), after Saudi Arabia. The majority of these reserves are found in Alberta's oil sands located in the Athabasca Basin in northeastern Alberta, Canada. In 2004, Alberta Energy reported that production of crude oil from these reserves had reached 1.1 million barrels per day (Department of Energy Government of Alberta 2007).

Oil sand is comprised of bitumen, mineral content including sands and clays and water (Department of Energy Government of Alberta 2007). The bitumen is extracted from the oil sands ore with a hot water extraction process which produces large volumes of slurry waste. Approximately, 0.1 to 0.2 m^3 of tailings pond water is accumulated for each tonne of oil sands processed (Lo et al., 2003). In 2003, it was estimated that approximately 4×10^8 m^3 of tailings comprised of a slurry of sand, clay, water and unrecovered bitumen in a stable aqueous suspension are retained in the Athabasca region and that the total volume was expected to increase to 10^9 m^3 by 2020 (Lo et al., 2003). The toxicity of the liquid wastes from the tailings pond water has been largely attributed to the salinity and presence of organic compounds which are believed to be a complex mixture of organic acid surfactants referred to collectively as naphthenic acids (NAs) (Hsu et al., 2000; Headley et al., 2002a; Lo et al., 2003; Quagraine et al., 2005b; Bataineh et al., 2006; Headley et al., 2007).

NAs are a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+z}O_2$, where (n) indicates the carbon number and *Z* specifies the number of hydrogen atoms that are lost as the structure becomes more compact when compared with those with linear hydrocarbon chains.

NAs are extracted from crude oil during the refining process. There are many different classes of carboxylic acids present in crude oils (Meredith et al., 2000). The commercial applications for NAs are varied and include: fuel additives; wood

preservatives; paint driers; anti-wear additives in lubricants; high aluminum ceramics; enhancement of the water proofing properties in cement; and in the manufacturing of tires (Lower, 1987; Brient et al., 1995; Deinko et al., 1994; Clemente et al., 2003a).

Low molecular weight components in oil sands naphthenic acids, such as 4-methyl-1-cyclohexane carboxylic acid (4MCHCA) are known to be biodegradable in aquatic environments. The trans-isomer of 4MCHCA (referred to herein as trans-4MCHCA) was thus investigated in this study, to elucidate factors (concentration, pH and temperature) controlling the kinetics of biodegradation using batch cultures. An earlier research study indicated that the trans-isomer would be more amenable to biodegradation than the cis-isomer (Headley et al., 2002b). The chemical formula for trans-4MCHCA is $CH_3C_6H_{10}CO_2H$ with a formula weight of 142.2 $g \cdot mol^{-1}$. It has a single ring structure fitting the formula of NAs where the *Z* series equals -2 (CAS Number 13064-83-0).

3.2 Materials and Methods

3.2.1 Microbial Culture and Medium

The original culture utilized throughout this study was isolated using a commercially prepared NA mixture available as Fluka technical NAs (manufactured and supplied by Sigma-Aldrich, CAS No. 1338-24-5) as substrate. The culture was then used to inoculate trans-4MCHCA prepared in modified McKinney's medium at concentrations of 100 $mg \cdot l^{-1}$.

McKinney's modified medium containing non-growth rate limiting concentrations of all required mineral nutrients was used for the growth and maintenance of the microbial consortium and biodegradation studies (Hill and Robinson, 1975; Yu et al., 2006). The medium was prepared in 2 litre batches of reverse osmosis water and had the following composition: KH_2PO_4 (840 $mg \cdot l^{-1}$); K_2HPO_4 (750 $mg \cdot l^{-1}$); $(NH_4)_2HPO_4$ (474 $mg \cdot l^{-1}$); NaCl (60 $mg \cdot l^{-1}$); $CaCl_2$ (60 $mg \cdot l^{-1}$); $MgSO_4 \cdot 7H_2O$ (60 $mg \cdot l^{-1}$); $Fe(NH_4)_2SO_4 \cdot 6H_2O$ (20 $mg \cdot l^{-1}$). Trace mineral medium was added to the macronutrients at a concentration of 0.1% on a volumetric basis. The trace mineral medium was

comprised of: H_3BO_3 (600 mg·l^{-1}); $CoCl_3$ (400 mg·l^{-1}); $ZnSO_4 \cdot 7H_2O$ (200 mg·l^{-1}); $MnCl_2$ (60 mg·l^{-1}); $NaMoO_4 \cdot 2H_2O$ (60 mg·l^{-1}); $NiCl_2$ (40 mg·l^{-1}); $CuCl_2$ (20 mg·l^{-1}).

The resultant solution, a buffered medium with pH 7, was sterilized at 121^{o}C for 30 minutes prior to use. The cultures were maintained in 250 ml flasks each containing 100 ml of medium with 100 mg·l^{-1} of trans-4MCHCA. The trans-4MCHCA (substrate) was dissolved in the medium by vigorous mixing on a magnetic stirrer at 200 rpm. This was followed by adjustment of pH between 7.0 and 7.2 using a 1.0 and/or 0.1 M solution of sodium hydroxide, as required. The medium was then inoculated with 10 ml of the designated culture (10% on a volumetric basis). Optical densities were monitored and recorded daily over several days with a spectrophotometer. Upon complete biodegradation of the substrate, the culture was used as an inoculum for subculturing of fresh medium. Subculturing was initially carried out every 10 to 14 days. Frequent subculturing eventually resulted in shorter lag phases of approximately 5 to 6 days at room temperature (23^{o}C with a standard deviation of ±2^{o}C) and as a result the subculturing was carried-out every 7 days. After several months of experimentation in shake flasks testing a variety of inoculum sources, the culture originating from the Fluka NA standard was selected as the most suitable candidate for biodegradation of the trans-4MCHCA studies.

In order to identify the dominant species of the developed microbial consortium used in this study a sample taken from a culture in the exponential phase of growth was plated on aseptic agar. Agar mixture was prepared by dissolving 3 g of Difco® Bacto agar, 3 g Difco® Bacto tryptose phosphate broth in 100 ml of reverse osmosis (RO) water under sterile conditions. Trans-4MCHCA (250 mg·l^{-1}) was added to this mixture to support the microbial growth. The prepared mixture was then poured into Petri dishes. Microbial identification was conducted at a commercial laboratory (EPCOR – Quality Assurance Lab, Edmonton, Canada). The MIDI® Microbial Identification System utilizing a fatty acid profile for a fingerprint technique was used. The microbial consortium was also preserved and examined by scanning electron microscopy (SEM) using a JEOL 840A scanning electron microscope.

3.2.2 Experimental Systems and Procedures for Biodegradation Studies

Following the establishment of a suitable microbial culture for biodegradation of trans-4MCHCA, batch experiments were conducted to study the kinetics of microbial growth and biodegradation. The effects of trans-4MCHCA initial concentration, temperature and pH were investigated.

Batch experiments were carried out in 500 ml shake flasks each containing 150 ml of medium described previously, containing substrate (trans-4MCHCA) at various initial concentrations of 50, 100, 250, 500, 750 and 800 $mg \cdot l^{-1}$, with an initial pH range 7.0 to 7.2. Each flask was inoculated by a 5 day old culture (5% on a volumetric basis). Flasks were maintained at room temperature ($23 \pm 2^{o}C$) on a magnetic stirrer (200 rpm) to achieve sufficient mixing and oxygen transfer. pH was monitored daily and adjusted to the designated value as required with a 0.1 M solution of NaOH. In all cases, samples were taken on a daily basis and tested for optical density (biomass concentration), substrate concentration, and pH. The sampling frequency was increased during the exponential phase and decreased once stationary growth was established. Progressive experiments were carried out under similar and/or modified conditions using the preceding batch culture as an inoculum to permit adaptation of the consortium to the higher substrate concentrations. For example, upon completion of batch experiments with an initial substrate concentration of 100 $mg \cdot l^{-1}$, the consortium capable of degrading this concentration was then used as an inoculum in the subsequent batch experiments with an initial substrate concentration of 250 $mg \cdot l^{-1}$.

Samples required for various analyses were collected from the reactor using a stainless steel needle and glass hypodermic syringe. Prior to determination of substrate concentration, the liquid sample was filtered through 0.22 μm nylon microfilters using a stainless steel cartridge. The samples were collected in duplicate in 2 ml amber vials and immediately stored at $-17^{o}C$ until the time of analyses.

To assess the effect of temperature, experiments were carried out in a temperature controlled environmental chamber where the temperature was incrementally lowered from room temperature 23^{o}C to 16, 12, 10, 8 and 4^{o}C and similarly raised to 30, 37 and 40^{o}C. The initial substrate concentration used in these experiments was 500 mg·l^{-1} and the pH was initially set at a value of 7.0 to 7.2. All other conditions and procedures were similar to those described earlier.

Batch experiments were also carried-out to assess the effects of initial pH (5.5, 7.0, 8.5, 10.0, 11.0, and 13.0) on the activity of the bacterial culture and biodegradation of trans-4MCHCA. During these experiments the pH of the culture was adjusted once a day to the designated experimental pH. The initial substrate concentrations and temperature for these experiments were 500 mg·l^{-1} and 23±2^{o}C, respectively. All other conditions and procedures were similar to those described earlier.

3.2.3 Analytical Methods

The concentration of biomass was determined by direct measurement of the optical density (OD) of the samples taken from the flasks at a wavelength of 620 nm (Shuler and Kargi, 1992; Gadekar et al., 2006; Panikov et al., 2007; Rampinelli et al., 2007). An Ultraviolet (UV) spectrophotometer (Mini Shimadzu, Model 1240) was used for the determination of the optical density. The optical density was then related to dry-weight using a calibration curve developed in this work.

The concentration of substrate (trans-4MCHCA) was measured throughout the course of this work using an Agilent 6890N gas chromatograph (GC) equipped with a flame-ionization detector (FID) and a split/splitless injector. The injector was operated in splitless mode with an initial column inlet temperature of 90^{o}C. The oven was heated to a final temperature of 180^{o}C at a rate of 30^{o}C per minute. The column was an Agilent 19091N-113 HP_INNOWAX Polyethylene Glycol capillary column with a length of 30 meters and a nominal diameter of 320 μm. Calibration was carried out with 8 standard solutions prepared in nutrient medium at concentrations covering those used in the batch

reactor experiments (2 to 1,000 $mg \cdot l^{-1}$ trans-4MCHCA). The upper range of calibration represents the solubility limit of trans-4MCHCA in the medium as determined experimentally. Samples of standard solutions were filtered and prepared in a manner similar to that of the actual samples taken from the experimental system. The standards were stored in amber vials at -17^{o}C. Two working standards were tested each time a set of samples were analyzed to confirm the GC calibration. The retention time for trans-4MCHCA was determined to be 4.20 minutes. Each sampling event included the collection of 3 samples using a clean glass syringe with a stainless steel needle. The injection volume was 0.5 μL. Each sample was analyzed in triplicate. Millipore water was injected into the column following the analyses of a maximum of 5 samples (15 injections) to prevent the build up of substrate or medium components in the column. The injection syringe was cleaned with Millipore water prior to analyses. The inlet was heated to a temperature greater than the operating conditions prior to each batch of analyses (maximum of 12 samples) for a minimum of 1 hour in order to clear the inlet of any residual NAs and/or medium minerals. The column inlet was cleaned or replaced as required when the Millipore blanks injected between samples were not sufficient to return to the method detection limit of 11 $mg \cdot l^{-1}$. The effectiveness of this procedure in clearing the inlet and column of any residual mineral or substrate build-up was confirmed by a minimum of 3 injections of Millipore water prior to analyses to ensure a background concentration of <10 $mg \cdot l^{-1}$. This maintenance procedure was performed as required in order to ensure minimal interference of the medium minerals on the inlet liner.

3.3 Results and Discussion

Microbial identification carried out for the developed biological consortium capable of degrading trans-4MCHCA at room temperature indicated that the consortium was comprised of two bacterial species. The first bacterial species which was dominant on the agar plates and formed colourless (transparent) colonies was identified as *Variovorax paradoxus* (previously referred to as *Alcaligenes paradoxus*). The second species which formed larger colonies with a beige or brown colour and were present in smaller numbers was identified as *Pseudomonas aeruginosa*. Figure 3.1 presents the

scanning electron micrographs of the bacteria. Microbial identification of the culture capable of biodegradation of trans-4MCHCA at 8^{o}C indicated that the dominant bacteria were *Pseudomonas putida*. It was observed through plating of the microbial consortium at 12^{o}C and 4^{o}C that the predominant genera at these temperatures were represented by white, small, uniform colonies similar to those observed in the culture observed at 8^{o}C.

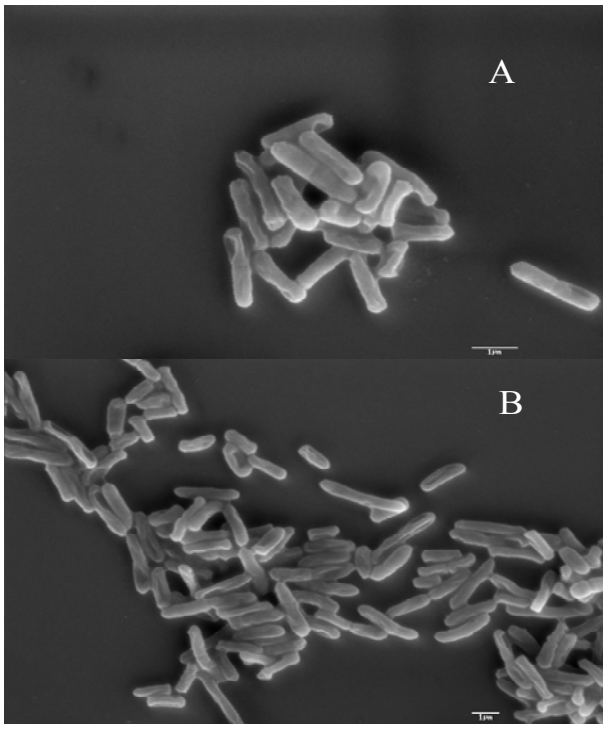

Figure 3.1 SEM image of microbial consortium developed at room temperature.
Panel (A) 16,000X magnification, **Panel (B)** 10,000X magnification.

Figure 3.2 illustrates the results of substrate biodegradation and biomass growth in batch reactors at four selected initial substrate concentrations (50 mg·l^{-1}, 250 mg·l^{-1}, 500 mg·l^{-1} and 750 mg·l^{-1}). In all cases a direct relationship between the microbial growth and substrate utilization was seen (i.e. the maximum substrate utilization corresponded to the exponential growth phase of the biomass). It can also be observed that the lag phase generally increased with increase in initial concentration of substrate.

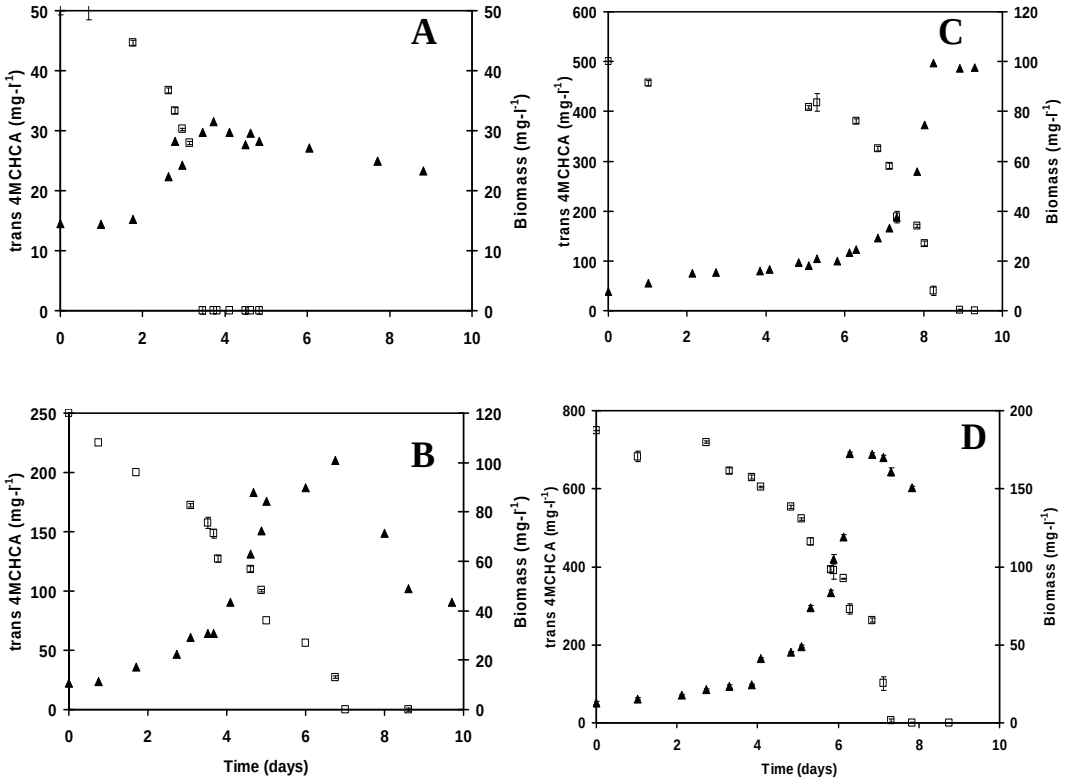

Figure 3.2 Substrate biodegradation and biomass growth with time for various initial substrate concentrations (pH 7; 23°C). **Panel (A)** Initial substrate concentration of 50 mg·l^{-1}, **Panel (B)** 250 mg·l^{-1}, Panel (C) 500 mg·l^{-1}, Panel (D) 750 mg·l^{-1}. ▲Biomass concentration (mg·l^{-1}); □ Substrate concentration (mg·l^{-1}).
Note: Error bars represent 95% confidence interval and may not be visible in some cases.

Table 3.1 presents the biodegradation rates observed at different initial concentrations of substrate. In order to calculate the rate of substrate utilization the lag phase was excluded. Included in this table are also the calculated values of specific growth rate and biomass yield. The observed specific growth rates ranged between 0.31±0.05 d^{-1} for the lowest initial substrate concentration (50 mg·l^{-1}) and 0.52±0.04 d^{-1} observed at the highest initial concentration (750 mg·l^{-1}). Biodegradation did not occur with an initial substrate concentration of 800 mg·l^{-1}. The mean value of specific growth rate was 0.45 d^{-1} with a standard deviation of ±0.09 d^{-1}. The overall yield ranged between 0.21 mg·mg^{-1} (at 500 mg·l^{-1}) and 0.34 mg·mg^{-1} (at 50 mg·l^{-1}) with the average value being 0.26 mg·mg^{-1} with a standard deviation of ±0.08 mg·mg^{-1}. The rates of observed

biodegradation (Table 3.1) were calculated by substrate utilization over the degradation time (lag phase excluded) in the batch reactor. The biodegradation rate (r_A) for each experiment increased proportional to the initial substrate (S_i) concentration following a linear trend represented by Equation 3.1:

$$r_A = (0.192 \pm 0.027) \times S_i \quad (3.1)$$

Where: r_A = biodegradation rate ($mg \cdot (l \cdot d)^{-1}$)

S_i = initial substrate concentration ($mg \cdot l^{-1}$)

Similar biodegradation studies with simpler contaminants have reported maximum specific growth rates which are significantly higher than those determined in the present work (i.e. up to 6 times greater). For example, for phenol and naphthalene, the maximum specific growth rate was reported to be 0.09 h^{-1} (2.2 d^{-1}) (Nikakhtari and Hill, 2006) and 0.13 h^{-1} (3.1 d^{-1}) (Purwaningsih, 2002), respectively. Research on biodegradation of trans-4MCHCA in Athabasca River water (both non-amended and pH amended) resulted in a maximum specific growth rate of 0.067 d^{-1} for an initial substrate concentration of 9 $mg \cdot l^{-1}$ (Tanapat, 2001). The average maximum specific growth rate observed in this study was 6.4 times higher than previously reported. However, the specific growth rates for pure naphthenic acid compounds are not as high as those for simpler organic compounds. Future studies involving environmental or cosubstrate factors may lead to further enhancements in biodegradation rates.

Table 3.1 Summary of specific growth rate, biodegradation rate and overall yield determined at various initial substrate concentrations for trans-4MCHCA at room temperature (23°C) and neutral pH (7)[1]

Initial Substrate Concentration ($mg \cdot l^{-1}$)	Average Specific Growth Rate (d^{-1})	Biodegradation Rate ($mg \cdot (l \cdot d^{-1})$)	Overall Yield (mg biomass· mg substrate^{-1})
50	0.31±0.05	11.0±2.5	0.34±0.08
100	0.45±0.02	27.8±3.2	0.28±0.03
250	0.51±0.07	31.1±3.2	0.31±0.08
500	0.45±0.17	114±7	0.21±0.11
750	0.52±0.04	134±15	0.22±0.03
800[2]	–	–	–

Notes:
1 – standard error represented by ± where applicable
2 – biodegradation did not occur

Similar trends in biodegradation performance were observed for various temperatures as represented in Figure 3.3 and Table 3.2. A decreased lag phase was observed in the biomass growth and corresponding substrate utilization upon increasing the temperature from 23°C to 37°C. However, biodegradation was not achieved at 40°C and 42°C. Interestingly, the developed microbial consortium was able to grow and utilize substrate at temperatures as low as 4°C. However, the lag phase did increase considerably with each incremental decrease in temperature (23 to 16, 12, 10, 8 and 4°C). As presented in Table 3.2, the specific growth rates observed reached the maximum value of 0.45±0.17 d^{-1} at room temperature (23°C) which was 4.5 times greater than the specific

growth rate observed at 4°C. Similarly, the maximum values of cell yield and biodegradation rate were observed at room temperature. Cell yield was decreased as temperature increased or decreased above room temperature. Cell yield coefficients ranged from 0.13 (at 37°C) to 0.21 mg of biomass·mg^{-1} of substrate (at 23°C). However, the decrease in cell yield between 23°C and 4°C did not exceed the standard deviation of the mean cell yield (0.20±0.09 mg·mg^{-1}). The typical reported values for *Pseudomonas* grown with various carbon sources such as glucose, ethanol and methanol ranges between 0.4 and 0.6 mg·mg^{-1}. The yield for *Pseudomonas* in acetate has been reported as 0.28 mg·mg^{-1} (Shuler and Kargi, 1992). This value is more comparable with the cell yield coefficients observed with trans-4MCHCA at room temperature.

