ANAEROBIC CO-BIODEGRADATION OF LINEAR AND CYCLIC MODEL NAPHTHENIC ACIDS UNDER DENITRIFYING CONDITIONS

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DECLARATION

This is certified that the dissertation entitled "**ANAEROBIC CO-BIODEGRADATION OF LINEAR AND CYCLIC MODEL OF NAPHTHENIC ACIDS UNDER DENITRIFYING CONDITIONS,** is an authentic record of my own work carried out as requirements for the award of the degree of M.Tech(Chemical Engineering) at Thapar University, Patiala(India),under the guidance of Dr. Nemati Mehdi (Graduate chair and Professor,ChED,) and Dr. P.K. Bajpai(Distinguished Professor,ChED) during July,2012 to June 2013.

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ABSTRACT

Large volumes of oil sand process water are generated as part of the Clarke caustic hot water process used for extraction of bitumen from shallow oil sand reserves. These process waters contain naphthenic acids in high concentrations (40-120 mg/L), which are persistent in the environment for decades. The toxic nature of naphthenic acids has been found to endanger aquatic biota and terrestrial habitat. Reclamation of these oil sand process waters has also come to the forefront due to the increasing future demand for water consumption in the oil sand industry and the need for sustainable use of water. Bioremediation as a cost effective technology for treatment of these process affected waters is gaining impetus.

In earlier works, the biodegradation of single naphthenic acids was studied in the batch and continuous modes. In this work, biodegradation of individual naphthenic acid as well as co-biodegradation of mixture of naphthenic acid (linear and cyclic) namely octanoic acid, trans 4-methylcyclohexanecarboxylic acid (4MCHCA) and a mixture of octanoic acid and 4MCHCA used to study in batch and continuous mode. The batch studies were completed to evaluate the kinetics of biodegradation process in anaerobic conditions. Mixed culture was used to conduct the biodegradation process. Nitrate ions were utilized to replace the oxygen as electron acceptor to oxidize the organic substrate.

The production of nitrite was observed during biodegradation process, which indicated that denitrifying process was happening. However, at the end of the process, concentration of nitrite decreased and reached to zero, suggesting that nitrite was utilized together with nitrate for additional electron acceptor. The method was suggested as an efficient treatment for both naphthenic acids and nitrate as water pollutants. In addition, the effect of temperature in co-biodegradation, ranged from 10^0C to 35^0C, was also monitored.

The maximum removal rates of the cyclic naphthenic acid (trans-4MCHCA) were found to be lower than that of octanoic acid (linear) irrespective of the presence of the other compound in the mixture.

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Abbreviations

4MCHAA - 4-methylcyclohexane acetic acid

cis-4MCHAA - *cis*-isomer of 4-methylcyclohexane acetic acid

CSTR - continuous stirred tank reactor

ESI - electrospray ionization

FID - flame ionization detection

FTIR - fourier transform infrared

GC - gas chromatography

IC – ion exchange chromatography

HPLC - high-performance liquid chromatography

LSI - liquid secondary ion

MS - mass spectrometry

MEUF – micellar-enhanced ultrafiltration

NA - naphthenic acid

NAs - naphthenic acids

OD - optical density

OSPW – oil sand process water

QTOF - quantitative quadrupole time of flight

RO - reverse osmosis

TAN – total acid number

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

trans-4MCHAA - *trans*-isomer of 4-methylcyclohexane acetic acid

μ - specific growth rate

Chapter 1

INTRODUCTION

The quality of life on Earth is linked inextricably to the overall quality of the environment. In early times, we believed that we had an unlimited abundance of land and resources; today, however, the resources in the world show, in greater or lesser degree, our carelessness and negligence in using them. Increase in populations of the world and decline in conventional oils deposits have given the unconventional oil reserves an important role to help meeting the demand in energy source. The decline in deposit of conventional oils made the unconventional oils playe an important role to help meeting the demand in energy source. One example of unconventional oils is oil sand. Located in one of the provinces in Canada, Northern part of Alberta is considered to be one of the largest oil deposits in the world (Alberta Energy and Utilities Board, 2005). There are three major regions of oil sands recoverable resources in northern Alberta: Athabasca, Cold Lake, and Peace River. Canadian Association of Petroleum Producers reported that in 2010, Canada reserved about 170 billion barrels of oil sand (CAPP, 2012). There are two main processes of recovering the bitumen: surface mining and in-situ extraction (CAPP, 2012). Bitumen recovered from surface mining was extracted using Clark hot water extraction (CHWE) process (Han et al., 2009), a method which combines hot water, steam, and caustic addition (eg. NaOH) to separate bitumen from oil sands (Clark, 1939). On the other hand, in-situ extraction required drilling methods and wells to extract the oil sand. The example of commonly used in-situ methods are steam-assisted drainage gravity (SAGD) (Suncor Energy, 2013) and cyclic steam stimulation (CSS) (Imperial Oil, 2013). Both surface mining and in-situ methods use a large amount of water to extract and separate oil sand from bitumen. A report from CAPP showed that in 2011, drilling method required approximately 0.4 barrels of fresh water to produce one barrel of bitumen, while surface mining required approximately 2.7 barrels of fresh water to produce one barrel of bitumen (CAPP, 2012).
Many researches were performed to find solutions to treat tailings ponds water, especially to decrease the toxicity of NAs. Furthermore, NAs were found to be susceptible to natural degradation by micro-organisms (Herman et al., 1994; Holowenko et al., 2002). However, most of the studies on biodegradation of NAs were done in aerobic conditions.

On the basis of existing literatures, information regarding the impact of NA structure on the biodegradation rate is limited. Only a few studies have included the information

about the dependency of biodegradation kinetics on the molecular structure of individual NA (Tanapat, 2001; Paslawski, 2008). The existing literature data also suggests that the use of certain microbial cultures in a controlled environment (i.e. a properly designed bioreactor) results in faster biodegradation rates and as such a number of studies have focused on biodegradation of surrogate NAs and those extracted from the oil sand process water. However, the amount of information regarding the biokinetics and the impact of bioreactor design on the biodegradation kinetics is very limited, as majority of these studies have been carried out in microcosms such as serum bottles with their main focus being on development and characterization of suitable microbial cultures, effect of molecular structure of NAs on biodegradation, as well as identification of governing pathways.

Therefore, further research on biodegradation of single NA with various structures is needed. Additionally, biodegradation of NAs can be enhanced in an improved bioreactor deign. Further investigations on the *ex-situ* process were necessary in order to evaluate the performance of these novel bioreactors and the influential factors influencing the degradation process.

The present work thus aims at studying the co-biodegradation of linear and cyclic NAs which could be scaled-up and implemented in a large-scale bio-treatment plant. As a part of this work, a mixed culture was developed in our laboratory to study the co-biodegradation of several cyclic model NA compounds including *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA) with a linear NA (octanoic acid) under batch and continuous modes of operation with the aim of generating biokinetic data and to assess the potential for improving the biodegradation of recalcitrant NAs through co-biodegradation with linear NAs which are known to be amenable to biodegradation.

Chapter 2
LITERATURE REVIEW

2.1 Overview of Oil Sands

Oil sands reserves found in the Northern part of Alberta are estimated to hold around 174 billion barrels of bitumen (Allen, 2008; Alberta Energy and Utilities Board, 2005). Athabasca, Peace River