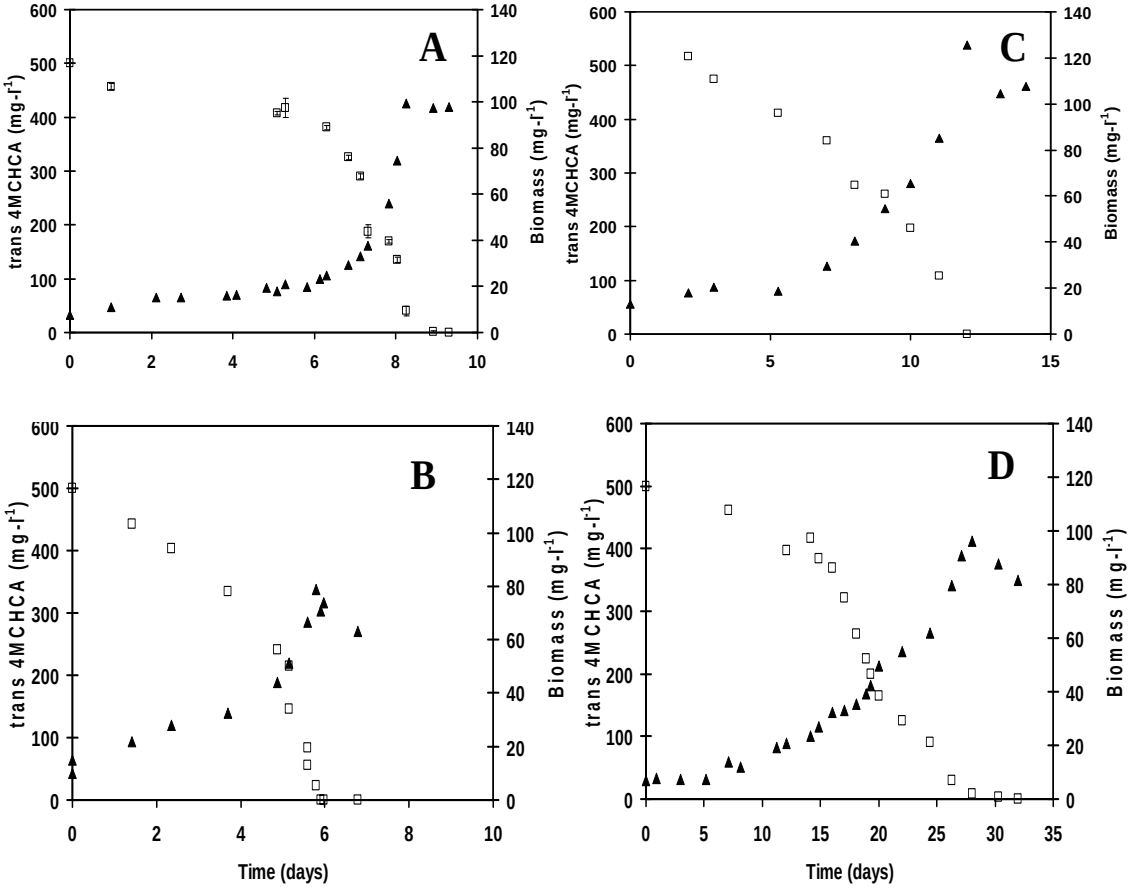

Figure 3.3 Substrate biodegradation and biomass growth with time at various temperatures (pH 7; initial concentration 500 mg·l^{-1}). Panel (A) 23°C, Panel (B) 37°C, Panel (C) 12°C, Panel (D) 4°C. ▲Biomass concentration (mg·l^{-1}); □ Substrate concentration (mg·l^{-1}). Note: Error bars represent 95% confidence interval and may not be visible in some cases.

Table 3.2 Summary of specific growth rate, biodegradation rate and overall yield determined at various temperatures for initial substrate concentrations of 500 $mg \cdot l^{-1}$ and neutral pH (7)[1]

Batch Experiment Temperature (^oC)	Average Specific Growth Rate (day^{-1})	Biodegradation Rate ($mg \cdot l^{-1} \cdot day^{-1}$)	Overall Yield (mg biomass· mg substrate^{-1})
4	0.091±0.003	25.1±1.8	0.18±0.13
8	0.19±0.11	40.3±3.6	0.16±0.03
12	0.11±0.01	28.1±1.3	0.16±0.03
16	0.27±0.05	21.2±1.4	0.16±0.03
23±2	0.45±0.17	114±8	0.21±0.11
30	0.16±0.01	26.9±1.6	0.14±0.01
37	0.29±0.03	86.3±8.0	0.13±0.01
40[2]	–	–	–

Notes:
1 – standard error represented by ± where applicable
2 – biodegradation did not occur

Figure 3.4 shows the Arrhenius type dependency of the specific growth rates of trans-4MCHCA on temperature. The activation energy calculated on the basis of the Arrhenius equation was 6.7±0.4 $kcal \cdot mol^{-1}$ (28±2 kilojoules per mole). Higher values of activation energy indicate a limitation in the biochemical reaction at low temperatures. Lower values of activation energy are associated with diffusion control. The activation energy of molecular diffusion is approximately 6 $kcal \cdot mol^{-1}$ (Shuler and Kargi, 1992).

Reactions with high activation energies are very temperature-sensitive, while those with low activation energies are relatively temperature insensitive (Levenspiel,

1972). Using a different culture than that used in here, Tanapat (2001) reported an activation energy of 17 kcal·mol^{-1} for biodegradation of trans-4MCHCA. However, the reported activation energy for the cis-isomer of 4MCHCA was 7.4 kcal·mol^{-1}.

The effect of pH on the microbial growth and substrate utilization is shown in Figure 3.5. The calculated values of specific growth rate, cell yield and biodegradation rate are presented in Table 3.3. During these experiments the pH of the culture was adjusted to the designated experimental pH on a daily basis. For the cultures with initial pH values above 8, pH decreased to values in the range 7.7 to 8.0 which was then readjusted to the designated values. For the culture with an initial pH of the 7.0 the change in pH was not significant (from 7.0 to 6.8). Biodegradation was successfully achieved within the pH range of 7.0 to 11.0 with a maximum specific growth rate, biodegradation rate and cell yield at pH 10 (See Table 3.3). Biodegradation did not occur in the culture with a pH of 5.5 which represents the ambient pH of a solution of 500 mg·l^{-1} of trans-4MCHCA in the medium.

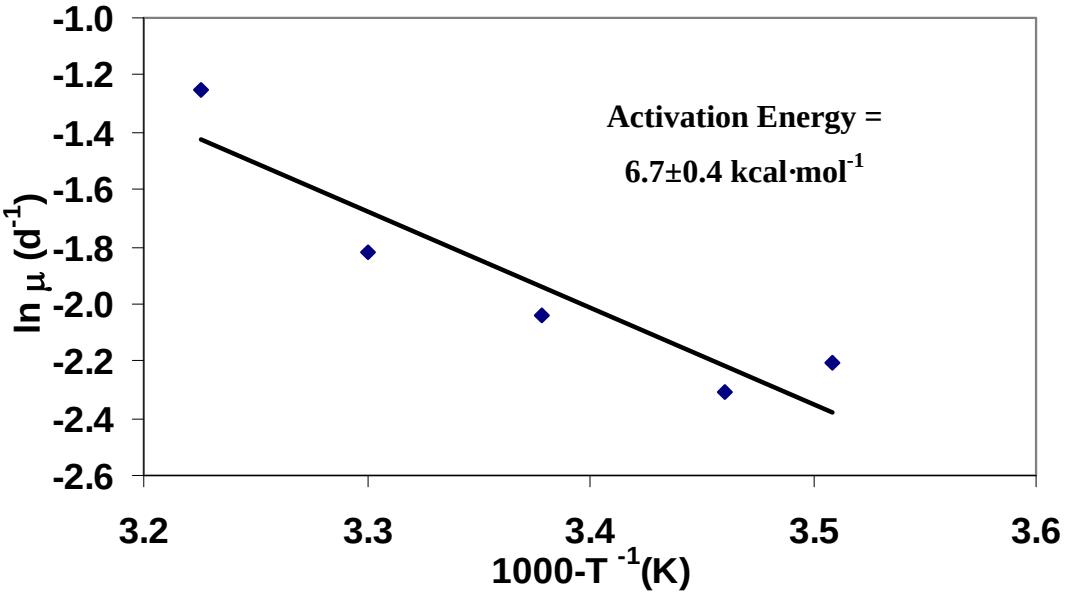

Figure 3.4 Arrhenius dependency of specific growth rates (μ) on temperature.

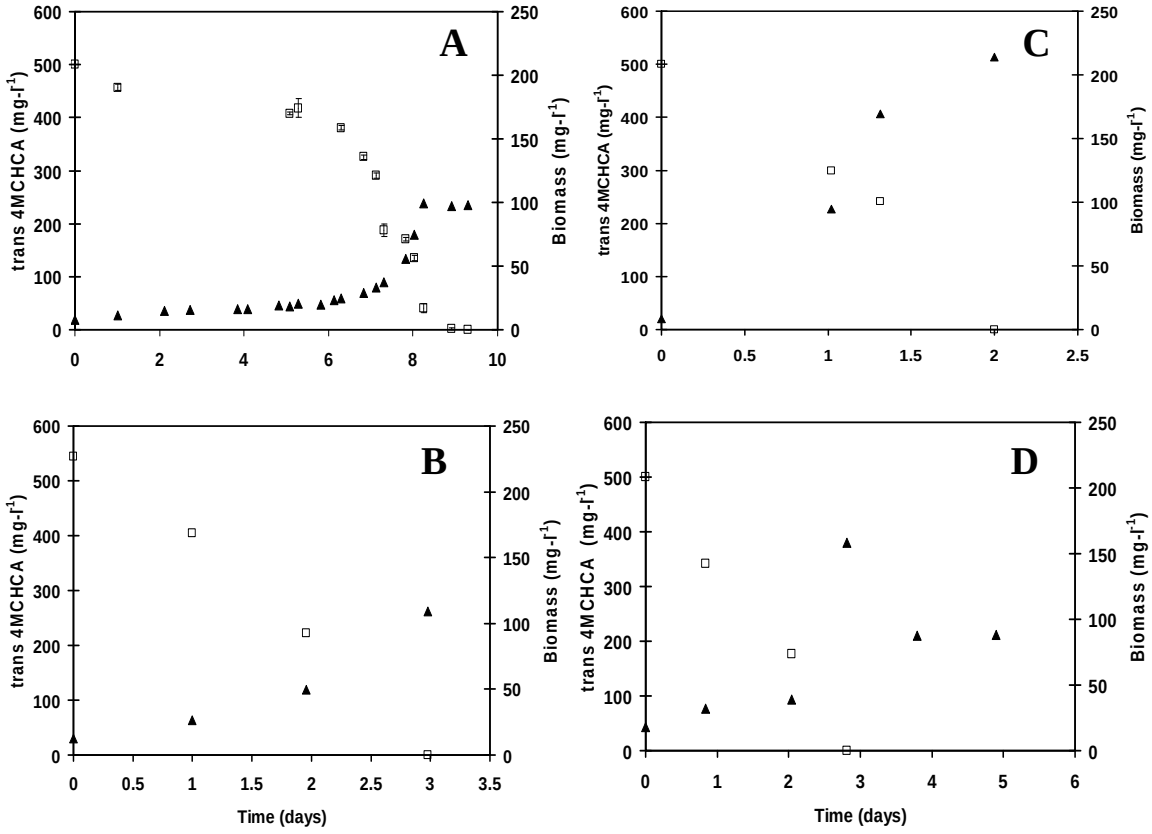

Figure 3.5 Substrate biodegradation and biomass growth with time at various pH conditions (23±2°C; initial substrate concentration 500 mg·l^{-1}). **Panel (A)** pH 7, **Panel (B)** pH 8.5, **Panel (C)** pH 10, **Panel (D)** pH 11. ▲Biomass concentration (mg·l^{-1}); □ Substrate concentration (mg·l^{-1}) Note: Error bars represent 95% confidence interval and may not be visible in some cases.

Table 3.3 Summary of specific growth rates, biodegradation rates and yield determined at various pH for initial substrate concentrations of 500 $mg \cdot l^{-1}$ at room temperature (23^oC)[1]

Batch Experiment pH	Average Specific Growth Rate (day^{-1})	Biodegradation Rate ($mg \cdot l^{-1} \cdot day^{-1}$)	Overall Yield (mg biomass· mg substrate^{-1})
5.5[2]	–	–	–
7	0.45±0.17	114±8	0.21±0.11
8.5	0.71±0.02	184±12	0.19±0.03
10	1.69±0.40	244±30	0.41±0.06
11	0.35±0.13	171±11	0.28±0.17
13[2]	–	–	–

Notes:
1 – standard error represented by ± where applicable
2 – biodegradation did not occur

3.4 Conclusions

The biokinetics of a single ringed NA compound (trans-4MCHCA) was studied under batch conditions in order to determine the rates of biodegradation of trans-4MCHCA and the environmental factors affecting the biodegradation. This study served to develop a viable consortium capable of biodegradation of trans-4MCHCA and to verify the influential factors influencing the activity of this culture.

Biodegradation was achieved at pH values in of the range 7 to 11. The maximum specific growth rate (1.69±0.40 d^{-1}), biodegradation rate (244±30 $mg \cdot (l \cdot d)^{-1}$) and cell yield (0.41±0.04 $mg \cdot mg^{-1}$) were obtained at pH 10.

The microbial consortium capable of biodegradation of trans-4MCHCA at moderate temperatures (23±2^{o}C) was dominated by *Variovorax paradoxus*, while *Pseudomonas putida* was the dominant species when biodegradation was carried out at low temperatures (4^{o}C to 12^{o}C). The microbial growth and biodegradation of the pure trans-4MCHCA was achieved at a concentrations as high as 750 mg·l^{-1}, with a corresponding maximum specific growth rate of 0.52±0.04 d^{-1} and biodegradation rate of 134±15 mg·(l·d)$^{-1}$. Biodegradation was influenced with temperature ranging between 4^{o}C and 37^{o}C with the highest rate of 114±8 mg·(l·d)$^{-1}$ achieved at a temperature of 23±2^{o}C for initial substrate concentrations of 500 mg·l^{-1}. The activation energy calculated on the basis of the Arrhenius equation was 6.7±0.4 kcal·mol^{-1}. Cell yield decreased slightly as temperature increased or decreased from 23^{o}C.

The maximum specific growth rate observed at neutral pH and room temperature in this study (0.52±0.04 d^{-1}) was greater than that observed in a previous study of trans-4MCHCA (0.067 d^{-1}). However, this specific growth rate is lower than that reported for simpler organic compounds such as phenol and naphthalene.

The results of the present study indicate that biodegradation of the candidate naphthenic acid is influenced by the environmental conditions in decreasing order of importance: pH, temperature and concentration of the naphthenic acid compound.

CHAPTER 4
BIODEGRADATION KINETICS OF TRANS 4-METHYL-1-CYCLOHEXANE CARBOXYLIC ACID IN A CONTINUOUSLY STIRRED TANK REACTOR AND IMMOBILIZED CELL REACTORS

A similar version of this chapter has been submitted to the Journal of Microbiology and Biotechnology in May 2008.

Contribution of the PhD Candidate

The bioreactor set-up, experimental design and modelling were developed for the purposes of this study by Janice Paslawski with technical input and guidance from Dr. M. Nemati and Dr. G.A. Hill. All written text was prepared by Janice Paslawski with editorial guidance from Dr. M. Nemati and Dr. G.A. Hill.

Contribution of this Chapter to the Overall Study

This chapter presents the fundamental evaluation of biodegradation kinetics for trans-4MCHCA studied herein. Evaluation of the biodegradation of trans-4MCHCA in a continuously stirred tank reactor utilizing the microbial consortium developed throughout the batch reactor investigation is presented in Chapter 3. The analytical technique for quantification of the substrate concentration is described in Chapter 2. The kinetic results form the basis for the remainder of this study involving the development of an immobilized cell system in a packed bed reactor configuration, and the modelling of this bioreactor system for future biokinetic studies and reactor design.

Key words: biodegradation, continuously stirred tank reactor, immobilized cell reactor, Monod kinetics, naphthenic acids, modelling

4.1 Introduction

Naphthenic acids (NAs) are complex mixtures of organic acid surfactant compounds that naturally occur in crude oils (Lower, 1987; Lower, 1988; Meredith et al., 2000). NAs encompass a large range of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+z}O_2$, where (n) indicates the carbon number and Z specifies the number of hydrogen atoms that are lost as the structure becomes more compact. NAs are present in crude oils with widely varying concentrations. As a result of their corrosivity, NAs must be removed from crude oil during processing. There are many commercial uses for naphthenic acids including: fuel additives; wood preservatives; paint driers; aluminum ceramics; anti-wear lubricants and in the manufacturing of tires (Lower, 1987; Deinko et al., 1994; Brient et al., 1995; Clemente et al., 2003b).

NAs are of importance in the mining of the Canadian oil sands from the Athabasca region. Bitumen is extracted from the oil sands ore using hot water. Approximately, 0.1 to 0.2 m^3 of tailings pond water is accumulated for each tonne of oil sands processed. The total volume of tailings water is expected to reach 10^9 m^3 by 2020 (Lo et al., 2003). The toxicity of the tailings pond water has been largely attributed to the salinity and presence of NAs (Hsu et al. 2000; Headley et al., 2002a; Quagraine et al. 2005a; Bataineh et al., 2006; Headley et al. 2007). Currently, in Canada, environmental regulations require the tailings to be retained in large ponds to prevent release into the environment due to concerns about their toxicity.

Bioremediation as a treatment method is non-destructive, cost- and treatment-effective and often a logistically favourable cleanup technology. Recent attempts to biodegrade NAs in tailing ponds waters have been unsuccessful using laboratory cultures of tailings pond water bacteria (Scott et al., 2005). Leung et al. (2003) found that under aerobic conditions in the laboratory, NAs undergo microbial biodegradation and a reduction in relative toxicity can be achieved but the influential factors are not well characterized. Laboratory studies of weathered light crude exposed to microbial degradation observed an increase in the concentrations of cyclic carboxylic acids with (n)

greater than 20 showing that biodegradation of the lower carbon numbers was prevalent. These results concurred with previous studies (Holowenko et al., 2001).

Research on toxicity and biodegradation has been conducted on commercial preparations of NA mixtures, model compounds, tailings waters and NAs extracted from the tailings waters (Herman et al. 1994; Lai et al., 1996; Clemente and Fedorak., 2005). Although these studies were primarily focused on identifying the components of NAs responsible for the observed toxicity, biodegradation was also monitored. Lai et al. (1996) studied the impact of a single nutrient (phosphate) addition to tailings pond water. The results of this study indicated that phosphate addition did enhance the biodegradation for the first four weeks in a closed system. However, the subsequent four weeks showed a decrease in biodegradation rates which was attributed to either limitations of another nutrient or accumulation of metabolic by-products. This study also noted no decrease in toxicity for either treated or untreated effluents by Microtox™ analyses. Nonetheless, this study did show that large variations exist in toxicity and degradation rates for linear and bicyclic NAs. The study indicated the importance of identifying the specific components of the effluent responsible for toxicity and how this toxicity is linked to bioremediation.

Only a few studies have included biodegradation of individual compounds of commercially available NAs and more specifically single ringed compounds (Tanapat, 2001; Peng et al. 2002; Paslawski et al., 2008). Studies with individual NAs have reported the impact of molecular structure on biodegradation and adsorption of NAs on soils. Studies of the toxicity of NAs and its individual components have revealed a need to better understand the factors controlling the kinetics of NA biodegradation.

In similar cases involving environmentally persistent contaminants related to petroleum operations such as polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs), biodegradation has been studied with model PAHs such as phenol, naphthalene, methyl naphthalenes and pyrene, and VOCs such as ethanol (Quail and Hill, 1991; Bi et al., 2004; Nikakhtari and Hill, 2006; Yu et al., 2006; Clarke et al., 2007). The influential factor on biodegradation can be determined under controlled

settings and the biodegradation can be improved by nutrient additions and/or conducting the process in properly designed bioreactors (Folsom et al., 1990). The design of novel bioreactors has often been used to enhance the biodegradation rates of environmental contaminants in wastewaters (Quail and Hill, 1991). These cases have evaluated the biodegradation kinetics with the use of various bioreactor designs such as batch reactors, continuously stirred tank reactors, roller reactors, packed bed reactors, and fluidized bed reactors. In recent years, biodegradation research has shown that superior performance can be achieved in reactors supporting high biomass hold-ups such as packed bed, fluidized bed reactors and other types of immobilized cell systems (Quail and Hill, 1991; Nikakhtari and Hill, 2006; Clarke et al., 2007). High biomass concentrations are achieved by immobilization of the cells on the surface of a proper carrier matrix (Quail, 1990). Bioreactors with fixed biomass or biofilm reactors have been used for over 100 years primarily in the treatment of wastewater. The primary advantage of the biofilm reactor is the larger biomass hold-up in the bioreactor as compared with suspended cultures, decoupling of biomass residence time from the hydraulic residence time and therefore higher volumetric reaction rates (Karamanev and Nikolov, 1991).

In the present work, a culture suitable for biodegradation of NAs developed in previous work (Paslawski et al., 2008) was used in a continuously stirred tank reactor (CSTR) to study the biodegradation of a candidate NA and to determine the important kinetic coefficients for microbial growth and biodegradation kinetics. Subsequently and as an alternative for improving the biodegradation rate, an immobilized cell reactor (ICR) was operated continuously under identical environmental conditions with varying input flow rates as well as two initial trans-4MCHCA feed concentrations. Toxicity evaluation tests have been conducted on medium, influent and treated effluents from these bioreactors to asses the effectiveness of the biotreatment process for reducing the toxicity of the effluent.

4.2 Materials and Methods

4.2.1 Selection of Candidate Compound and Biological Medium

The trans-isomer of 4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA, Sigma-Aldrich, 98% purity) was selected as a candidate NA compound due to its availability and potential for biodegradation. The molecular formula for this compound is $CH_3C_6H_{10}CO_2H$ (CAS Number 13064-83-0) with a molecular weight of 142.2 Da. McKinney's modified medium containing non-growth rate limiting concentrations of all required mineral nutrients was used for the growth and maintenance of the microbial consortium and biodegradation studies (Hill and Robinson, 1975; Purwaningsih, 2002; Yu et al., 2006). The medium was prepared as described in Paslawski et al. (2008). The medium used for the growth and maintenance of the cultures and the medium used as the feed for the bioreactors were prepared by aseptically adding trans-4MCHCA (substrate) at the designed initial concentration. The trans-4MCHCA was dissolved in the medium by vigorous mixing for 24 h prior to use.

4.2.2 Microbial Consortium

The microbial consortium used in this study was a mixed culture capable of degrading trans-4MCHCA previously developed in our laboratory and was dominated by *Variovorax paradoxus*. The detail of the development of this culture and procedure and conditions for the growth and maintenance of the cultures are described in detail in Chapter 3 and reported in Paslawski et al., 2008. The consortiums from both the CSTR and ICR were plated on 3g of Difco® Bacto agar, 3 g Difco® Bacto tryptose phosphate broth in 100 ml of RO water under sterile conditions. Trans-4MCHCA (250 $mg \cdot l^{-1}$) was added to this mixture to support the microbial growth. The developed colonies were then used for microbial identification. Microbial identification was conducted at a commercial laboratory (EPCOR – Quality Assurance Lab, Edmonton, Canada) using the MIDI® Microbial Identification System. The microbial consortiums and biofilm were also preserved and examined by scanning electron microscopy (SEM) using a JEOL 840A scanning electron microscope.

4.2.3 Experimental Set Up

Two bioreactor systems, a CSTR and an ICR both operated continuously, were used to study the biodegradation of trans-4MCHCA. A BIOFLOWTM Model C30 reactor (New Brunswick Scientific, Inc.) was used as the continuous flow bioreactor (CSTR). The CSTR vessel was constructed of a clear glass with a diameter of 11 cm and a total volume of 1.5 l. A working volume of 550 ml was used to minimize the volume of stock solution required. The working volume was at a liquid depth of 5.8 cm. Filtered air was pumped into the headspace of the CSTR using the internal pump system of the BIOFLOW™ at a constant rate of 0.2 $l \cdot min^{-1}$. The CSTR was vigorously stirred with a magnetic stirrer. The dissolved oxygen level in the bioreactors was monitored at key intervals throughout the course of experimentation using a Hach oxygen meter (Model 50175) and membrane probe (Model 50180). Initially the CSTR was operated in batch mode for 15 days with modified McKinney's medium and a trans-4MCHCA initial concentration of 500 $mg \cdot l^{-1}$. The bioreactor was inoculated with 5% (v/v) of a 15 day old batch culture with a biomass concentration of 41 $mg \cdot l^{-1}$. Inoculating cultures were maintained throughout the course of this study in batch systems (Paslawski et al., 2008). Once complete substrate removal was achieved, the bioreactor was switched to continuous mode and operated in this mode throughout the duration of experimentation. Fresh sterile substrate and nutrient medium were pumped into the bioreactor and fermentation broth was removed at identical flow rates. Feed stock solution was pumped into the bioreactor at an initial flow rate of 1.0 $ml \cdot h^{-1}$ using a peristaltic pump (Amersham Biosciences No. 18-1110-91). The flow rate was set based on the calibration of the pump and then verified daily by weighing the collected effluent over a certain period. The CSTR was operated at room temperature (23^{o}C) over a period of 7 months. The experimental set up for the CSTR is presented in Figure 4.1. The CSTR was monitored daily for pH, optical density and substrate concentration. At each flow rate sufficient time was given for establishment of steady state conditions which was verified by stability in the residual substrate concentration and optical density. The bioreactor was run following the establishment of each steady state for an additional period equal to the greater of 3 residence times or 3 days before increasing the flow rate to the next level. The increase in flow rate continued until the system approached wash-out conditions. The tested flow

rates corresponded to residence times of 22.9, 10.0, 8.5, 4.8, 3.1, 2.5, 2.2, 1.6, 1.4, 1.2, 1.1 and 1.0 d). Following the establishment of steady state at a residence time of 1.0 d, the flow rate of the feed was decreased and reproducibility of the data was checked by maintaining the bioreactor at residence times of 3.2 and 2.1 d.

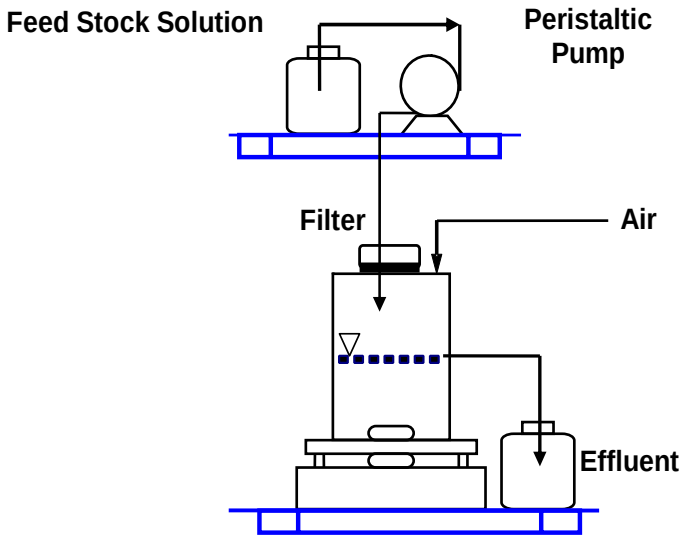

Figure 4.1 Schematic diagram of the experimental continuously stirred tank reactor set up.

Figure 4.2 shows the experimental set up for the ICR. Three ICRs were used to study the suitability of a biofilm for biodegradation of trans-4MCHCA. The glass bioreactors had a 0.04 m inside diameter and 0.44 m length. Ports were devised for air input and sampling. Sterile air was supplied at an average rate of 220±40 $cm^3 \cdot min^{-1}$ (Aalborg Mass Flow Meter, GFM-170-GFMS-011260) through a check valve attached to the lower sampling port. This port was located approximately 0.05 m above the bottom of the bioreactor. Air was vented through the open top of the bioreactor. The trans-MCHCA sterile stock solution was fed into the immobilized cell bioreactor with a peristaltic pump system as described above for the CSTR.

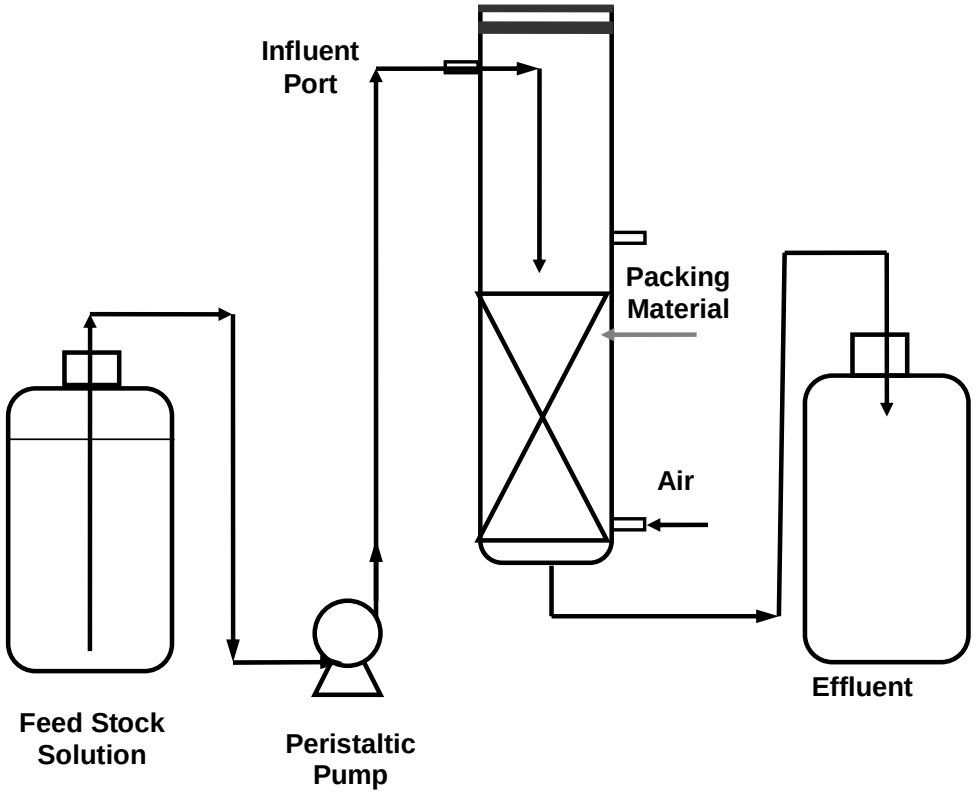

Figure 4.2 Schematic diagram of the experimental immobilized cell reactor set up.

Stainless steel coiled packing material was chosen on the basis of enhanced performance in the study of a packed bed bioreactor used in the biodegradation of phenol polluted air (Nikakhtari and Hill, 2005b). The measured density of the packing was 7.95 g·cm^{-3}. The packing was purchased in 35 g coiled packages from a local distributor. The strands were 0.8 mm wide and the thickness of the strand was approximately 10% of the width. The length of coil was determined on the basis of the weight and density of the material.

The biofilm was developed by trickling partially degraded effluent over 30 g of stainless steel packing material (packed-bed density of 0.99) placed in one column over a length of 25 cm for a two month period. Recycling of partially degraded CSTR effluent at an average flow rate of 20 ml·h^{-1} provided both viable organisms and substrate. This

allowed establishment of a biofilm with the ability of degrading trans-4MCHCA in the bioreactor.

After establishment of biofilm, the packing was removed and divided into three sections and used in three ICRs operated in submerged mode with air bubbled through the bed. The dry weights of clean packing in reactors #1, #2, and #3, as determined at the end of experiments, were 9.8, 11.4 and 9.4 g, respectively, and the packing heights were 8.0, 8.5 and 8.5 cm for these bioreactors, respectively. The bed porosity in all three bioreactors was 99%, determined by calculating the working volume of the bioreactor and the volumes of the packing material based on the measured weight and density. ICR #2 was operated concurrently during this period with a medium containing 250 $mg \cdot l^{-1}$ of trans-4MCHCA. ICR #3 was operated with a medium containing 500 $mg \cdot l^{-1}$ of trans-4MCHCA for 2 months. Following completion of the 250 $mg \cdot l^{-1}$ experiments, ICR #2 was operated with an initial substrate concentration of 500 $mg \cdot l^{-1}$ for an additional month and the generated data was compared with the data obtained in ICR #3 to assess the reproducibility of the results. During the entire study, ICR #1 was operated with effluent feed which contained any residual substrate from both the CSTR and/or the ICRs for the purposes of complete biodegradation to permit acceptable effluent disposal. This bioreactor was also operated to duplicate one dilution rate condition for ICR #3.

Sterile feed medium was prepared in quantities suited to the flow rate being evaluated such that a sterile feed supply was renewed at intervals of 24 hours. The frequent preparation of the feed medium reduced the likelihood of contamination of the feed. The pump tubing was connected to the feed stock tubing with adapted sterile plastic flexible connectors. The concentration of substrate at the delivery point to the bioreactor was checked frequently to ensure that no reaction occurred in the delivery tubing. Flow rates for both bioreactor systems were verified at intervals not exceeding 24 hours by monitoring the weight of the effluent. If necessary, the peristaltic pump was adjusted accordingly to meet the desired experimental flow rate. Both systems were also monitored daily for optical density, pH and sampled in duplicate for analysis of substrate concentration. Each bioreactor was operated at a predetermined flow rate until steady

state conditions were reached, using the same criteria as described for the CSTR. The initial flow rate for ICR #3 was set at the critical dilution rate determined for the CSTR (0.6 d^{-1} or a theoretical residence time of 1.4 d). Following the establishment of steady state, the bioreactor was maintained at that flow rate for a period greater than 3 days or 3 residence times. At steady state conditions, the effluent was monitored for residual substrate concentration and freely suspended biomass in the liquid effluent. Upon observation of the steady state conditions, the flow rate was increased incrementally. For ICR #3 operated at 500 $mg \cdot l^{-1}$, the residence time increments were: 7.6, 3.4, 2.4, 2.0, 1.3, 0.93, 0.71, 0.59 and 0.43 h with duplicate steady state conditions measured in ICR #1 for 1.2 h. For ICR #2 operated at 250 $mg \cdot l^{-1}$, the residence time increments were: 6.5, 4.4, 2.2, 1.5, 0.53, 0.40, 0.27 h. A decrease in the calculated volumetric reaction rate was observed in ICR #3 at the residence time of 0.6 h. ICR #2 showed a decrease from 100% conversion at the lowest residence time. ICR #2 was then operated with a 500 $mg \cdot l^{-1}$ feed concentration for 0.43, 0.33 and 0.25 h residence times. The shorter residence times (<0.59 h) for 500 $mg \cdot l^{-1}$ in ICR #2 also showed a decrease in the calculated volumetric reaction rates.

Upon disassembly of the ICRs, the bioreactors were drained for 1 hour and the packing material was removed from the glass columns. The drained liquid was collected and weighed to determine the liquid hold-up in each bioreactor. The wet biofilm packing material from each bioreactor was then placed in pre-weighed aluminum weighing dishes and weighed. The packing for each bioreactor was dried at 65^{o}C in a vacuum oven with a vacuum pressure of -70 kPa for 24 hours. The packing was carefully removed and weighed to obtain the weight of the dry biomass for each bioreactor.

4.2.4 Analytical Methods and Toxicity Tests

In this study, GC-FID (gas chromatograph-flame ionization detection) analyses of trans-4MCHCA concentrations in the biological medium were performed with a dedicated Hewlett Packard (HP) GC/FID (5890 HP Series II with Chemstation operating software). The analytical procedures and conditions have been presented in detail in Chapter 2.

The concentration of biomass was determined by direct measurement of the optical density (OD) of samples collected from the bioreactor systems in glass syringes with stainless steel needles. The OD was measured at a wavelength of 620 nm (Janikowski et al., 2002; Shuler and Kargi, 2002; Gadekar et al., 2006) using a spectrophotometer (Shimadzu, Model 1240). The OD was related to dry-weight using a calibration curve presented in Appendix A (Figure A.1).

Biomass concentration was also monitored at each steady state by most probable number (MPN) method and plate counts. In these methods biomass concentration is represented in terms of colony forming units (CFU) in each sample (Spiegelman et al., 2005). The MPN method of monitoring biomass concentration was conducted in accordance with accepted procedures (Thomas, 1942). Samples were collected in duplicate and diluted with 0.85% saline solution through an appropriate range and distributed into a sterile 96 well plate with well volumes of 400 µl. Each plate was prepared with a minimum of two blank rows with 90 µl of sterile Difco® Bacto Nutrient Broth (5 g of peptone and 5 g of beef extract per litre of RO water) in each well. The dilutions were added to the remaining rows in 10 µl quantities per well added to the 90 µl nutrient broth. Duplicate plates were prepared. The tops of the microplates were sealed with sterile covers to prevent cross contamination. Microplates were vigorously mixed (setting 2) on a Jitterbug incubator-shaker (Model 130000, Boekel Scientific) for a minimum of 36 hours at a temperature of 25^{o}C. The plates were then read with an automated microplate reader (Bio-Tek Elx808 with KC4 Software by Bio-Tek Instruments, Inc.). The Bio-Tek reader was attached to a laptop computer housing the software for direct data recording. The reader recorded optical densities in each of the 96 microplate wells at a selected (pre-set) wavelength of 630 nm.

Three of the dilutions were selected for plating on aseptic agar. The agar mixture was prepared in advance by dissolving 3 g of Difco® Bacto Plate Count Agar in 100 ml of RO water under sterile conditions. Each of the selected dilutions, representative of cell counts ranging in the order of 30 to 100 CFUs per ml of the bioreactor effluent, was

plated in duplicate. The plates were counted 48 hours after incubation at room temperature. Diversity of colony shapes and colours was also noted.

Toxicity reduction evaluation tests for the medium, substrate stock solution and bioreactor effluents were carried out in accordance with the methods outlined in Chapter 1. *Artemia salina* tests were conducted for all bioreactor effluents. *Daphnia magna* and Microtox™ tests were carried out by ALS Laboratories as required in accordance with the appropriate regulatory protocols (Alberta Energy and Utility Board, 1996; Environment Canada, 2000).

4.3 Results

4.3.1 Continuously Stirred Tank Reactor

The steady-state profiles of the trans-4MCHCA and biomass concentrations observed at various dilution rates are shown in Figure 4.3. Applying dilution rates ranging between 0.04 to 0.56 d^{-1} led to the increase in residual steady state substrate concentration from 0 to 443 $mg \cdot l^{-1}$. This was accompanied by a gradual but continuous decrease in concentration of biomass. The maximum biomass concentration (145 $mg \cdot l^{-1}$) was observed at steady state conditions for 100% conversion of the substrate in the bioreactor at a dilution rate of 0.10 d^{-1} or an equivalent residence time of 10 days and decreased to a minimum of 40 $mg \cdot l^{-1}$ at the highest dilution rate (0.95 d^{-1}) immediately prior to wash-out (1 day residence time). A comparison of the biomass concentration data using the three methods described in Section 4.3.4 for the CSTR is presented in Table 4.1 together with the biomass data for the ICRs. Biomass concentration as measured by OD indicated a decreasing trend with increase in dilution rate. However; this trend was not observed in the MPN or plate count data.

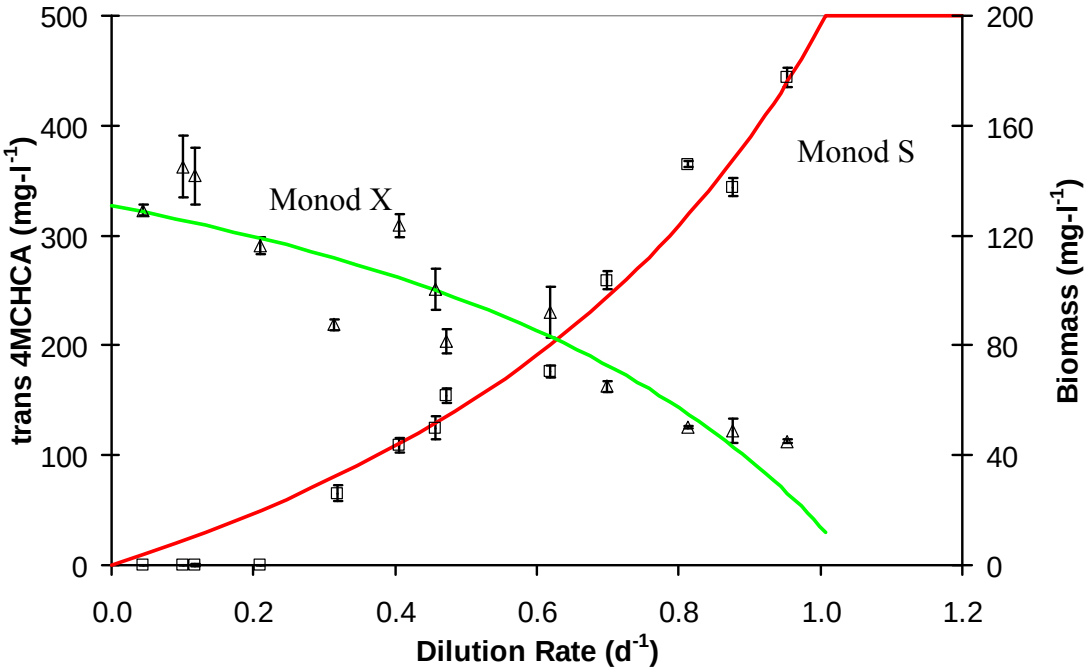

Figure 4.3 Profiles of trans-4MCHCA and biomass concentrations observed in the continuously stirred tank reactor under steady state conditions at 23°C. (□ : average substrate concentration; Δ: average biomass concentration; error bars represent 95% confidence interval in measured substrate and biomass concentrations; ▬: represents theoretical D (μ based on Monod best fit parameters for μ_m and Ks); ▬: represents theoretical biomass concentration based on Monod best fit parameters for μ_m, Y and Ks)

4.3.2 Evaluation of Kinetic Parameters Based on CSTR Data

The continuous stirred reactor provides data regarding the steady state conditions at variable dilution rates (D). The general material balance equations for substrate (trans-4MCHCA) and biomass show that in a continuous system, assuming Monod kinetics and under steady state conditions (Shuler and Kargi, 2002):

$$\mu_{net} = u_g - k_e = D = \frac{F}{V} \qquad (4.1)$$

Table 4.1 Summary of comparison of MPN, plate count and biomass data

	MPN ($CFUs \cdot ml^{-1}$)		PC ($CFUs \cdot ml^{-1}$)		Biomass ($mg \cdot l^{-1}$)	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
CSTR	1.3×10^{8}	3.6×10^{10}	3.5×10^{7}	6.1×10^{9}	45	145
ICR #2	9.0×10^{6}	1.4×10^{9}	9×10^{6}	1.7×10^{8}	31	116
ICR #3	6.3×10^{7}	1.3×10^{9}	1.0×10^{7}	4.6×10^{8}	36	301

Notes:
No trends in MPN or plate count data were observed with increasing flow rate for any of the bioreactors.
CSTR – plate count data are an average of 1.22±0.25 orders of magnitude less than MPN data; biomass followed a linear trend with increased flow rate of -480 mg biomass$\cdot l^{-1}$ per 100 ml$\cdot h^{-1}$ ($R^2 = 0.91$)
ICR #2 – plate count data are an average of 0.93±0.12 orders of magnitude less than MPN data; biomass data showed an increase with flow rate and over operating time by
+70 mg biomass$\cdot l^{-1}$ per 100 ml$\cdot h^{-1}$ in flow rate ($R^2 = 0.61$)
ICR #3 – plate count data are an average of 0.54±0.24 orders of magnitude less than MPN data; biomass data did not indicate a trend

The dependency of μ_{net} on substrate concentration can be described by a variety of unstructured, non-segregated models such as Monod, Tessier, Moser, Contois (Gadekar et al., 2006) or Haldane which incorporates substrate inhibition to the Monod model (Nikakhtari and Hill, 2006). The most commonly used kinetic expression relating the rates of bacterial growth and substrate removal to the concentration of substrate is the Monod expression (Lin et al., 2007; Karamanev and Nikolov, 1991). Monod proposed a simple equation (4.2) which applies whenever a single essential substrate is a growth-limiting factor (Purwaningsih, 2002). The Monod equation describes substrate-limited growth best when growth is slow and population density is low (Shuler and Kargi, 2002). Other equations have been proposed to describe the substrate limiting growth phase. Specifically, the Contois equation (Eq. 4.3) has a saturation constant proportional to cell concentrations and best describes substrate-limited growth at high cell densities (Chen et al., 1980; Gadekar et al., 2006). Finally, in cases where the substrate presents an inhibitory degradation environment, the simplest of specific growth rate models is the Haldane model (Quail and Hill, 1991; Tarighian et al., 2001). This model was also

evaluated as little is known on the inhibitory effects of trans-4MCHCA nor naphthenic acids in general. Given their recalcitrant properties, the Haldane inhibition model was also evaluated to determine the potential inhibitory effect of substrate. The models used are summarized in Equations 4.2 through 4.4.

$$\mu_{net} = \frac{\mu_m S}{K_s + S} - k_e \qquad \text{Monod expression} \qquad (4.2)$$

$$\mu_{net} = \frac{\mu_m S}{K_{sx} X + S} - k_e \qquad \text{Contois expression} \qquad (4.3)$$

$$\mu_{net} = \frac{\mu_m S}{K_s + S + S^2 / K_i} - k_e \qquad \text{Haldane expression} \qquad (4.4)$$

The value of various coefficients for these models were determined by fitting the experimental data to the models and performing non-linear regression using least-squares minimization together with the Solver tool available in Excel™ software. The values for endogenous decay (k_e) and yield (Y) were also determined graphically based on the experimental data to verify the theoretical value of yield and to support the assumption that k_e is significantly less than the gross specific growth rate (μ_g).

Best fit values for kinetic coefficients (Ks and μ_m) for the selected substrate-limited growth kinetic models are summarized in Table 4.2. A comparison of the sum of residual squares singled-out the Monod expression as the most suitable model. Interestingly among the tested models, the Monod expression is the simplest form with only two coefficients as opposed to other expressions having three coefficients. Figure 4.3 shows the goodness of fit for both biomass and substrate concentrations for Monod model with a μ_m value of 1.74±0.004 d^{-1} and Ks of 363±17 $mg \cdot l^{-1}$ which represented the best fit kinetic coefficients.

Endogenous decay and yield coefficient were determined using Equation 4.5 which results from manipulation of the material balances for steady state biomass and substrate concentrations for a CSTR (Reynolds and Richards, 1996).

$$X = \frac{Y(S_o - S_{ss})}{1 + k_e \theta_i} \tag{4.5}$$

θ_i represents the residence time for a given flow rate. The values of endogenous decay and yield were 0.0019 d^{-1} and 0.24±0.01 mg cells·(mg substrate) $^{-1}$, respectively. The yield coefficient corresponded well with the overall yield (0.26±0.08 $mg \cdot mg^{-1}$) determined in batch culture experiments reported in Chapter 3.

4.3.3 Immobilized Cell Reactors

The profiles of biomass and substrate concentrations at steady state conditions for a range of dilution rates up to 95 d^{-1} and 56 d^{-1} are presented in Figures 4.4 and 4.5 for the ICR #2 and #3 reactors, respectively. Complete conversion of the substrate below the detection limit of the analytical method (3 $mg \cdot l^{-1}$) by the immobilized cell biofilm was achieved at dilution rates up to 60 d^{-1} for the 250 $mg \cdot l^{-1}$ (ICR #2). Complete conversion of the substrate for the 500 $mg \cdot l^{-1}$ (ICR #3) was observed up to a dilution rate of 40 d-1 corresponding to 36 minute residence time. Application of higher dilution rates led to partial biodegradation with the minimal biodegradation being observed at a dilution rate of 56 d^{-1} (26 minute residence time) where steady state conditions showed a decrease in degradation. The reported dilution rates are calculated on the basis of the actual volume of free liquid in the bioreactor determined at the end of the experimentation.

Table 4.2 Summary of best fit kinetic parameters using CSTR data and three growth models

	μ_m	SE	K_s	K_{sx}[1]	SE	K_i	SE	SSE	SSE_w	W_x
	(d^{-1})	(d^{-1})	($mg \cdot l^{-1}$)	($mg \cdot mg^{-1}$)	($mg \cdot l^{-1}$)	($kg \cdot l^{-1}$)	($kg \cdot l^{-1}$)			
Monod[2]	**1.74**	**0.0044**	**363**	–	**17**	–	–	11,037	**15,805**	**1.6**
Monod[3]	1.66	0.0047	332	–	19	–	–	**11,011**	15,987	1.6
Contois[2]	1.17	0.078	–	2.17	0.57	–	–	29,796	50,093	2.3
Contois[3]	1.15	0.072	–	2.19	0.54	–	–	29,587	50,575	2.3
Haldane[2]	1.74	0.0044	363	–	17	160[4]	7.9	11,037	15,805	1.6
Haldane[3]	1.66	0.0047	332	–	19	160[4]	9.4	11,012	15,988	1.6

Notes:
1 – K_{sx} is the Contois saturation constant (mg trans-4MCHCA per mg biomass)
2– standard error based on best fit of minimizing the SSE for X weighted and S; based on best fit for μ_m, K_s and Y (0.24 mg biomass per mg substrate)
3– standard error based on best fit of minimizing the SSE for S
4– inhibition constant (K_i) best fit exceeds the solubility limit
Bold – indicates best fit model based on minimum SE and SSE
SE – standard error in parameter reported to the left; SE for the saturation constant applies to relevant constant (K_s, K_{sx} or K_i)
SSE – sum of the squares error of biomass and substrate concentration prior to weighting
SSE_w – sum of the squares error of biomass weighted and substrate concentration
W_x – model weighting factor for equating the error in X with the error in S

The bioreactor fed with a medium containing 250 $mg \cdot l^{-1}$ of trans-4MCHCA (ICR #2) was initially operated with a flow rate of 4.2 $ml \cdot h^{-1}$ and 100% conversion was achieved within 5 days. The flow rate was increased by 1.5 $ml \cdot h^{-1}$ every 5 to 7 days until complete conversion of substrate was achieved. Over this period of incremental flow increases, the optical density observed in the free liquid increased. The bioreactor eventually plugged due to complete filling of the pores with biofilm after 1 month of

operation and had to be dismantled and cleaned by passing a mild solution of NaOH through the bed. The bioreactor was then restarted and continued to operate for an additional month until the dilution rate was increased to 90 d^{-1} (100 $ml \cdot h^{-1}$). Subsequent to this experimentation, ICR#2 was operated with an initial feed concentration of 500 $mg \cdot l^{-1}$. This served to assess the reproducibility of the experimental results and to find the performance of the system at a maximum dilution rate of 56 $ml \cdot h^{-1}$. ICR #2 also plugged after approximately 1 month of operation. The biomass concentration observed during steady state generally increased with dilution rate and extent of operation.

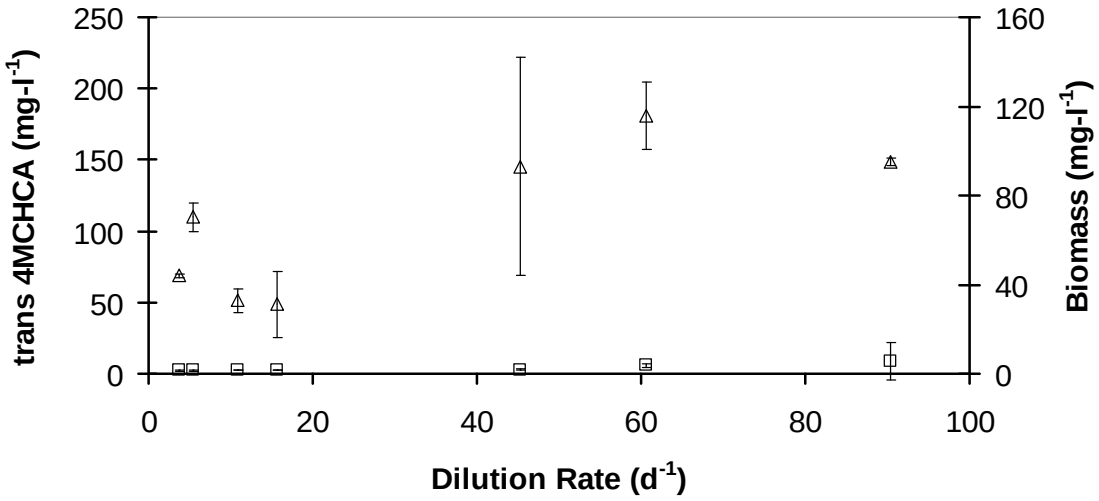

Figure 4.4 Immobilized cell reactor (ICR #2) with feed stock at 250 $mg \cdot l^{-1}$ for steady state conditions throughout the range of evaluated flow rates. (□: represents average substrate concentration at steady state conditions; Δ: represents average biomass concentration at steady state conditions; error bars represent 95% confidence interval in measured substrate concentrations and biomass at steady state).

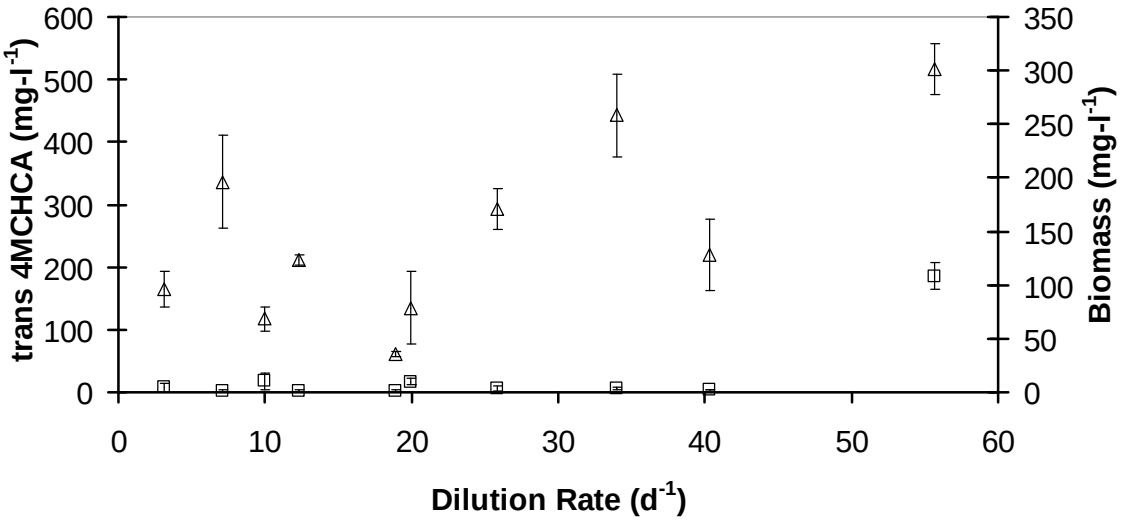

Figure 4.5 Immobilized cell reactor (ICR#3) with feed stock at 500 mg·l^{-1} for steady state conditions throughout the range of evaluated flow rates. (□: represents average substrate concentration at steady state conditions; Δ: represents average biomass concentration at steady state conditions; error bars represent 95% confidence interval in measured substrate concentrations and biomass at steady state).

ICR #3 was operated initially with a flow rate of 3 ml·h^{-1} which increased by increments of 3 ml·h^{-1} initially. At 19 ml·h^{-1}, 100% conversion was still being achieved and the flow rates were adjusted by approximately 8 ml·h^{-1} increments to a flow rate of 40 ml·h^{-1} where 100% conversion was observed. At this time consistent plugging was observed in ICR #3 so the experimentation was continued on ICR #2 as described above. The change in feed concentration initially resulted in excess biomass being released from the bioreactor presenting an increase in observed optical density. Initially, the bioreactor operated with no conversion of the feed substrate. However, as the bioreactor feed rates were lowered, the biofilm stabilized and the substrate conversion increased. In general, the maximum observed biomass concentrations were 300 mg·l^{-1} and 116 mg·l^{-1} for the feeds containing 500 mg·l^{-1} and 250 mg·l^{-1} of substrate, respectively. The biomass concentration as determined by OD for ICR #3 decreased with increase in flow rate of the feed and followed a linear trend of -480±47 mg biomass·l^{-1} per 100 ml·h^{-1}.

A summary of the biomass concentration data for the ICRs compared with the CSTR data is presented in Table 4.1. As observed with the CSTR biomass data, a trend was not observed in the MPN or plate count data. In general the MPN method resulted in biomass concentrations approximately one order of magnitude greater than that of the plate count method. This could be attributed to differences in the growth medium used for support of the microorganisms in each method.

The biofilm for the 250 $mg \cdot l^{-1}$ reactor (ICR #2) had a dry mass of 25% (mg of dry biomass per mg of clean dry packing material) and the volume of the liquid in the bioreactor was 27 ml. The dry biomass was measured subsequent to completion of the 500 $mg \cdot l^{-1}$ experimentation (5 weeks additional operation for ICR #2). In ICR #3, dry biomass was 18% (mg dry biomass per mg clean dry packing material) and the volume of the liquid in this bioreactor was 24 ml.

4.3.4 Comparison of Biodegradation Rates in the CSTR and ICR

In this study the performances of the bioreactors were compared using the volumetric reaction rate given by Equation 4.6:

$$V_r = (S_i - S_{ss}) \times D \tag{4.6}$$

The volumetric reaction rate profiles for each of the ICRs operating with feed substrate concentration of 500 and 250 $mg \cdot l^{-1}$ and for the CSTR operated with 500 $mg \cdot l^{-1}$ are presented in Figure 4.6. In ICR #3, operated with 500 $mg \cdot l^{-1}$ trans-4MCHCA, the maximum volumetric reaction rate of 22,000 $mg \cdot (l \cdot d)^{-1}$ was observed at a dilution rate 40 d^{-1} (corresponding to a residence time of 36 minutes). This volumetric reaction rate was approximately 65 times faster than that observed in the CSTR operated with freely suspended cells (230 $mg \cdot (l \cdot d)^{-1}$) at a dilution rate of 0.62 d^{-1} rate or a 1.6 d residence time and a 68% conversion rate. In ICR #2, operated with 250 $mg \cdot l^{-1}$ of substrate, 100% conversion of the substrate was achieved over the entire range of applied dilution rates (4 to 90 d^{-1}) with a maximum volumetric reaction rate of 22,000 $mg \cdot (l \cdot d)^{-1}$ similar to that

observed in ICR #3. This maximum dilution rate for ICR #2 corresponded to a 16 minute residence time.

4.3.5 SEM Results

A SEM image for a segment of unused stainless steel packing material prior to construction of the ICR system is presented in Panel A of Figure 4.7. Panel B presents the SEM (3,300X magnification) of a section of the same packing material removed from the ICR #3 after approximately 5 weeks of operation. For comparison purposes, a SEM of a sample obtained from the liquid culture used in the CSTR is presented in Panel C at 10,000X magnification.

4.3.6 Microbial Identification

Microbial identification carried out for the initial CSTR inoculum and the resultant biofilm developed on the packing material for the ICRs indicated that the consortium was dominated by two aerobic bacterial species. The initial inoculum for the CSTR from a fully degraded batch culture was primarily comprised of *Variovorax paradoxus* (*Alcaligenes paradoxus*). A secondary microbial community of Pseudomonas aeruginosa was also present. The primary colony formed in the ICR operated at a substrate concentration of 500 $mg \cdot l^{-1}$ was identified to be *Chryseobacterium indologenes* (previously identified as *Flavobacterium indologenes*). The second colony which was identified after operating the ICR at an initial substrate concentration of 250 $mg \cdot l^{-1}$ for two months was identified to be *Delftia acidovorans* (formerly *Comamonas*, *Pseudomonas acidovorans*.

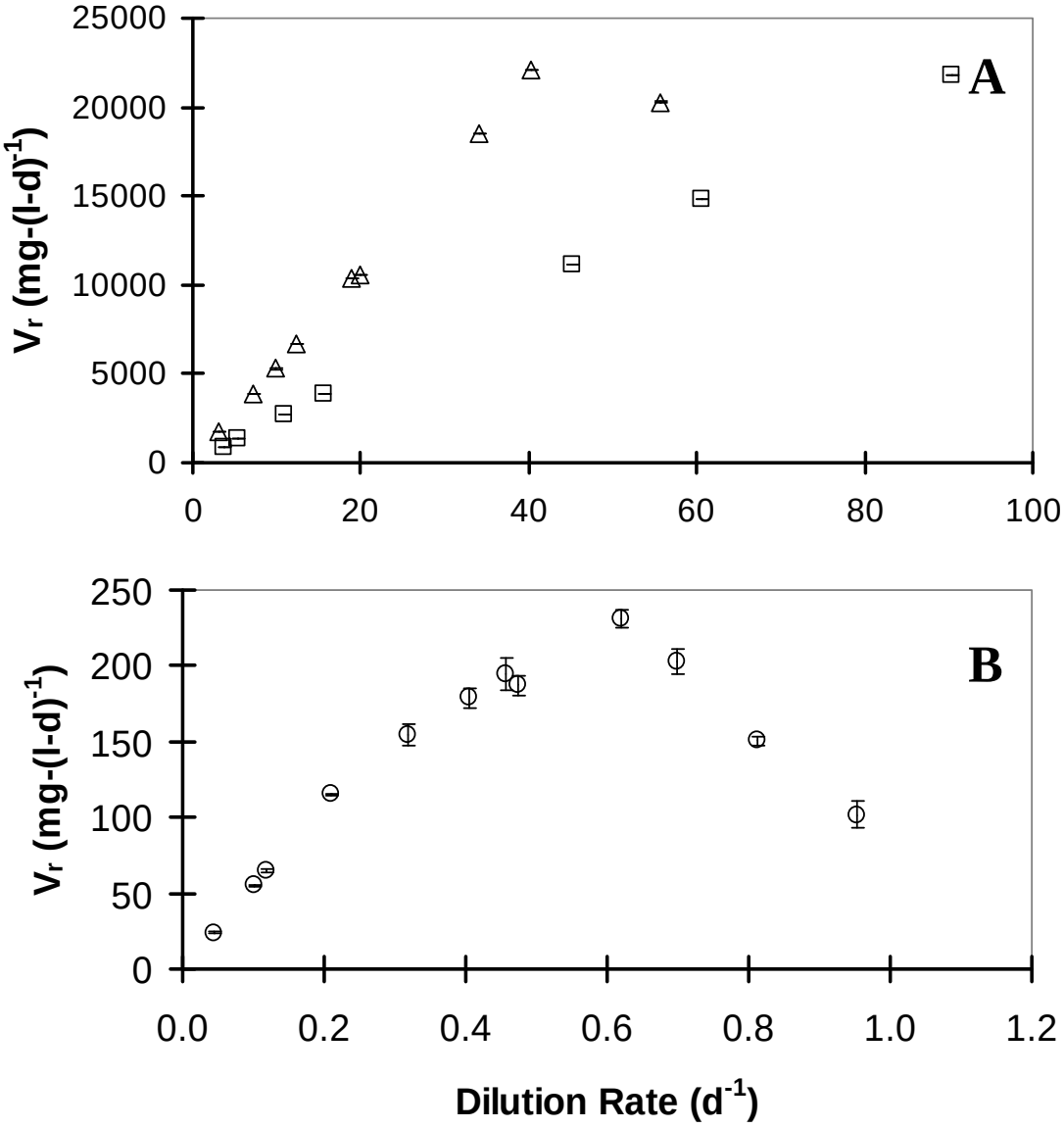

Figure 4.6 Volumetric reaction rates for bioreactors. **Panel A**: ICRs Δ: operating at an initial feed concentration of 500 mg·l^{-1}; □: operating at an initial feed concentration 250 mg·l^{-1}; **Panel B**: ○: CSTR operating at an initial feed concentration of 500 mg·l^{-1} (error bars represent 95% CI based on substrate concentrations measured over the period of steady state).

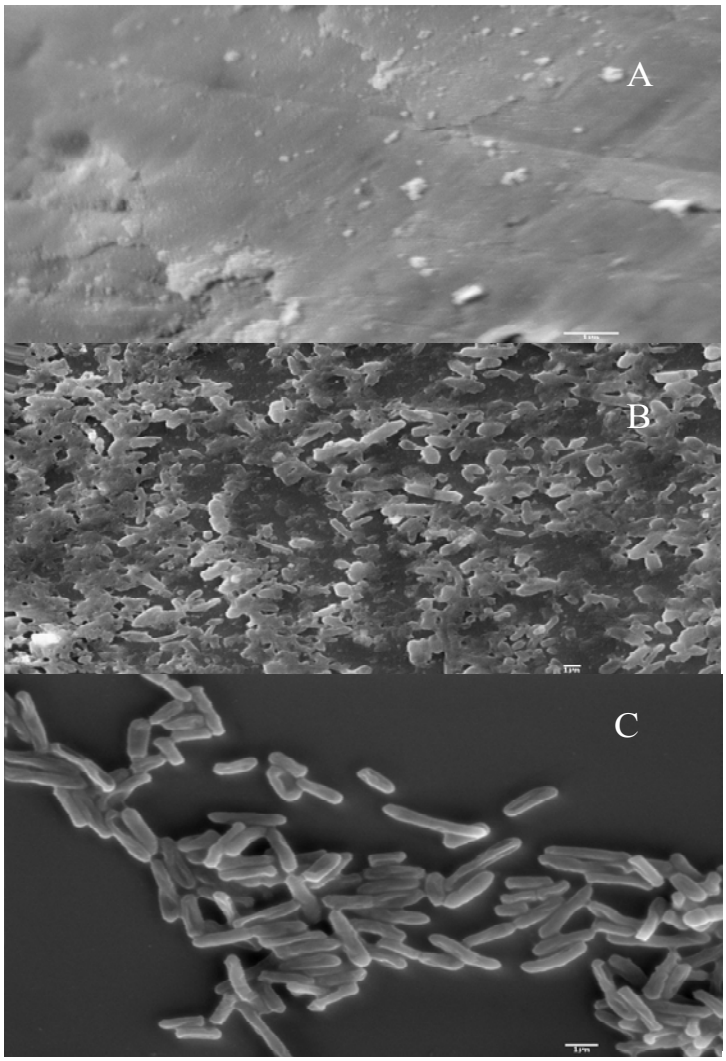

Figure 4.7 SEM results. **Panel A** Clean stainless steel packing material (SEM at various locations 10,000X); **Panel B** SEM 3,300X of biofilm samples from immobilized cell system (500 $mg \cdot l^{-1}$); **Panel C** SEM 10,000X of liquid culture from batch reactor (initial substrate concentration 500 $mg \cdot l^{-1}$).

4.3.7 Toxicity Reduction Evaluation

The results of the toxicity reduction evaluation are summarized in Table 4.3. The *A. salina* test revealed (Tarighian, 1999; Tarighian et al., 2003) that the survival rate was significantly higher in the treated effluent from ICR (>80%) as compared with the untreated feed (<20%). The response to the CSTR effluent collected after approximately 6 months of continuous operation was slightly less based on survival (65%). For

comparison purposes a partially degraded effluent sample collected from ICR #3 in transient mode prior to steady state conditions was tested. The sample contained 160 $mg \cdot l^{-1}$ of residual substrate and resulted in a 67% survival in *A. salina*. The *A. salina* was not sensitive to the Modified McKinney's medium, indicating that the mineral nutrients at the level present in this medium did not impose a toxic effect.

Toxicity reduction tests carried out by ALS Laboratories comprised Microtox™ conducted in accordance with Directive 050 of the Alberta Energy and Utility Board Appendix 4, (1996) and *D. magna* carried out in accordance with Environment Canada (2000) were more sensitive to the nutrient media constituents as well as the CSTR effluent which led to further investigation of the medium as compared with trans-4MCHCA in Millipore water. This revealed that the *D. magna* was less adversely affected by the buffered stock solution than the 500 $mg \cdot l^{-1}$ trans-4MCHCA in Millipore water. The ICR effluent served to be more amenable for both organisms considered (*A. salina* and *D. magna*). Considering that Microtox™ tests conducted on CSTR effluent did not indicate a detectable toxicity and the results of tests with *A. sal*ina which resulted in a higher survival rate in the presence of the effluent from ICR than that in the presence of CSTR effluent further testing of the ICR effluent by Microtox™ was not carried out.

Table 4.3 Summary of toxicity reduction evaluation results

Test Name	Endpoint	Medium	trans-4MCHCA (500 mg·l^{-1}) (in media)	Bioreactor Effluent CSTR	ICR
A. salina[1]	% survival	>80[2]	<20[3]	65	>80[4] 67[5]
Microtox™	IC50 (%)	>82[6]	14	>82[6]	NA
	IC20 (%)	>82[6]	6.0	>82[6]	
D. magna	LC50 (%)	64.5	35	35	80

Notes:
1 − *A. salina* results represent % survival at end of test
2 – a series of 6 tests conducted on medium resulted in a survival of greater than 80% in all cases
3 − a series of 6 tests conducted on trans-4MCHCA stock solution in media resulted in a survival of less than 20% in all cases
4 – a series of 3 tests conducted for completely degraded ICR effluent (ICR #2 and ICR #3) resulted in a survival of greater than 80% in all cases
5 – an additional test was conducted for comparison purposes on partially degraded effluent (160 mg·l^{-1}) during the transient stage of operation for ICR #3 (Flow rate 7.1 ml·h^{-1}; after 1 day of operation)
6 – represents the upper detection limit for test (ALS Laboratories)

NA - not analyzed
Microtox™ and *D. magna* results represent % of original test sample producing the noted endpoint; CSTR effluent was collected from a continuously stirred reactor after approximately 6 months operation; ICR effluent was collected from a packed bed reactors operating over 3 months; medium is prepared with several nutrients for the support of the micro organisms; for the purposes of the biodegradation study, the McKinney's modified medium was used at a concentration of 2:1

4.4 Discussion

The results of this study show that kinetics for microbial growth and biodegradation of trans-4MCHCA can be described by Monod expression. As indicated in the literature, the Monod expression is often suited to slow degrading organic contaminants where substrate-limited cell growth is slow and the population densities are low. Kinetic parameters were determined by best-fitting three simple kinetic models (Monod, Contois and Haldane) to the continuously stirred reactor data. The Monod model was found to be the best model with a maximum specific growth rate of 1.74±0.004 d^{-1} and a half saturation constant of 363±17 mg·l^{-1}. Previously reported

kinetic information for trans-4MCHCA was in unamended river water in a laboratory setting with a specific growth rate of 0.067 d^{-1} (Headley et al., 2002b). The maximum specific growth observed in this study with the use of a CSTR is 26 times higher. The maximum specific growth rate observed in batch reactors was 0.52±0.04 d^{-1} (Chapter 3).

Previous study of enhanced biodegradation of candidate NAs with the addition of phosphate nutrients to tailings pond waters showed no enhancement of biodegradation (Lai et al., 1996), while in laboratory experiments with trans-4MCHCA provision of nutrients for microbial activity has improved the biodegradation rate (Headley et al., 2002b; Paslawski et al., 2008). The results of the present study indicate that maintaining the optimum conditions is not the only contributing factor and the configuration of the bioreactor used for the ex-situ biodegradation of NAs could play a crucial role in the enhancement of biodegradation rate of these compounds.

Biodegradation studies in two bioreactor configurations conducted herein show that the biodegradation of trans-4MCHCA can be enhanced by choosing a proper bioreactor design. A packed-column with established biofilm is able to enhance the biodegradation rate of trans-4MCHCA up to 95 times when compared with a freely suspended cell bioreactor. The volumetric reaction rates in the CSTR, reaching up to a maximum of 230 $mg\cdot(l\cdot d)^{-1}$, are significantly lower than those seen for simpler air pollutants such as toluene reportedly achieving removal rates of 2,300 $mg\cdot(l\cdot d)^{-1}$ with 93% efficiency (Bi et al., 2004). A bioreactor designed to improve the biodegradation rates of naphthalene and 2-methylnaphalene achieved degradation rates of 1,500 and 720 $mg\cdot(l\cdot d)^{-1}$, respectively (Riess et al., 2005; Yu et al., 2006). This reduction in biodegradation rate for the more complex PAH structure may be more comparable with the rates observed with trans-4MCHCA in a freely suspended system.

The highest observed biodegradation rate achieved in this study was 22,000 $mg\cdot(l\cdot d)^{-1}$ which is one-fifth of reactor performance achieved for a much simpler organic pollutant. Phenol degradation in a similar packed bed column with glass spheres as carrier matrix resulted in a biodegradation rate of 105,000 $mg\cdot(l\cdot d)^{-1}$ which was much

higher than that reported in the literature for other packed bed designs (Quail and Hill, 1991). The reaction rate observed for the candidate NA as compared to that of a much simpler organic contaminant by bioreactor design indicates the potential of a reactor enhanced biodegradation system. This bioreactor design is a useful tool for modelling the biodegradation parameters and studying the effects of parameter variations and scale-up design.

The results of this study with rapid screening tests reveal a significant reduction in the toxicity of an effluent resulted from biodegradation of a soluble naphthenic acid (trans-4MCHCA) when compared with the feed containing 500 $mg \cdot l^{-1}$ of trans-4MCHCA. The toxicity reductions observed by both the *A. salina* and the *D. magna* tests indicate that the effluent from the ICR treatment system is significantly less toxic than from the CSTR treatment system.

CHAPTER 5
A PLUG FLOW MODEL FOR BIODEGRADATION OF TRANS 4-METHYL-1-CYCLOHEXANE CARBOXYLIC ACID IN AN IMMOBILIZED CELL REACTOR

A similar version of this chapter has been prepared for submission to the Canadian Journal of Chemical Engineering.

Contribution of the PhD Candidate

Modelling and experimental verification were conducted by Janice Paslawski with technical input and guidance from Dr. M. Nemati and Dr. G.A. Hill. All written text was prepared by Janice Paslawski with editorial guidance from Dr. M. Nemati and Dr. G.A. Hill.

Contribution of this Chapter to the Overall Study

This chapter describes the development of a model for biodegradation kinetics of trans-4MCHCA in an immobilized cell reactor using the kinetic data presented in Chapter 4. The use of a plug flow model is considered for predicting the biodegradation of trans-4MCHCA in an immobilized cell reactor with two input concentrations. Using biokinetic parameters (μ_{max}, K_s and Y) from the CSTR experiments presented in Chapter 4, the bioreactor parameters are evaluated using the experimental data for the immobilized cell reactors (also presented in Chapter 4). Consideration of the assumptions in terms of improvements to the model as an optimization tool for bioreactor design is discussed. The model is used to predict the performance of the immobilized cell reactors over a range of operating conditions.

Key words: biodegradation, immobilized cell reactor, plug flow model, Monod kinetics, naphthenic acids

Nomenclature

A – cross-sectional area of the reactor (cm^2)

A_b – cross-sectional area of biofilm (cm^2)

A_p – cross-sectional area of packing fibre (cm^2)

$\mathcal{D}$ – axial dispersion coefficient ($cm^2 \cdot s^{-1}$)

D – dilution rate (d^{-1})

F – liquid flow rate of the substrate medium ($ml \cdot h^{-1}$)

K_s – Monod saturation constant ($mg \cdot l^{-1}$)

L – length of reactor column (cm)

Pe – Peclet number (unitless)

S_i – substrate feed concentration ($mg \cdot l^{-1}$)

S_{ss} – steady state substrate concentration at the bottom of the reactor ($mg \cdot l^{-1}$)

S_z – steady state substrate concentration at any point z in the axial direction of liquid flow ($mg \cdot l^{-1}$)

SA_p – packing surface area (cm^2)

t – thickness of the packing (cm)

V_r – volumetric reaction rate ($mg \cdot (l \cdot d)^{-1}$)

V_w – working volume of reactor (l)

V_{wbiof} – volume of wet biofilm (l)

w – width of stainless steel packing fibre (cm)

W_b – dry weight of biomass in biofilm (mg)

W_p – weight of clean stainless steel packing (mg)

X_l – biomass concentration in the free liquid effluent ($mg \cdot l^{-1}$)

X_{biof} – biomass concentration in the biofilm ($mg \cdot l^{-1}$)

X_T – total biomass concentration in the reactor ($mg \cdot l^{-1}$)

Y – biomass yield coefficient (mg biomass·mg $substrate^{-1}$)

z – axial distance in direction of flow (cm)

Greek Symbols

ρ_{ss} – density of stainless steel ($mg \cdot cm^{-3}$)

δ – biofilm thickness (cm)

δ_{eff} – effective biofilm thickness (cm)

α – coefficient for effective biomass concentration in biofilm (unitless)

η – porosity of packing bed (unitless)

ϕ_L – liquid hold up in the reactor (unitless)

μ – specific growth rate (d^{-1})

μ_m – maximum specific growth rate (d^{-1})

Abbreviations

CSTR – continuously stirred tank reactor

EPS – extracellular polymer substance

ICR – immobilized cell reactor

OD – optical density

RO – reverse osmosis

SE – standard error

SSE – sum of the squares of the error

trans-4MCHCA – trans 4-methyl-1-cyclohexane carboxylic acid

5.1 Introduction

Environmental pollutants in wastewaters are reportedly unique in the fact that they contain high concentrations of toxic chemicals. Environment Canada and the United States Environmental Protection Agency have identified priority pollutants and update this list on an ongoing basis since the early 1980s (CEPA, 1999, EPA, 2008). These priority pollutants in waste waters tend to be water-soluble compounds resulting from chemical processes and are recalcitrant in the environment and toxic to aquatic organisms (Quail and Hill, 1991). Bioremediation has been reported as an effective strategy for removal of environmental pollutants since the 1950s (Yu et al., 2006). Immobilized cell column reactors are widely utilized in situations where micro organisms need to be used over extended periods and the treatment system is run continuously and at high flow rates (Ranjan et al., 2008).

Naphthenic acids (NAs) are a large group of individual compounds collectively following a single chemical formula which naturally occur in crude oil. NAs have been identified as one of the major sources of toxicity to aquatic life in the Athabasca region oil sands tailings water. The toxicity has been reported to decrease with biodegradation of the NAs. However, in-situ biodegradation rates have not met with the increases in production. Studies on the biodegradability of NAs as a mixture in tailing ponds waters have been unsuccessful using laboratory cultures of tailings pond water bacteria (Scott et al., 2005). In order to make bioremediation a viable option for total NAs, alternatives to natural degradation such as bioaugmentation must be considered.

Mathematical models have been used to predict biodegradation of environmental pollutants in lab-scale reactors for the purpose of designing large scale systems and predicting the performances of such systems. A number of researchers have developed various models for packed bed reactor models for the prediction of reactor performance and maximum loading rates (Quail, 1990; Tarighian et al., 2001; Clarke et al., 2005; Nikakhtari and Hill, 2006).

Modelling of the immobilized cell reactor (ICR) performance reported in Chapter 4 is necessary for effective reactor design for future work. The objective of this study was to develop a suitable mathematical model capable of predicting the reactor performance under steady state conditions and to evaluate this model using the kinetic data for biodegradation of the trans isomer of 4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA) generated in the ICR.

5.2 Experimental

5.2.1 Substrate, Microbial consortium and Media

A single substrate was used in the biodegradation experimentation for the evaluation of the immobilized cell reactors. A candidate naphthenic acid, trans-4MCHCA, was used at two initial concentrations (250 $mg \cdot l^{-1}$ and 500 $mg \cdot l^{-1}$) for the evaluation of the biokinetics in a biofilm reactor system. A mixed culture of micro organisms capable of degrading the substrate was used in the experimental section of this

study. The consortium was comprised of two predominant species identified to be *Chryseobacterium indologenes* and *Delftia acidovorans*. McKinney's modified medium (Paslawski et al., 2008) containing non-growth rate limiting concentrations of all required mineral nutrients was used for the growth and maintenance of the microbial consortium and biodegradation studies.

5.2.2 Immobilized Cell Reactors

Immobilized cell reactors reported in detail in Chapter 4 were used as the experimental reactors for continuously fed biodegradation of trans-4MCHCA at two initial substrate concentrations (Figure 5.1). Selected reactor properties together with the packing material specifications are summarized in Table 5.1. Three reactors were constructed similarly comprising columns constructed of glass and packed with stainless steel mesh material. At the time of experimentation the reactors were operated in continuous submerged and fully aerated modes. Initially one reactor was operated in trickle mode as a continuously fed packed bed containing a large section of packing material. Recycling of partially degraded effluent from previous work at an average flow rate of 20 $ml \cdot h^{-1}$ provided both viable organisms and substrate. This allowed establishment of a biofilm with the ability of degrading trans-4MCHCA in the bioreactor prior to disposal. The biofilm developed over a two month period.

For experimentation of the submerged immobilized cell reactors, the established biofilm was sectioned into three equal portions and placed in individual reactors labelled ICR #1 through #3. ICR #1, the initial trickle column, was used to biodegrade unused substrate over the experimentation period. Two clean reactors were then set up (ICR #2 and #3) for experimentation. ICR #2 was operated with a medium containing 250 $mg \cdot l^{-1}$ of trans-4MCHCA. ICR #2 operated with an initial flow rate of 4.2 $ml \cdot h^{-1}$ and was incrementally increased through 6.1, 12, 51, 63 and 100 $ml \cdot h^{-1}$. Each flow rate was operated continuously for a minimum of 5 days and maintained until a steady state condition of substrate conversion was observed. Throughout experimentation, the optical density was observed in the free liquid phase at the base of the reactor ($z = 0.085$ m).

Table 5.1 Specification of the immobilized cell reactors

Parameter	**ICR #2**	**ICR #3**
Column height (cm)	8.5	8.5
Column diameter (cm)	4.0	4.0
Weight of stainless steel packing (g)	11.42	9.44
Dry weight of biofilm (g)	2.82	1.68
Density of stainless steel, ρ_{ss} ($g \cdot cm^{-3}$)	7.95	7.95
Porosity, η (unitless)	0.99	0.99
Volume of free liquid at completion, V_l (ml)	27.1	24.0
Reactor volume (cm^3)	107	107

A second reactor with a similar configuration (ICR #3) was operated with a feed substrate concentration of 500 $mg \cdot l^{-1}$. This reactor was initially operated in a continuously fed mode with a flow rate of 3.1 $ml \cdot h^{-1}$. Upon 100% conversion of the substrate, the flow rate was increased step-wise through 7.1, 9.9, 12, 19, 26, 34, 40 and 56 $ml \cdot h^{-1}$. A duplicate steady state condition on ICR #1 was evaluated at 20 $ml \cdot h^{-1}$.

As determined at the end of the experimentation, the biofilm for the 250 $mg \cdot l^{-1}$ reactor (ICR #2) had a total measured dry mass of 25% (mg of dry biomass per mg of clean dry packing material) and the volume of the liquid in the reactor was 27 ml. The dry biomass was measured subsequent to completion of the 500 $mg \cdot l^{-1}$ experimentation (5 weeks additional operation for ICR #2). In ICR #3, dry biomass was 18% (mg dry biomass per mg clean dry packing material) and the volume of the liquid in this reactor was 24 ml.

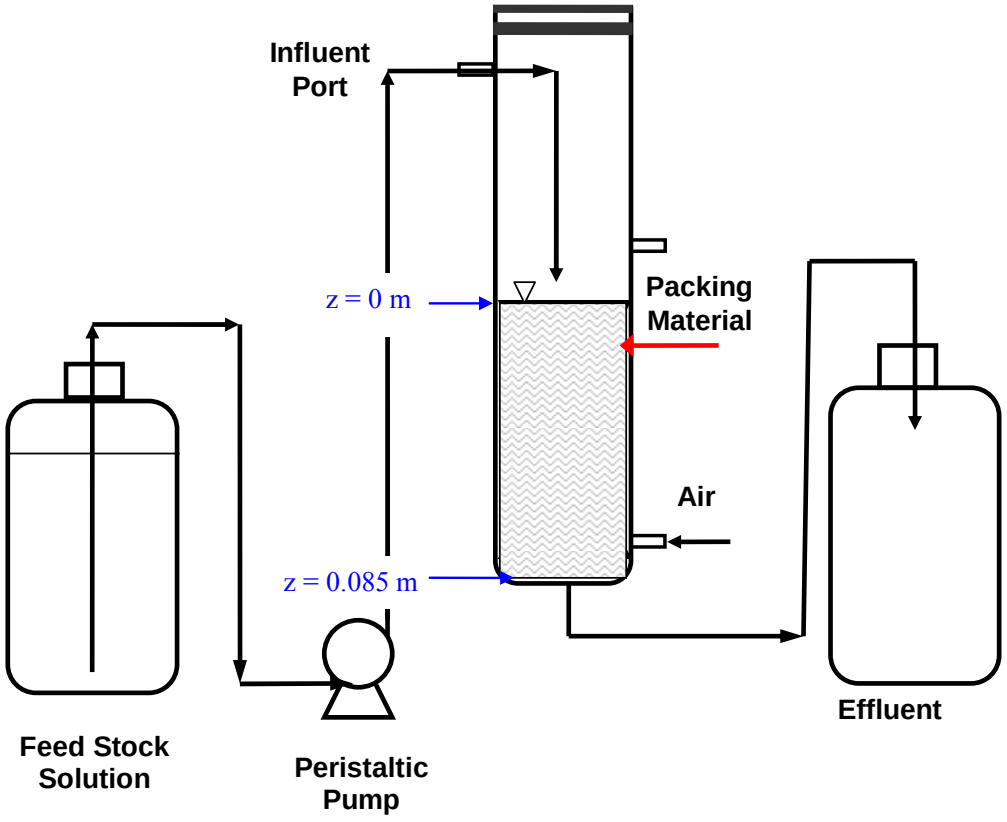

Figure 5.1 Schematic of immobilized cell reactor.

5.2.3 Model

Mass balance equations for biomass and substrate for a thin section at any point (z) along the axial direction of flow of a tubular reactor are developed for the Monod kinetic model in Bailey and Ollis (1977). Figure 5.2 illustrates a thin section over which the mass balance equations are integrated to give the substrate concentration (S_z) at steady state in the liquid phase at any point (z) along the axial direction. The following non-linear differential equation governing concentration distributions in an immobilized cell reactor are used to predict the performance of the reactor during the continuous steady-state biodegradation process, which are similar to equations used in previous reactor performance models (Quail and Hill, 2001; Nikakhtari and Hill, 2006). The general equation for a dispersed plug flow model is given by Equation 5.1:

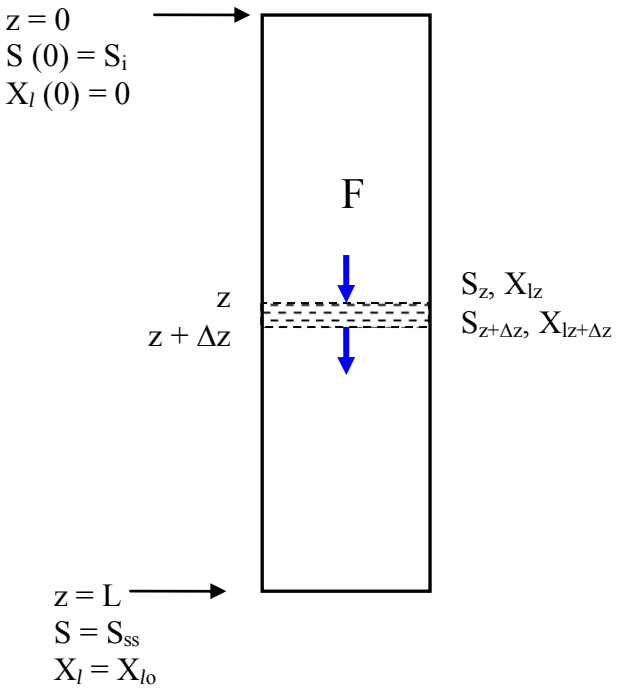

Figure 5.2 Schematic of tubular flow reactor.

$$\mathcal{D}\frac{d^2S_z}{dz^2} - \frac{F}{A\phi_L}\frac{dS_z}{dz} - \frac{\mu X_T}{Y} = 0 \qquad (5.1)$$

Where ϕ_L is the liquid hold up in the biofilm determined by the ratio of the free liquid in the reactor to the total working volume of the reactor (total volume of pore space):

$$\phi_L = V_l / V_w \qquad (5.2)$$

Based on our previous study, the dependency of specific growth rate on the substrate concentration was shown to follow the Monod expression (Chapter 4) given by Equation 5.3.

$$\mu = \frac{\mu_m S_z}{K_s + S_z} \qquad \text{Monod expression} \qquad (5.3)$$

The change in substrate concentration in the axial direction causes the change of biomass concentration in the free liquid phase. The total biomass concentration is the sum of the active biomass concentration in the biofilm and the concentration of the free biomass in the liquid phase (Nikakhtari and Hill, 2006):

$$X_T = X_{biof} + X_l \qquad (5.4)$$

For calculation of X_{biof}, it is assumed that the packing material is uniformly covered by a layer of biofilm and the available biomass is given by:

$$X_{biof} = \alpha W_{biof} / V_w \qquad (5.5)$$

Where an effectiveness coefficient (α) is applied to the dry weight of biofilm to allow for the extracellular polymer substance surrounding the microorganisms in the biofilm.

Biomass in the free liquid phase (X_l) was calculated at any point along the column length by relating it to substrate concentration:

$$\frac{dX_l}{dz} = Y(-\frac{dS_z}{dz}) \qquad (5.6)$$

Values for best-fit coefficients (K_s, μ_m and Y) for the Monod kinetic model determined using the data from continuously stirred tank reactor (Chapter 4) were used in this reactor model.

$\mu_m = 1.74\ d^{-1}$

$K_s = 363\ mg \cdot l^{-1}$

Y = 0.24 mg biomass per mg of substrate

In developing the mathematical model the following assumptions were made:

1. Dispersion is evaluated in the axial direction in the liquid phase.
2. Dispersion of the substrate in the radial and angular directions is assumed to be negligible.
3. The mass transfer into the gas phase of the bioreactor is assumed to be negligible as the substrate is not volatile.
4. The stainless steel packing material, made of coiled strands with measured width and thickness, is uniformly packed throughout the column.
5. The biofilm covers the packing uniformly and is a homogenous phase within which nutrients diffuse and are consumed.
6. The microbial growth kinetics are assumed to follow the Monod kinetic expression.
7. The biofilm is assumed to be in a steady state condition where the growth rate and the death rate are balanced and sloughing of the cells maintains a constant biofilm thickness.
8. The substrate is consumed by the total biomass (X_T) but only the biomass in the free liquid phase is assumed to vary in the axial direction.
9. The maintenance requirements of the biofilm are negligible.
10. The reactor operates under aerobic conditions throughout the course of experimentation (i.e. oxygen is not a limiting substrate).
11. Dispersion of the substrate in the radial and angular directions is assumed to be negligible.

Equations 5.1 through 5.6 were solved for each reactor simultaneously using a fourth-order Runge-Kutta numerical procedure in an Excel™ 2003 spreadsheet. Boundary conditions for Equation 5.1 are as follows:

$$\left.\frac{dS}{dz}\right|_{(z=L)} = 0 \qquad (5.7)$$

The inlet concentrations of substrate (S_i) and biomass (X_I) at z = 0 in the liquid feed are known.

Because the equations are highly non-linear, the finite steps calculated along the length of the column were chosen at small, 1 mm increments. The accuracy of the computations was monitored by comparing with more simplistic kinetic estimations of the parameters. The theoretical substrate utilization along the length of the column was also monitored graphically to identify potential numerical instability. The value of coefficients such as effective biofilm thickness for these models were determined by fitting the experimental data to the models and performing non-linear regression using least-squares minimization together with the Solver routine within Excel™ software.

To model the reactor performance, both dispersed plug flow and plug flow regimes were evaluated. The results from each model were compared with the experimental data for each ICR independently and best fit by varying the dispersion and/or effective biofilm coefficients. For the dispersed plug flow model, consideration was given to axial dispersion ($\mathcal{D}$) in Equation 5.1 together with the other relevant substrate flow and substrate utilization terms. The dispersed plug flow model was adjusted to each set of experimental ICR data by simultaneously optimizing the dispersion coefficient and the effectiveness coefficient to evaluate the value for dispersion. Based on these results the model was then simplified to a plug flow model in which dispersion was neglected. Performance of the reactors was evaluated for the experimental dilutions rates.

5.3 Results and Discussion

In this study the experimental performances of the reactors were compared with the model predictions using the volumetric reaction rate given by Equation 5.8:

$$V_r = (S_i - S_{ss}) \times D \tag{5.8}$$

Biofilm thickness was calculated using the volume of wet biofilm divided by the total surface area of the packing as determined by the weight and density of the packing material. Where:

$$SA_p = \frac{2W_p(w+t)}{\rho_{ss} wt} \tag{5.9}$$

Specific parameters of the reactors and the packing material are summarized in Table 5.1. Both the dispersed plug flow and plug flow models were optimized by minimization of the sum of the squares of error for the prediction of both the steady state experimental biomass and substrate concentrations in the liquid phase at the outlet of the reactor. This involved varying the values of the dispersion and effectiveness coefficients by the Solver routine within Excel™ software.

Optimization of the dispersed plug flow model revealed a best fit dispersion coefficient of 2.5 x 10^{-4} $cm^2 \cdot s^{-1}$. The range of axial dispersion coefficients related to flow rate as described by Quail (1990) and Quail and Hill (1991) is 1.9 x 10^{-3} to 1.0 x 10^{-6} $cm^2 \cdot s^{-1}$ for the experimental range of flow rates used. The Peclet number (Pe) is regarded as a measure of the importance of convective mass transport relative to mass transport by dispersion and is defined by Equation 5.10 (Bailey and Ollis, 1977).

$$Pe^{-1} = \frac{\mathcal{D}}{F/(A\phi L)L} \tag{5.10}$$

As dispersion becomes insignificant, the Pe^{-1} approaches 0 and a simple tubular plug flow model best represents the reactor. For the range of flow rates evaluated (3 to 100 $ml \cdot h^{-1}$), Pe^{-1} is between 0.1 and 0.003, therefore, a plug flow model (Equation 5.1 without the axial dispersion term) was used to represent the system.

The biofilm thickness (δ) and the effective biofilm thickness (δ_{eff}), assumed to be the outermost portion of the biofilm at the interface of the biofilm and the free liquid,

were assumed to be of uniform distribution over the entire surface of the stainless steel packing. The biofilm effectiveness coefficient (α), optimized in evaluation of the plug flow model for each experimental reactor, was used to calculate the effective biomass available in the biofilm for biodegradation of the substrate. The effectiveness coefficient was applied to the wet biofilm volume and the effective outer biofilm thickness was solved geometrically based on the cross section schematic illustrated in Figure 5.3.

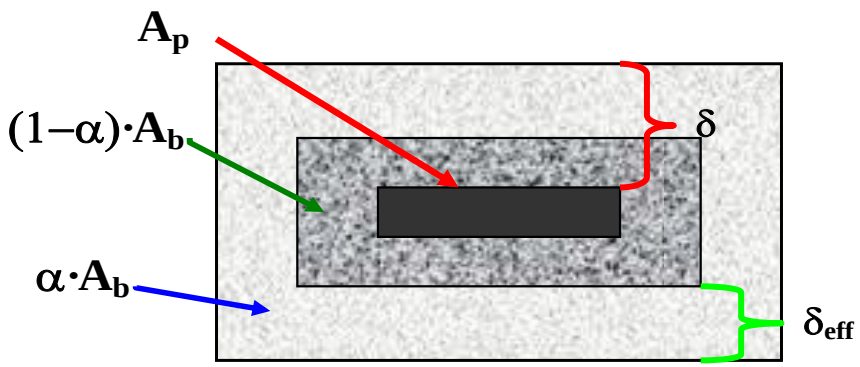

Figure 5.3 Cross section schematic of biofilm surrounding stainless steel packing fibre.

Notes:
: represents effective portion of biofilm ($\alpha \cdot A_b$)
: represents ineffective portion of biofilm (1-α)
: represents cross section of stainless steel packing fibre
A_b : cross-sectional area of biofilm; A_p : cross-sectional area of packing fibre
δ : total biofilm thickness; δ_{eff} : effective biofilm thickness

Figure 5.4 presents the profiles of biomass and substrate concentrations at steady state for experimental data for ICR #2 (250 $mg \cdot l^{-1}$) as compared with the best fit theoretical plug flow model. Complete conversion of the substrate is predicted by the model for the range of dilution rates evaluated (up to 90 d^{-1}). The experimental and predicted theoretical residual substrate concentrations were less than or equal to 10 $mg \cdot l^{-1}$ for the range of dilution rates evaluated. This represents approximately 3 times the analytical method detection limit of 3 $mg \cdot l^{-1}$. The best fit model indicated a biofilm effectiveness coefficient of 0.66 with a standard error of 0.17. The measured weight of wet biofilm present at the end of experimentation is calculated to represent a uniform biofilm over the packing material equivalent to a total biofilm thickness (δ) of 0.33 mm.

The calculated effective biofilm thickness (δ_{eff}) corresponding with a 66% effectiveness coefficient is 0.18 mm assuming that the outer layer of biofilm metabolizes the substrate.

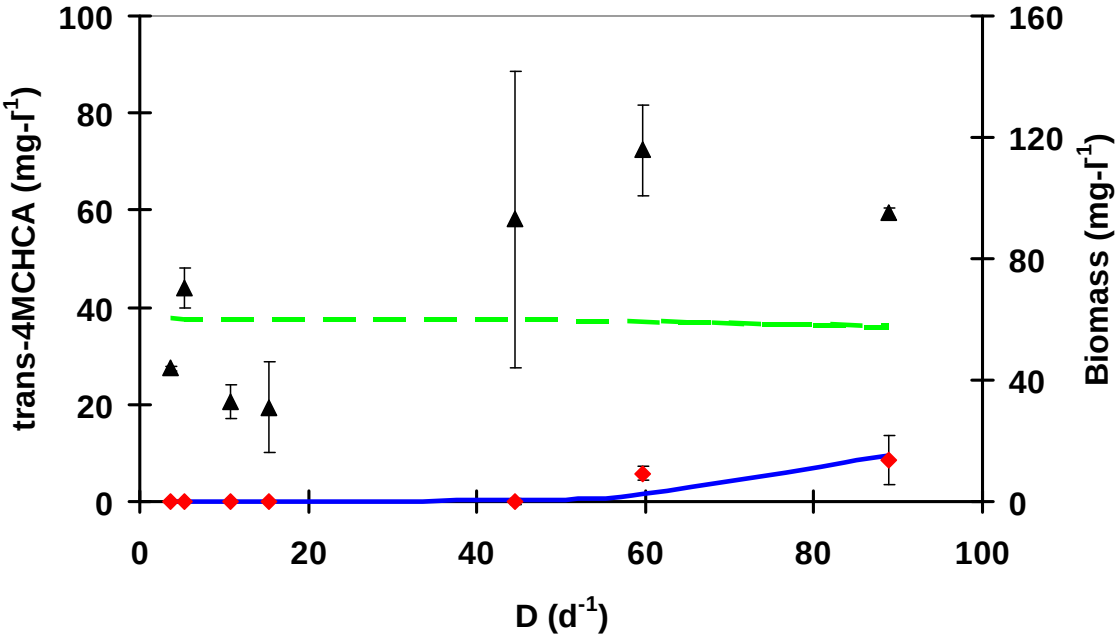

Figure 5.4 Immobilized cell reactor (ICR #2) with feed stock at 250 mg·l^{-1} for steady state conditions throughout the range of evaluated flow rates versus theoretical plug flow model. ▬: represents theoretical substrate concentration for plug flow at reactor outlet; ♦: represents experimental average substrate concentration at steady state conditions; ▲: represents average biomass concentration at steady state conditions; ▬ ▬: represents theoretical biomass concentration in the free liquid; error bars represent 95% confidence interval in experimental substrate concentrations and biomass at steady state; $\alpha = 0.66$.

Figure 5.5 presents the best fit plug flow model for steady state substrate concentration and biomass with the experimental data for ICR #3 over the range of dilution rates studied. The best fit biofilm effectiveness coefficient for the 500 mg·l^{-1} reactor shows that 45% (with a standard error of 17%) of the total dry weight biomass is active in biodegradation of the substrate flowing through the reactor.

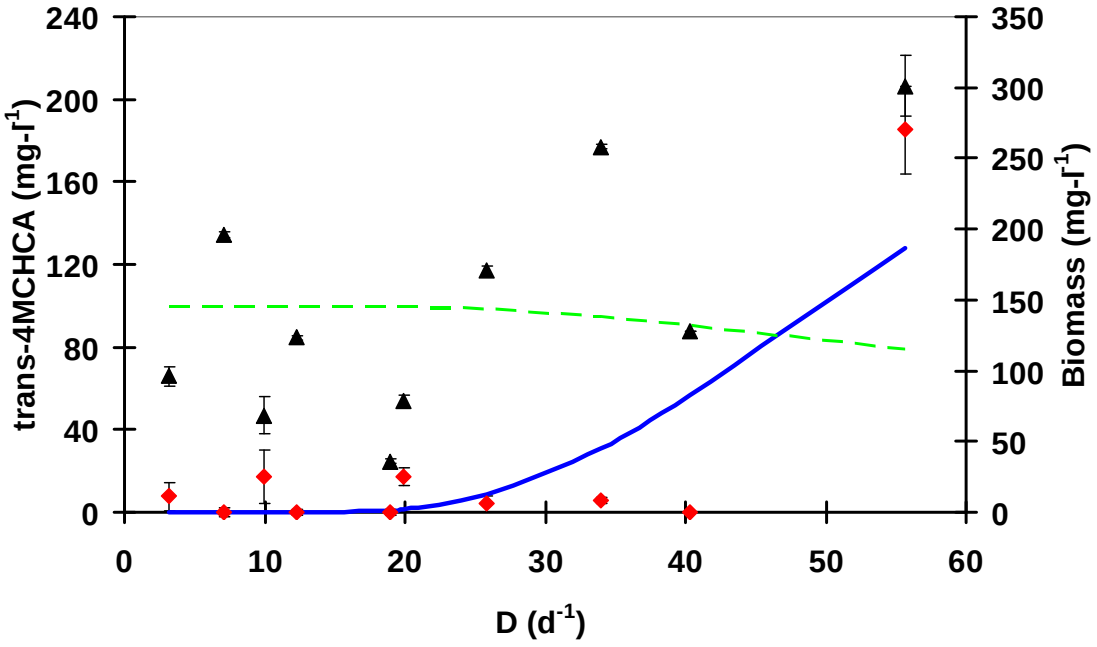

Figure 5.5 Immobilized cell reactor (ICR#3) with feed stock at 500 mg·l^{-1} for steady state conditions throughout the range of evaluated flow rates versus theoretical plug flow model. ▬ : represents theoretical substrate concentration for plug flow at reactor outlet; ♦: represents experimental average substrate concentration at steady state conditions; ▲: represents average biomass concentration at steady state conditions; ▬ ▬: represents theoretical biomass concentration in the free liquid; error bars represent 95% confidence interval in experimental substrate concentrations and biomass at steady state; $\alpha = 0.45$.

Complete conversion of the substrate for the 500 mg·l^{-1} (ICR #3) was observed up to a dilution rate of 40 d^{-1} corresponding to 36 minute residence time. The measured weight of wet biofilm present at the end of experimentation represents a uniform biofilm over the packing material equivalent to a total biofilm thickness of 0.38 mm. This relates to an effective biofilm thickness of 0.13 mm for a 45% effectiveness coefficient. Nikakhtari and Hill (2006) reported that in an external loop airlift bioreactor with a packed bed for degradation of phenol polluted air, 11.2% of the biofilm dry weight was actively available biomass. The range of effective biofilm thickness (0.13 to 0.18 mm) calculated in this study is comparable with Quail and Hill (1991) estimation that the majority of biodegradation of a biofilm occurs within 0.2 mm of the outer biofilm.

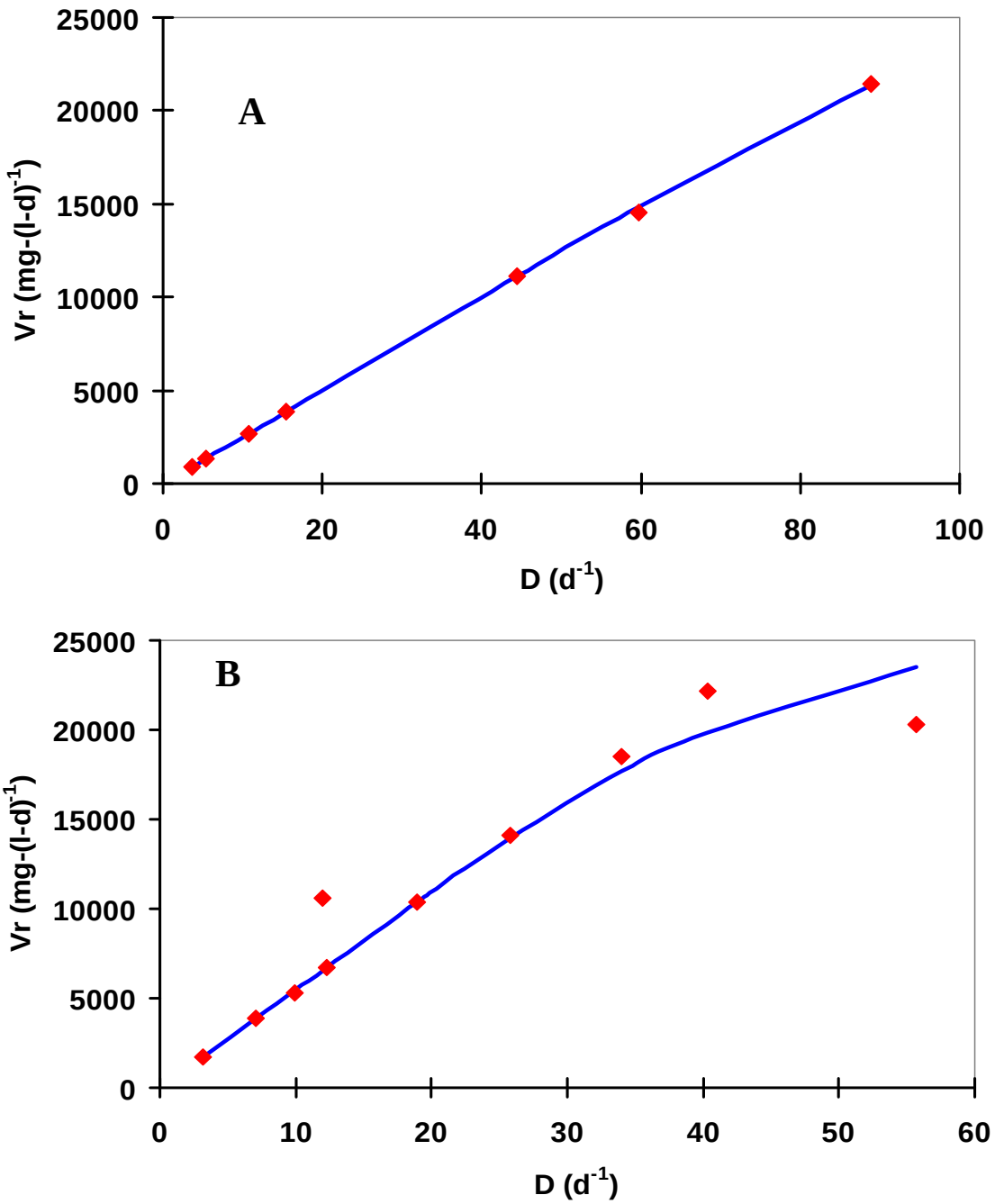

Figure 5.6 Volumetric reaction rates for reactors. **Panel A**: ICR #2 operating at an initial feed concentration of 250 mg·l^{-1}; ♦: experimental; —: theoretical plug flow; α = 0.66; **Panel B**: ICR #3 operating at an initial feed concentration of 500 mg·l^{-1}; α = 0.45.

The experimental and theoretical volumetric reaction rate profiles representing the performance for each ICR operating with feed substrate concentrations of 250 mg·l^{-1} and 500 mg·l^{-1} are presented in Figure 5.6. The maximum theoretical volumetric reaction rate

over the range of dilution rates studied experimentally for the 250 $mg \cdot l^{-1}$ reactor is 21,400 $mg \cdot (l \cdot d)^{-1}$ corresponding with the observed maximum volumetric reaction rate of 21,500 $mg \cdot (l \cdot d)^{-1}$.

The effect of variation in the coefficient of biofilm effectiveness on the substrate concentration at steady state at the outlet of the reactor is demonstrated in Figure 5.7.

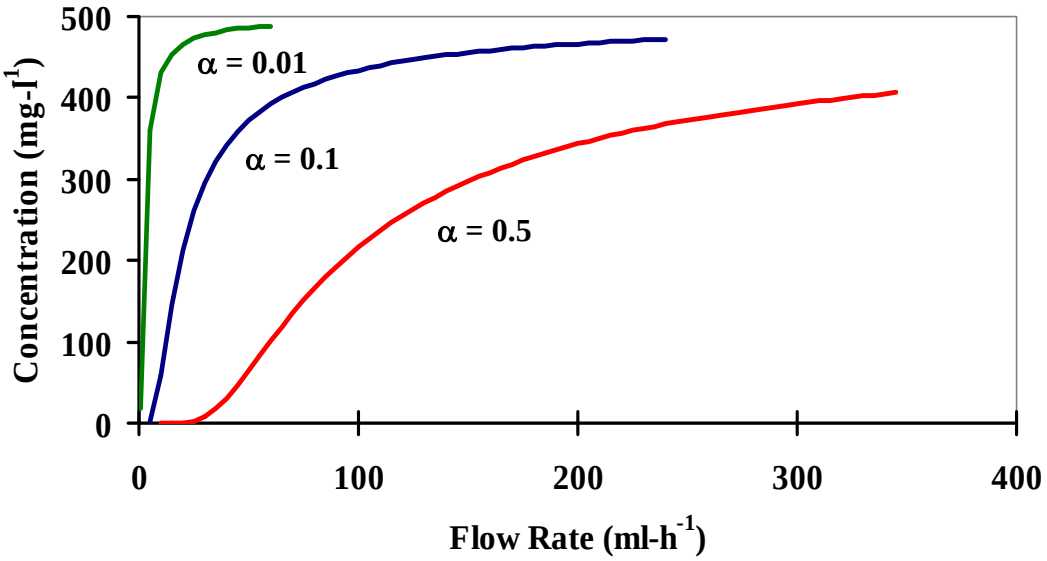

Figure 5.7 Sensitivity in steady state concentration at reactor outlet to coefficient of effective biofilm (α). — : represents $\alpha = 0.01$; — : represents $\alpha = 0.1$; — : represents $\alpha = 0.5$.

In the ICR (#3) operated with 500 $mg \cdot l^{-1}$ trans-4MCHCA, the maximum experimental volumetric reaction rate of 22,000 $mg \cdot (l \cdot d)^{-1}$ was observed at a dilution rate 40 d^{-1} after which the volumetric reaction rate appeared to stabilize. The theoretical plug flow model indicated a maximum volumetric reaction rate for the range of dilutions studied of 23,000 $mg \cdot (l \cdot d)^{-1}$ corresponding with a dilution rate of 56 d^{-1}. Extrapolation of the model for higher flow rates and various initial substrate concentrations compared with a constant biofilm coefficient is presented in Figure 5.8. The maximum theoretical performance for an initial substrate concentration of 500 $mg \cdot l^{-1}$ approaches 29,000 $mg \cdot (l \cdot d)^{-1}$ which is reached near a dilution rate of 120 d^{-1}. This indicates that the experimental capacity of the reactor was at 76% of the maximum capacity. A similar

trend is observed for lower initial substrate concentrations. For an initial substrate concentration of 250 mg·l^{-1}, the maximum performance (approx. 22,000 mg·(l·d)$^{-1}$) could be maintained through much higher dilution rates. Extrapolating the model to a lower initial substrate concentration follows this trend in maintaining high dilution rates. As well, the model is less sensitive to biofilm coefficient at lower initial substrate concentrations.

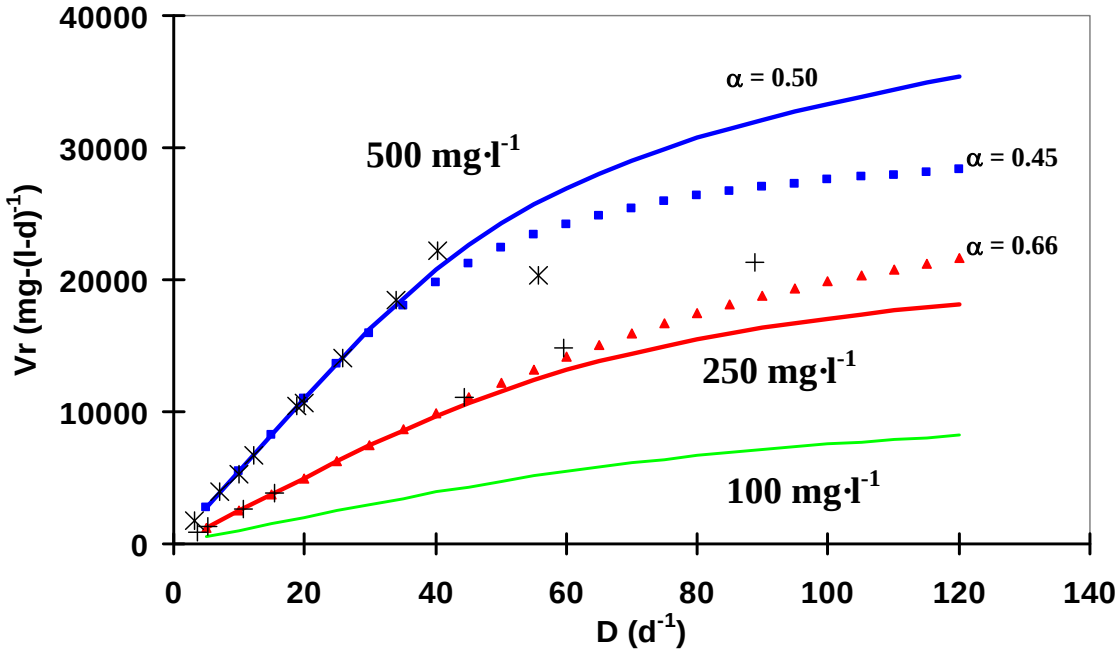

Figure 5.8 Volumetric reaction rates for theoretical plug flow at various initial feed concentrations and biofilm coefficients.

Notes: Model prediction values indicated by: ▬ : for initial feed concentration of 500 mg·l^{-1}; ▬ : for initial feed concentration of 250 mg·l^{-1} ; ▬ : for theoretical feed concentration of 100 mg·l^{-1} (α = 0.50); ■■■ : initial feed concentration of 500 mg·l^{-1} (α = 0.45); ▲▲▲ : initial feed concentration of 250 mg·l^{-1} (α = 0.66). Experimental data indicated by: ✳ : for initial feed concentration of 500 mg·l^{-1}; + : for initial feed concentration of 250 mg·l^{-1}.

These biodegradation studies in two reactor configurations show that the biodegradation of trans-4MCHCA can be enhanced by choosing the biofilm reactor design. This conclusion was also supported by Fava et al. (2000) in the evaluation of reactor configuration for the biotreatability of other recalcitrant environmental

contaminants. A plug flow model with a uniform biofilm coating the packing was fit to the experimental results of an immobilized cell reactor. The theoretical maximum volumetric reaction rates for various initial substrate concentrations are predicted to be sustained over a large range of flow rates as compared with the experimental data.

5.4 Conclusions

A high porosity packed bed immobilized cell reactor has been shown to effectively remove a single naphthenic acid substrate in continuously fed operation to dilution rates of 90 d^{-1}. Essentially 100% removal of trans-4MCHCA was removed from artificially polluted water at volumetric reaction rates up to 22,000 $mg\cdot(l\cdot d)^{-1}$ which were 95 times faster than CSTR rates. A plug flow model in which biokinetics of the substrate follow a Monod growth expression has been shown to accurately represent biodegradation in an immobilized cell reactor for two initial substrate concentrations. This model is demonstrated to be a useful tool for studying the effects of parameter variation and prediction of reactor performance.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The research activities conducted within the scope of this project included the study of the kinetics and the factors affecting biodegradation of a single naphthenic acid compound, the trans isomer of 4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA). The kinetic parameters associated with biodegradation of this compound were evaluated in three bioreactor systems: batch reactors, a continuously stirred tank reactor and immobilized cell reactors. The data generated in the continuous stirred tank reactor was used to determine the kinetic coefficients. These were then used to model the performance of the immobilized cell reactor.

Biodegradation experiments were completed first in shake flasks in order to develop a consortium of micro organisms capable of degrading the candidate naphthenic acid compound into carbon dioxide and water. These batch experiments were conducted at varying initial substrate concentrations as well as various pH and temperature conditions in order to assess the effect of these parameters on biodegradation in terms of kinetic rates. The results of the batch experiments were used to determine specific growth rates under the various conditions while identifying the dominant microbial species and optimal environmental conditions for biodegradation. The range of initial concentrations of trans-4MCHCA (50 to 750 $mg \cdot l^{-1}$) did not affect the maximum specific growth rate of the bacteria at 23^{o}C (0.52±0.04 d^{-1}). The maximum yield observed at this temperature and at a neutral pH of 7.0 was 0.34±0.08 mg of biomass per mg of substrate. Batch experiments indicated that biodegradation can be achieved at low temperatures; however, the biodegradation rate at room temperature (23^{o}C) and neutral pH was 4.5 times faster than at 4^{o}C and the same pH. Biodegradation at varying pH indicated a maximum

specific growth rate of 1.69±0.40 d^{-1} and yield (0.41±0.06 $mg \cdot mg^{-1}$) at a pH of 10, under alkaline conditions where the naphthenic acid was present predominantly as the naphthenate salt. The consortium developed during the batch experimentation work was dominated by *Alcaligenes paradoxus* and *Pseudomonas aeruginosa*.

Throughout the first stage of kinetics experimentation, a quantitative analytical tool was developed in order to monitor substrate utilization with time. A robust method using GC-FID was established for the purposes of this biodegradation study. This method provided timely results in order to design future experimental operating conditions. The primary advantage of the GC-FID method included direct injection of the compound in the medium used to support the biological activity without the need for complex extraction or derivatization methods. Other advantages of this method included consistency in the calibration and repeatability of measurements of the candidate compound. The sensitivity of the method was well suited for the monitoring and degradation of the candidate naphthenic acid compound and facilitated manipulation of the bioreactor operating conditions for evaluation of kinetics.

The results from the continuously stirred tank reactor showed that the biodegradation of trans-4MCHCA can be described by the Monod model. As indicated in the literature, the Monod model is often suited to degrading organic contaminants where substrate-limited cell growth is slow and the population densities are low. Kinetic parameters were determined by best-fitting the continuously stirred reactor data in various kinetic models. The Monod expression was found to be the best model with a maximum specific growth rate of 1.74±0.004 d^{-1} and a half saturation constant of 363±17 $mg \cdot l^{-1}$. Previously reported kinetic information for trans-4MCHCA in unamended river water in a laboratory reported a specific growth rate of only 0.067 d^{-1} (Headley et al., 2002b). The maximum specific growth observed in this study with the use of a CSTR and modified McKinney's medium was 26 times higher. The maximum specific growth rate observed in this study with batch reactors was 1.69±0.40 d^{-1} (pH 10).

The biodegradation rate of trans-4MCHCA in the immobilized cell reactors was almost two orders of magnitude higher than a continuously stirred tank reactor. The continuously stirred tank reactor achieved a maximum reaction rate of 230 $mg\cdot(l\cdot d)^{-1}$. The immobilized cell system operating with the same feed concentration (500 $mg\cdot l^{-1}$) attained a maximum reaction rate of 22,000 $mg\cdot(l\cdot d)^{-1}$. In a second immobilized cell system operating with a feed concentration of 250 $mg\cdot l^{-1}$, the maximum reaction rate of 21,800 $mg\cdot(l\cdot d)^{-1}$ was observed at the highest flow rate which corresponded to 100% conversion of the substrate. These biodegradation studies showed that the biodegradation of trans-4MCHCA can be enhanced by choosing a biofilm reactor design. A packed column with established biofilm was able to enhance the biodegradation rate of trans-4MCHCA up to 95 times when compared with a freely suspended cell reactor.

The results of the reactor modelling indicated that a plug flow model provided the best fit to the experimental data of the immobilized cell reactors. This study showed that maintaining the optimum conditions and design of an ex-situ biodegradation reactor are crucial to the successful enhancement of biodegradation rates of this naphthenic acid compound.

Evaluation of the toxicity reduction in the effluent conducted for this study included two screening methods, *Artemia salina* and Microtox™, and the *Daphnia magna* toxicity test. The Microtox™ screening test demonstrated a large reduction in toxicity of the effluent from the continuous stirred reactor as compared with the influent containing trans-4MCHCA. The *D. magna* toxicity test and the *A. salina* screening test also demonstrated a reduction in toxicity of the effluent from the continuously stirred reactor. Effluent from the immobilized cell reactor demonstrated a large reduction in toxicity for both the *A. salina* and the *D. magna* tests.

6.2 Recommendations

This study indicates that biodegradation of a single naphthenic acid compound under varying environmental and reactor design conditions may be significantly enhanced to increase the rate at which similar compounds may be degraded in the

environment. Further work with the reactor configurations developed in this work could be expanded for higher flow rates and in larger reactor systems in order to identify the full potential of the system.

There is little information about the dependency of biodegradation kinetics on the molecular structure of the various naphthenic acids. Further research should be conducted to study biodegradation of other simple compounds with varying structures in similar reactor systems to those studied in this research. Of particular interest, would be the comparison of a selected naphthenic acid compound with a more complex ring structure. Specific attention could be focused on optimizing pH conditions to further evaluate the effect of ionization on microbial growth. Also of interest would be the performance of an immobilized cell system at colder temperatures. Investigation of other single compounds with varying molecular structure and comparison with the parameter for the model developed in Chapter 5, as well as biodegradation studies with a mixture of naphthenic acids would provide a basis for the evaluation of potential ex-situ bioremediation of oil sands naphthenic acids.

Evaluation of the immobilized cell system model indicated that the reactor performance is significantly influenced by the fraction of the biofilm representing the active cells. Further microbial methods should be investigated as a means of determining the active biomass available for substrate removal in the biofilm as well as investigation in changes of the biofilm thickness as related to flow rates, and variations relative to the length of the reactor column. More detailed investigation of the microbial consortium and its changes with environmental conditions should be carried out. In particular, a better understanding of the composition of the consortium in a freely suspended system as compared with those species in the biofilm would be of value to further this area of research.

The reactor design could be modified to include a recycle stream where part of the liquid effluent is re-circulated through the packed bed at high flow rates to evaluate the effect of recycle on reactor performance. In future studies, the air effluent from the

bioreactors should be sampled to determine if volatile organic compounds are produced and released during the naphthenic acid biodegradation process. Also, consideration should be given to the effect of clay particles as potential hindrances to the effectiveness of the microbial activity and reactor performance.

This study only begins to reveal the requirement for detailed toxicological evaluation of naphthenic acids as both a mixture and individual molecular structures. As this is an initial investigation of the potential for bioreactors to enhance a difficult biodegradation process. The results highlight the need for concurrent toxicity studies. Toxicity and biodegradation studies of naphthenic acid groups (for example specific Z series) may provide an initial approach to evaluating mixtures.

With regard to quantification of the selected substrate, further evaluation of the GC-FID method utilizing a higher molecular weight compound as compared with a multiple ringed naphthenic acid structure would be of interest. The limitation of the GC-FID method could be determined with regard to molecular weight or naphthenic acid structure.

The insights on biodegradation of a single naphthenic acid compound gained by this study may not necessarily be directly applicable to other components in diverse mixtures such as those of oil sands naphthenic acids; nor should the results be extrapolated blindly. However, the use of a single compound as an initial kinetic assessment tool is of significant importance in future kinetic studies of other naphthenic acid compounds. This work highlights the potential of augmented bioremediation systems for the degradation of naphthenic acids.

LIST OF REFERENCES

Alberta Energy and Utility Board, "Guide 50 Drilling Waste Management," Alberta Energy and Utility Board. (October 1996 Edition).

Armstrong, S. A., J. V. Headley, K. M. Peru and J. J. Germida, "Phytotoxicity of Oil Sands Naphthenic Acids and Dissipation from Systems Planted with Emergent Aquatic Macrophyte," J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. A43, 36-42 (2008).

Bailey, J.E. and D.F. Ollis, "Biochemical Engineering Fundamentals," McGraw-Hill Inc. United States (1977).

Barrow, M. P., L. A. McDonnell, X. Feng, J. Walker and P. J. Derrick, "Determination of the Nature of Naphthenic Acids Present in Crude Oils using Nanospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: The Continued Battle Against Corrosion," Anal. Chem. **75,** 860-866 (2003).

Barrow, M. P., J. V. Headley, K. M. Peru and P. J. Derrick, "Fourier Transform Ion Cyclotron Resonance Mass Spectrometry of Principal Components in Oilsands Naphthenic Acids," J. Chromatogr. A. **1058,** 51-59 (2004).

Bataineh, M., A. C. Scott, P. M. Fedorak and J. W. Martin, "Capillary HPLCQTOF-MS for Characterizing Complex Naphthenic Acid Mixtures and their Microbial Transformation," Anal. Chem. **78,** 8351-8361 (2006).

Beck, J. V., "Parameter Estimation in Engineering and Science," John Wiley & Sons, New York, (1977).

Bi, Y., G. A. Hill and R. J. Sumner, "Continuous Toluene Vapor Remediation in a Well-Mixed Bioreactor," Can. J. Chem. Eng. **82,** 488-494 (2004).

List of References

Brient, J. A., P. J. Wessner and M. N. Doyle, "Naphthenic Acids," In: Encyclopedia of Chemical Technology 4th Edition, Edited by J.I. Kroschwitz, John Wiley & Sons, New York**, 16,** 1017-1029 (1995).

Bunce, N. J., "Introduction to Environmental Chemistry," Wuerz Publishing Ltd., Winnipeg, Canada, (1993).

Canadian Council of Ministers of the Environment (CCME), "A Protocol for the Derivation of Environmental and Human Health Soil Quality Guidelines," CCME (2006).

Canadian Environmental Protection Act (CEPA) 1999, "Environmental Registry," Environment Canada. http://www.ec.gc.ca/ceparegistry/the_act/Default.cfm. (Accessed 2008).

Chen, Y. R. and A. G. Hashimoto, "Substrate Utilization Kinetic Model for Biological Treatment Processes," Biotechnol. Bioeng. **22,** 2081-2095 (1980).

Clarke, K., G. A. Hill and T. S. Pugsley, "Direct Comparison of Fluidized and Packed Bed Bioreactors for Bioremediation of an Air Pollutant," Int. J. Chem. Reactor Eng. **5**, A11 (2007).

Clemente, J. S., N. G. N. Prasad, M. D. MacKinnon and P. M. Fedorak, "A Statistical Comparison of Naphthenic Acids Characterized by Gas Chromatography-Mass Spectrometry," Chemosphere. **50,** 1265-1274 (2003a).

Clemente, J. S., T. Yen and P. M. Fedorak, "Development of a High Performance Liquid Chromatography Method to Monitor the Biodegradation of Naphthenic Acids," J. Environ. Eng. Sci. **2,** 177-186 (2003b).

List of References

Clemente, J. S., M. D. MacKinnon and P. M. Fedorak, "Aerobic Biodegradation of Two Commercial Naphthenic Acids Preparations," Environ. Sci. Technol. **38,** 1009-1016 (2004).

Clemente, J. S. and P. M. Fedorak, "A Review of the Occurrence, Analyses, Toxicity, and Biodegradation of Naphthenic Acids," Chemosphere. **60,** 585-600 (2005).

Deeb, R. A., K. Scow M. and L. Alvarez-Cohen, "Aerobic MTBE Biodegradation: An Examination of Past Studies, Current Challenges and Future Research Directions," Biodegradation. **11,** 171-186 (2000).

Deineko, P. S., E. N. Vasil'eva, O. V. Popova and S. T. Bashkatova, "Naphthenic Acids as Antiwear Additives for Jet Fuels," Chem. Technol. Fuels Oils. **30,** 343-345 (1994).

Department of Energy, Government of Alberta, "Introduction to Oil Sands," http://www.Energy.Gov.Ab.ca/OilSands/oilsands.Asp Alberta Energy. (Accessed 2007).

Diamond, W. J., "Practical Experimental Designs for Engineers and Scientists," Van Nostrand Reinhold, New York, (1989).

Doran, P. M., "Bioprocess Engineering Principles," Academic Press Limited, San Diego, California, (1995).

Environment Canada, "Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*," Environmental Protection Series. EPS 1/RM/14 2nd Edition**,** (December 2000).

Environmental Protection Agency (EPA), Office of Water. Water Quality Standards Database. "List of priority pollutants," Clean Water Act.

http://oaspub.epa.gov/wqsdatabase/wqsi_epa_criteria.rep_parameter. (Accessed 2008).

Fava, F., D. Di Gioia and L. Marchetti, "Role of the Reactor Configuration in the Biological Detoxification of a Dump Site-Polychlorobiphenyl-Contaminated Soil in Lab-Scale Slurry Phase Conditions," Appl. Microbiol. Biotechnol. **53,** 243-248 (2000).

Fava, F., D. Di Gioia, L. Marchetti and G. Quattroni, "Aerobic Dechlorination of Low-Chlorinated Biphenyls by Bacterial Biofilms in Packed-Bed Batch Bioreactors," Appl. Microbiol. Biotechnol. **45,** 562-568 (1996).

Folsom, B. R., P. J. Chapman and P. H. Pritchard, "Phenol and Trichloroethylene Degradation by *Pseudomonas cepacia* G4: Kinetics and Interactions between Substrates," Appl. Environ. Microbiol. **56,** 1279-1285 (1990).

Gadekar, S., M. Nemati and G. A. Hill, "Batch and Continuous Biooxidation of Sulphide by *Thiomicrospira* Sp. CVO: Reaction Kinetics and Stoichiometry," Water Res. **40,** 2436-2446 (2006).

Gavala, H. N., F. Alatrise-Mondragon, R. Iranpour and B. K. Ahring, "Biodegradation of Phthalate Esters during the Mesophilic Anaerobic Digestion of Sludge," Chemosphere. **52,** 673-682 (2003).

Guieysse, B., P. Wikstromm, M. Forsman and B. Mattiason, "Biomonitoring of Continuous Microbial Community Adaptation Towards More Efficient Phenol-Degradation in a Fed Batch Bioreactor," Appl. Microbiol. Biotechnol. **56,** 780-787 (2001).

Hao, C., J. V. Headley, K. M. Peru, R. Frank, P. Yang and K. R. Solomon, "Characterization and Pattern Recognition of Oil-Sand Naphthenic Acids using

Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectormetry," J. Chromatogr. A. **1067,** 277-284 (2005).

Harborne, J. B., "Plant Phenolics," Academic Press, London, England, (1989).

Headley, J. V., K. M. Peru, D. W. McMartin and M. Winkler, "Determination of Dissolved Naphthenic Acids in Natural Waters by using Negative-Ion Electrospray Mass Spectrometry," J. AOAC Int. **85,** 182-187 (2002a).

Headley, J. V., S. Tanapat, G. Putz and K. M. Peru, "Biodegradation Kinetics of Geometric Isomers of Model Naphthenic Acids in Athabasca River Water," Can. Water Res. J. **27,** 25-42 (2002b).

Headley, J. V. and D. W. McMartin, "A Review of the Occurrence and Fate of Naphthenic Acids in Aquatic Environments," J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. **39,** 1989-2010 (2004).

Headley, J. V., K. M. Peru, M. P. Barrow and P. J. Derrick, "Characterization of Naphthenic Acids from Athabasca Oil Sands using Electrospray Ionization: The Significant Influence of Solvents," Anal. Chem. **79,** 6222-6229 (2007).

Herman, D. C., P. M. Fedorak, M. D. MacKinnon and J. W. Costerton, "Biodegradation of Naphthenic Acids by Microbial Populations Indigenous to Oil Sands Tailings," Can. J. Microbiol. **40,** 467-477 (1994).

Hill, G., "Kinetics of Phenol Degradation of *Pseudomonal putida*," M.Sc. Thesis, Univ. of Waterloo, Ontario, Canada (1974).

Hill, G. A. and C. W. Robinson, "Substrate Inhibition Kinetics: Phenol Degradation by *Pseudomonas putida*," Biotechnol. Bioeng. **17,** 1599-1615 (1975).

List of References

Holowenko, F., M., M. D. MacKinnon and P. M. Fedorak, "Naphthenic Acids and Surrogate Naphthenic Acids in Methanogenic Microcosms," Water Res. **35,** 2595-2606 (2001).

Holowenko, F., M., M. D. MacKinnon and P. M. Fedorak, "Characterization of Naphthenic Acids in Oil Sands Wastewaters by Gas Chromatography-Mass Spectrometry," Water Res. **36,** 2843-2855 (2002).

Hsu, C. S., G. J. Dechert, W. K. Robbins and E. K. Fukuda, "Naphthenic Acids in Crude Oils Characterized by Mass Spectrometry," Energy Fuels. **14,** 217-223 (2000).

Janfada, A., J. V. Headley, K. M. Peru and S. L. Barbour, "A Laboratory Evaluation of the Sorption of Oil Sands Naphthenic Acids on Organic Rich Soils," J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. **41,** 985-997 (2006).

Janikowski, T. B., D. Velicogna, M. Punt and A. J. Daugulis, "Use of a Two-Phase Partitioning Bioreactor for Degrading Polycyclic Aromatic Hydrocarbons by a *Sphingomonas* Sp.," Appl. Microbiol. Biotechnol. **59,** 368-376 (2002).

Karamanev, D. and L. Nikolov, "A Comparison between the Reaction Rates in Biofilm Reactors and Free Suspended Cells Bioreactors," Bioprocess. Eng. **6,** 127-130 (1991).

Kennedy, J. B. and A. M. Neville, "Basic Statistical Methods for Engineers and Scientists," 3rd Edition, Harper & Row Publishers Inc., New York, (1964).

Kharoune, L., M. Kharoune and J. M. Lebeault, "Aerobic Degradation of 2,4,6-Trichlorophenol by a Microbial Consortium - Selection and Characterization of Microbial Consortium," Appl. Microbiol. Biotechnol. **59,** 112-117 (2002).

List of References

Kim, D. J., J. W. Choi, N. C. Choi, B. Mahendran and C. E. Lee, "Modeling of Growth Kinetics for *Pseudomonas* Sp. during Benzene Degradation," Appl. Microbiol. Biotechnol. **69,** 456-462 (2005).

Kim, H. and D. W. Graham, "Effects of Oxygen and Nitrogen Conditions on the Transformation Kinetics of 1,2,-Dichloroethenes by *Methylosinus trichosporium* OB3b and its sMMO^{C} Mutant," Biodegradation. **14,** 407-414 (2003).

Kiviranta, J., "Detection of Toxicity of Cyanobacteria by *Artemia salina* Bioassay," Environ. Toxicol. Water Qual. **6,** 423 (1991).

Kotturi, G., W. C. Robinson and W. E. Inniss, "Phenol Degradation by a Psychrotrophic Strain of *Pseudomonas putida*," Appl. Microbiol. Biotechnol. **34,** 539-543 (1991).

Lai, J., W.S., L. Pinto J., E. Keihlmann, L. I. Bendell-Young and M. M. Moore, "Factors that Affect the Degradation of Naphthenic Acids in Oil Sands Wastewater by Indegenous Microbial Communities," Environ. Toxicol. Chem. **15,** 1482-1491 (1996).

Lapidus, L. and N. R. Amundson, "Chemical Reactor Theory A Review," Prentice-Hall, Inc., Englewood Cliffs, New Jersey, (1977).

Lee, C. C. and S. D. Lin, "Handbook of Environmental Engineering Calculations," McGraw-Hill Companies, New York, NY, (2007).

Leung, S. S., M. D. MacKinnon and R. E. H. Smith, "The Ecological Effects of Naphthenic Acids and Salts on Phytoplankton from the Athabasca Oil Sands Region," Aquat. Toxicol. **62,** 11-26 (2003).

Levenspiel, O., "Chemical Reactor Engineering," 2nd Edition, John Wiley & Sons, New York, NY, (1972).

Lin, C. and Y. Cheng, "Biodegradation Kinetics of Benzene, Methyl Tert-Butyl Ether, and Toluene as a Substrate under Various Substrate Concentrations," J. Chem. Technol. Biotechnol. **82,** 51-57 (2007).

Lo, C. C., B. G. Brownlee and N. J. Bunce, "Electrospray-Mass Spectrometric Analysis of Reference Carboxylic Acids and Athabasca Oil Sands Naphthenic Acids," Anal. Chem. **75,** 6394-6400 (2003).

Lo, C. C., B. G. Brownlee and N. J. Bunce, "Mass Spectrometric and Toxicological Assays of Athabasca Oil Sands Naphthenic Acids," Water Res. **40,** 655-664 (2006).

Lower, E. S., "Naphthenic Acid," Speciality Chemicals Magazine. **7,** 76-85 (1987).

Lower, E. S., "Naphthenic Acid III," Speciality Chemicals Magazine. **8,** 174-176 (1988).

McMartin, D. W., "Persistence and Fate of Acidic Hydrocarbons in Aquatic Environments: Naphthenic Acids and Resin Acids," Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (2003).

Meredith, W., S. J. Kelland and D. M. Jones, "Influence of Biodegradation on Crude Oil Acidity and Carboxylic Acid Composition," Org. Geochem. **31,** 1059-1073 (2000).

Merlin, M., S. E. Guigard and P. M. Fedorak, "Detecting Naphthenic Acids in Waters by Gas Chromatography-Mass Spectrometry," J. Chromatogr. A. **1140,** 225-229 (2007).

Nikakhtari, H., "Bioremediation of Industrial VOC Air Pollutants," Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (2005a).

Nikakhtari, H. and G. A. Hill, "Hydrodynamic and Oxygen Mass Transfer in an External Loop Airlift Bioreactor with a Stainless Steel Packed Bed," Biochem. Eng. J. **27,** 138-145 (2005b).

Nikakhtari, H. and G. A. Hill, "Continuous Bioremediation of Phenol-Polluted Air in an External Loop Airlift Bioreactor with a Packed Bed," J. Chem. Technol. Biotechnol. **81,** 1029-1038 (2006).

Panikov, N. S., M. V. Sizova, D. Ros, C. Christodoulatos, W. Balas and S. Nicholich, "Biodegradation Kinetics of the Nitramine Explosive CL-20 in Soil and Microbial Cultures," Biodegradation. **18,** 317-332 (2007).

Paslawski, J. C., J. V. Headley, G. A. Hill and M. Nemati, "Biodegradation Kinetics of Trans 4-Methyl-1-Cyclohexane Carboxylic Acid," Biodegradation. (Accepted July 2008).

Peachey, B., "Strategic Needs for Energy Related Water use Technologies," http://www.Aeri.Af.ca/sec/new_res/docs/EnergyINET_and_water_Feb2005.Pdf, Water and EnergyINet. **34** (2005).

Peng, J., J. V. Headley and S. L. Barbour, "Adsorption of Single-Ring Model Naphthenic Acids on Soils," Can. Geotech. J. **39,** 1419-1426 (2002).

Purwaningsih, I., "Mass Transfer and Bioremediation of Solid Polycyclic Aromatic Hydrocarbon (PAH) Particles in Bioreactors", Ph.D. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (2002).

Quagraine, E. K., J. V. Headley and H. G. Peterson, "Is Biodegradation of Bitumen a Source of Recalcitrant Naphthenic Acid Mixtures in Oil Sands Tailing Pond

Waters?" J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. **40,** 671-684 (2005a).

Quagraine, E. K., H. G. Peterson and J. V. Headley, "In Situ Bioremediation of Naphthenic Acids Contaminated Tailing Pond Waters in the Athabasca Oil Sands Region - Demonstrated Field Studies and Plausible Options: A Review," J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. **40,** 685-722 (2005b).

Quail, B. E., "Biodegradation of Phenol by *Pseudomonas putida* in a Countercurrent Packed Bed Bioreactor," M.Sc. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (1990).

Quail, B. E. and G. A. Hill, "A Packed-Column Bioreactor for Phenol Degradation: Model and Experimental Verification," J. Chem. Tech. Biotechnol. **52,** 545-557 (1991).

Rampinelli, L. R., R. D. Azeveda, M. C. Teixeira, R. Guerra-Sa and B. A. Leao, "A Sulfate-Reducing Bacterium with Unusual Growing Capacity in Moderately Acidic Conditions," Biodegradation. On-line publication**,** (2007).

Ranjan, A. P., C-H Su, W-T. Wu and J. Gomes, "An Alternative Method for Designing Immobilized Column Reactors: An Application to L-Methionine Production," Int. J. Chem. Reactor Eng. **6,** A34, (2008).

Rehm, H. J., G. Reed, A. Puhler and P. Stadler, "Biotechnology A Multi-Volume Comprehensive Treatise," VCH Wiley Canada (1986).

Reynolds, T. D. and P. A. Richards, "Unit Operations and Processes in Environmental Engineering," PWS Publishing Company, Boston MA, (1996).

Riess, R., M. Nemati, J. V. Headley and G. A. Hill, "Improved Mass Transfer and Biodegradation Rates of Naphthalene Particles using a Novel Bead Mill Bioreactor," J. Chem. Technol. Biotechnol. **80,** 662-668 (2005).

Rogers, V. V., K. Liber and M. D. MacKinnon, "Isolation and Characterization of Naphthenic Acids from Athabasca Oil Sands Tailings Pond Water," Chemosphere. **48,** 519-527 (2002).

Scott, A. C., M. D. MacKinnon and P. M. Fedorak, "Naphthenic Acids in Athabasca Oil Sands Tailings Waters are Less Biodegradable than Commercial Naphthenic Acids," Environ. Sci. Technol. **39,** 8388-8394 (2005).

Shuler, M. L. and F. Kargi, "Bioprocess Engineering Basic Concepts," Prentice-Hall, Inc., Englewood Cliffs, New Jersey, (1992).

Shuler, M. L. and F. Kargi, "Bioprocess Engineering Basic Concepts," Prentice-Hall, Inc., New Jersey, (2002).

Skoog, D. A., F. J. Holler and T. A. Nieman, "Principles of Instrumental Analysis, 5th Edition," Saunders College Publishing, Philadelphia, PA, (1998).

Tanapat, S., "Comparison of Biodegradation Kinetics of Geometric Isomers of Naphthenic Acids (NAs) in Athabasca River Water," M.Sc. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (2001).

Tarighian, A., "Cometabolic Biodegradation of Three Pulp and Paper Wastewater Components," M.Sc. Thesis, Univ. of Saskatchewan, Saskatoon, Canada (1999).

Tarighian, A., G. A. Hill and Y-H. Lin, "Lag Phase Model for Transient Growth of *Pseudomonas putida* on Phenol," Can. J. Chem. Eng. **79,** 732-736 (2001).

List of References

Tarighian, A., G. Hill, J. H. Headley and S. Pedras, "Enhancement of 4-Chlorophenol Biodegradation Using Glucose," Clean Technol. Environ. Policy. **5,** 61-65 (2003).

Thomas, H. A. Jr., "Bacterial Densities from Fermentation Tube Tests," J. Am. Water Works Assoc. **34,** 572-576 (1942).

Toussaint, M. W., T. R. Shedd and W.H. van der Schalie, "A Comparison of Standard Acute Toxicity Tests with Rapid-Screening Toxicity Tests," Environ. Toxicol. Chem. **14,** 907-915 (1995).

Yu, H., B. J. Kim and B. E. Rittmann, "A Two-Step Model for the Kinetics of BTX Degradation and Intermediate Formation by *Pseudomonas putida* F1," Biodegradation. **12,** 465-475 (2001).

Yu, R., M. Nemati, G. A. Hill and J. V. Headley, "Mass Transfer and Bioremediation of Naphthalene and Methyl Naphthalenes in Baffled and Bead Mill Bioreactors," Can. J. Chem. Eng. **84,** 349-355 (2006).

APPENDIX A

BIOMASS CALIBRATION

The concentration of biomass was determined by direct measurement of the optical density (OD) of the samples taken from the flasks at a wavelength of 620 nm (Shuler and Kargi, 1992; Gadekar et al., 2006; Rampinelli et al., 2007). An Ultraviolet (UV) spectrophotometer (Mini Shimadzu, Model 1240) was used for the determination of the optical density. The optical density was then related to dry-weight using a calibration curve presented in Figure A.1.

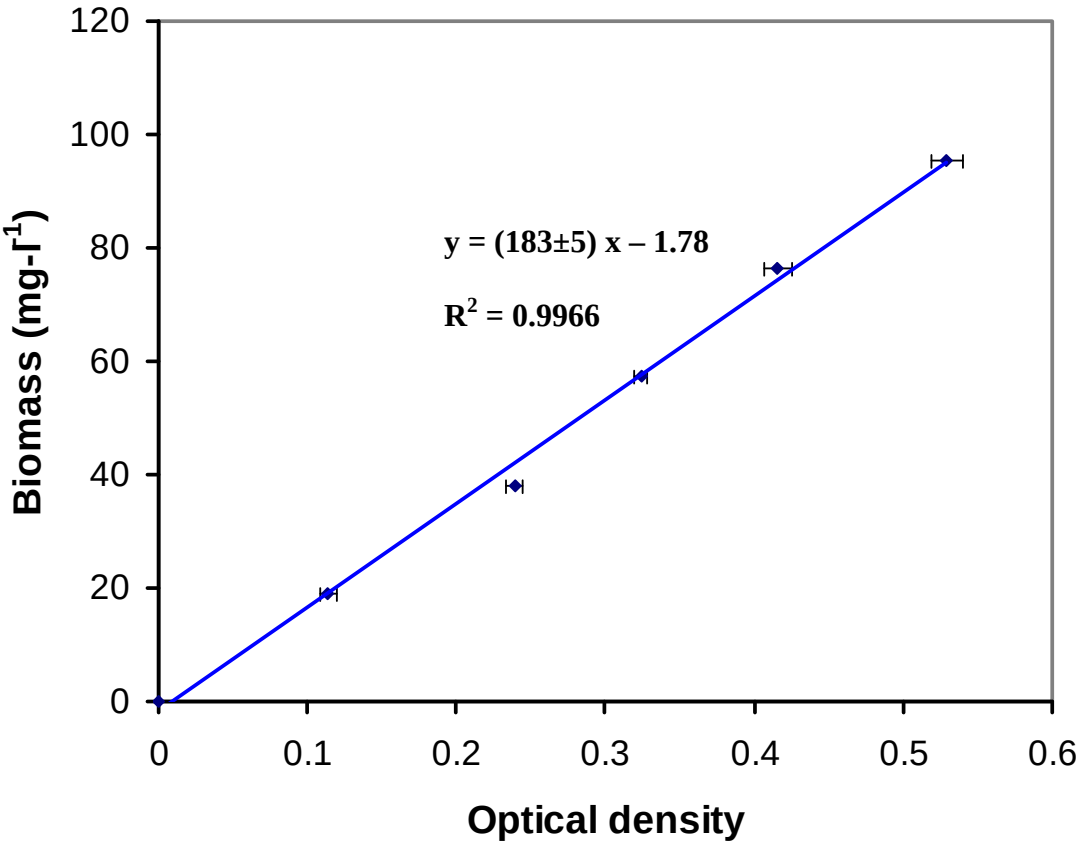

Figure A.1 Biomass calibration.

(error bars represent standard deviation in optical density readings)

APPENDIX B

APPLICATION OF TAILINGS POND NAPHTHENIC ACID EXTRACT TO IMMOBILIZED CELL REACTOR

Subsequent to the operation of the immobilized cell reactor operating continuously with trans-4MCHCA (ICR #2), the reactor was continued with a feed stock of mixture oil sands naphthenic acids extracted from Syncrude Canada Inc. tailings pond water. The objective of the experiment was to evaluate the potential for the existing biofilm capable of degrading trans-4MCHCA to adapt to an oil sands naphthenic acid mixture.

The naphthenic acid extract was previously extracted for other research conducted at the University of Saskatchewan (Armstrong et al., 2008). The analytical method used was electrospray ionization mass spectrometry (ESI-MS) in negative ion mode carried out by the Water Science and Technology Directorate of Environment Canada described in Armstrong et al., 2008. Solid phase extraction was not carried out prior to analyses. The concentration of the naphthenic acid extract was 6,800 $mg \cdot l^{-1}$. A shake flask with 100 $mg \cdot l^{-1}$ naphthenic acid extract was prepared in 100 ml of modified McKinney's and inoculated with 10% (v/v) of the consortium and effluent from ICR #2. The shake flask was placed on a magnetic stirred and mixed for 2 weeks.

At the same time, a feed stock of 100 $mg \cdot l^{-1}$ naphthenic acid extract was prepared in two 1 L amber bottles of modified McKinney's medium for continuous feed through ICR #2. The naphthenic acid mixture was fed into the reactor at an average rate of 5 $ml \cdot h^{-1}$ for 14 days. Samples were collected from the reactor on days 1, 5, 10 and 14. On these sampling occasions pH and optical density were also measured. Samples of the stock solution were collected at the beginning of experimentation as well as on day 5 and 10. At the end of the two week experiment, most probable number method and plate counts were conducted to observe the growth in the effluent in accordance with procedures described earlier (Chapters 3 and 4). At the end of the 2 week experiment samples were also collected from the shake flask batch system for comparison with the immobilized cell reactor.

The results of the change in optical density and naphthenic acid concentrations in the reactor(s) and the stock solution throughout the experiment are presented in Figure

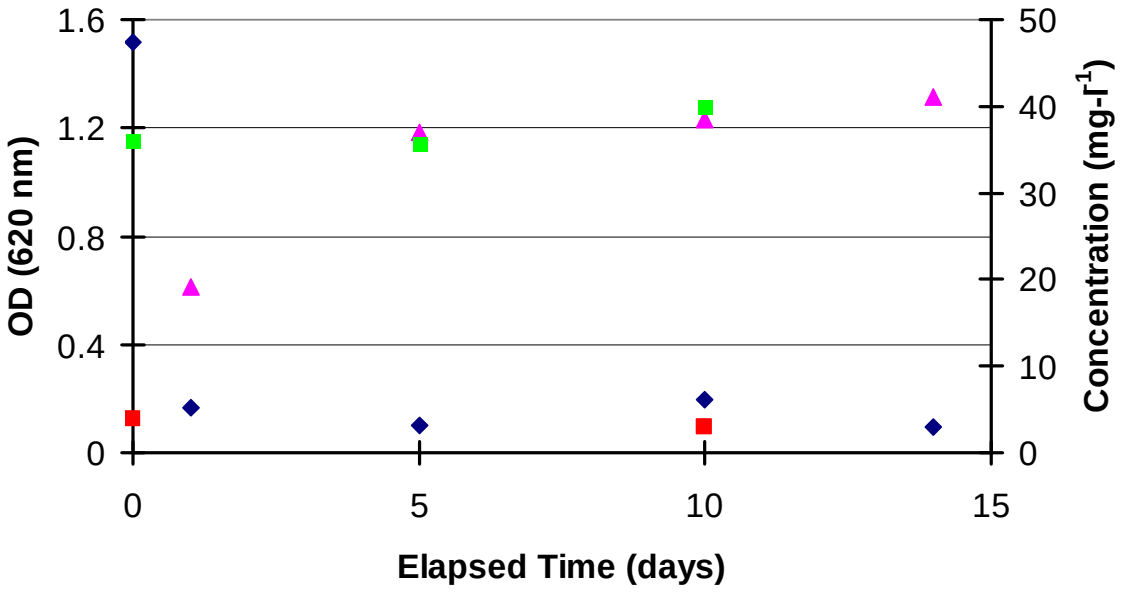

Figure B.1 Observed parameters for continuously fed naphthenic acid extract through the immobilized cell reactor (ICR #2). ♦: represents optical density in reactor effluent; ■ : represents optical density in stock solution; ▲ : represents naphthenic acid concentration in reactor effluent; ■ : represents naphthenic acid concentration in stock solution.

The results indicate that the optical density in the reactor effluent dropped significantly in the 1 to 5 days of operation and did not vary over the remaining two week period. The optical density at the end of the experiment was 0.098 which would be representative of medium with no microbial growth. The pH of the effluent remained stable between 7.5 and 7.8 and required no adjustment similar to that of the ICRs when operated with trans-4MCHCA. The measured concentration in the stock solution and the reactor effluent did not vary over the two weeks with the exception of a sample collected from the effluent at the base of the column after 1 day of operation. The average measured concentration of naphthenic acids in the stock solution as determined by ESI-MS was 37 $mg \cdot l^{-1}$. This measured percentage (37%) of the theoretical concentration (100 $mg \cdot l^{-1}$) was similar to the measured percentage (22%) of 100 $mg \cdot l^{-1}$ trans-4MCHCA in medium. The total

concentration is based on the calibration of the total ion current (TIC). Figure B.1 presents graphically the distribution of naphthenic acid over the expected molecular ranges. The graphs are represented by the percentage of ion current fitting the naphthenic acid general formula ($C_nH_{Z+2}O_2$) to the TIC assuming that the TIC represents 100 $mg \cdot l^{-1}$.

Although no measured change was observed the total concentration of the naphthenic acids after 2 weeks, subtle changes in the distribution of the molecular weight and degree of cyclicity of actual components within the mixture can occur after a given treatment. As the latter may not be observable based on the total measured naphthenic acid concentrations, results were reprocessed to show possible changes based on carbon number and Z series as illustrated in Figure B.2.

Based on the data for the ESI-MS analyses of the samples presented in Figure B.2, Table B.1 summarizes selected information pertaining to the distribution of the ion current (IC) for the single ringed naphthenic acids (Z=-2) and for the specific molecular weight of trans-4MCHCA (142) to identify any impact of the biofilm on biodegradation of this specific oil sands naphthenic acid in the ICR.

Table B.1 Summary of samples analyzed for immobilized cell reactor with naphthenic acid extract

Sample Name	S2	R1	R8	SF2
TIC	$7.4x10^7$	$3.7x10^7$	$7.3x10^7$	$5.3x10^7$
Measured Conc. ($mg \cdot l^{-1}$)	39	19	44	29
% IC for Z = -2	11.7	15.6	13.0	12.2
% IC for MW = 142	0.91	0.082	0.011	0.27

Notes: S2: represents the naphthenic acid stock solution (100 $mg \cdot l^{-1}$); R1: sample collected from reactor effluent after 1 day of operation; R8 sample collected from reactor effluent after 24 days of operation; SF2: sample collected for batch reactor after 14 days of operation.

Appendix B

Although there is no observable decrease in the percentage of ions falling within the Z=-2 series, there is a decrease in ion current measured for the naphthenic acid with a molecular weight of 142 which is the same the commercial naphthenic acid trans-4MCHCA. This may suggest that the consortium in the biofilm is capable of degrading a similar molecular weight compound within a naphthenic acid mixture.

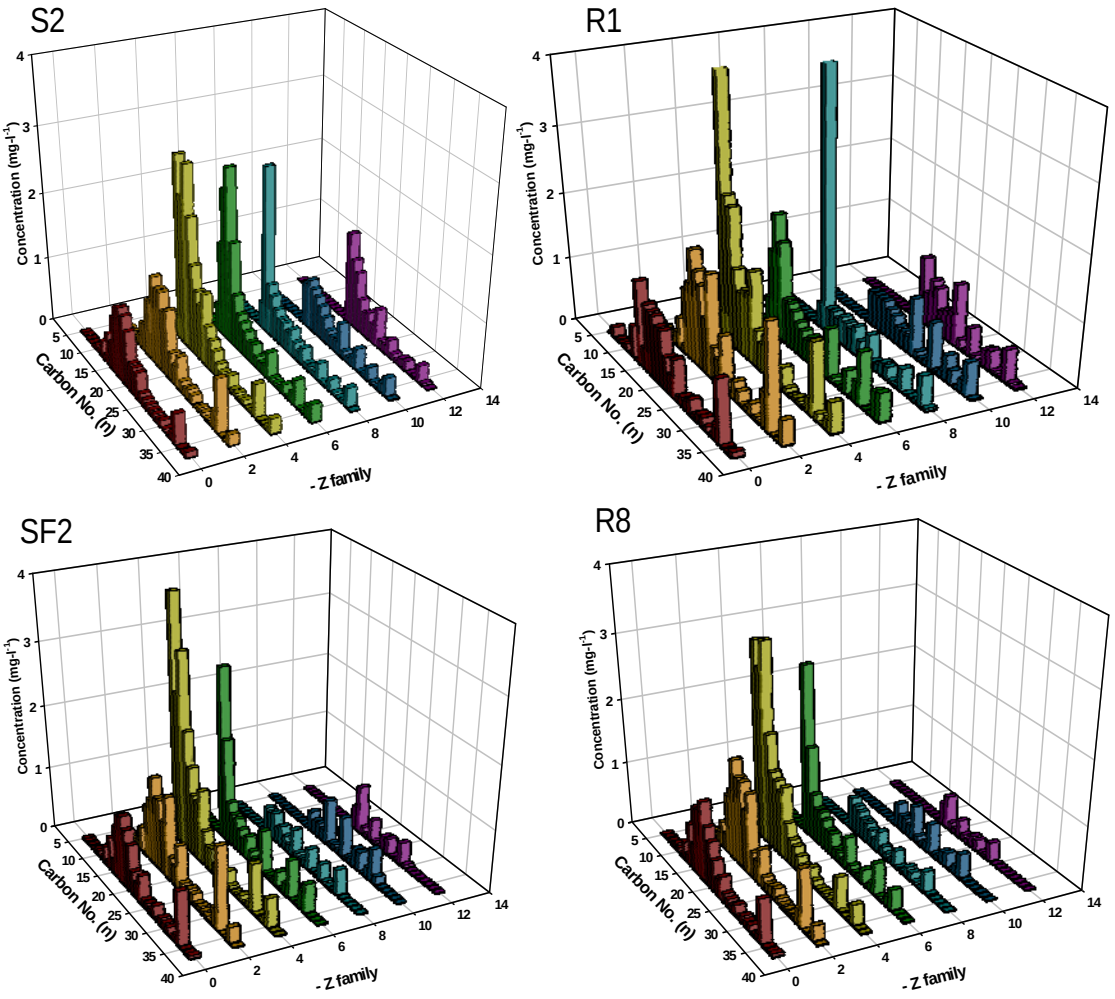

Figure B.2 ESI-MS characterized naphthenic acid results normalized to 100 mg·l^{-1}. S2: stock solution (S2); R1: reactor effluent sample after 1 day operation; SF2: shake flask sample after 14 days of operation in batch reactor; R8: reactor effluent after 14 days operation.

Another point of observation lies with the percentage of TIC fitting the naphthenic acid formula. This observation has been presented graphically in Figure B.3. This figure indicates that the total percentage of the TIC falling within the molecular weights (102 to 538) for naphthenic acids decreased over the 2 week period. A slight decrease in the percentage of the higher Z series (Z=8, 10 and 12) can be observed.

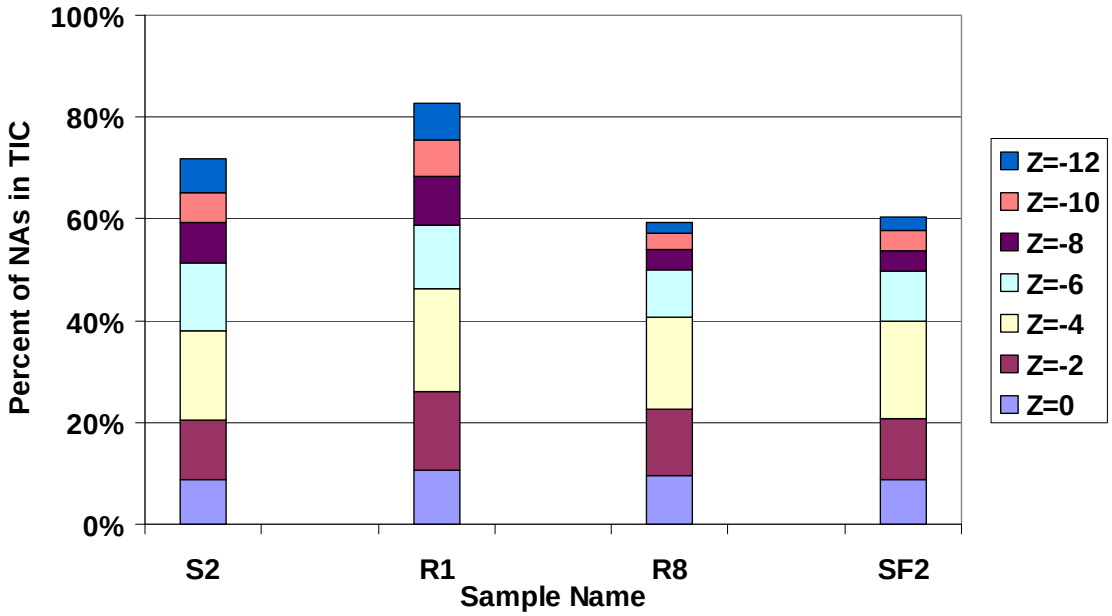

Figure B.3 Comparison of percentages of measured naphthenic acid concentrations within each Z-series to total ion current (TIC) for molecular weights 102 to 538. S2: represents a stock solution sample; R1: represents reactor effluent on Day 1; R8: represents reactor effluent on day 14; SF2: shake flask sample collected at the end of experimentation.

MPN and plate counts for the reactor effluent after 2 weeks indicated that 6×10^6 colony forming units per ml of effluent were present on plates with a similar proportion of dominant small white colonies (56×10^5 CFUs·ml^{-1}) to larger yellow colonies (6×10^5 CFUs·ml^{-1}). A similar trend to the research conducted herein was observed with the higher observed CFUs by the MPN method where 1×10^8 colony forming units per ml of effluent was observed.

The results of this experiment with naphthenic acid extracted for oil sands tailing pond waters indicate that the biofilm was able to survive in the 2 week period of continuous flow. The consortium in the liquid effluent resembled that of the consortium

capable of degrading the single naphthenic acid trans-4MCHCA. Finally, the fingerprint of the naphthenic acids after treatment in both the batch reactor and the biofilm indicated some reduction in naphthenic acids in the immobilized cell reactor effluent after 2 weeks. More detailed sampling and longer operation of an immobilized cell reactor would be required to determine if any of the reported trends are significant.

VITA

Janice Paslawski is a native Saskatchewanian who has returned from several years of consulting in the field of environmental engineering to complete a Ph.D. program at the University of Saskatchewan in the Division of Environmental Engineering. Her field of study is bioremediation of naphthenic acids associated with the tailings wastewater produced from oil sands mining in Northern Alberta. Her graduate research was conducted under the supervision of Drs. Gordon Hill and Mehdi Nemati of the Department of Chemical Engineering and Dr. John Headley of the Water Science and Technology Directorate of Environment Canada. Janice is a practising professional engineer in Alberta and Saskatchewan and is licensed as an environmental engineering consultant with the Association of Professional Engineers and Geoscientists of Saskatchewan.

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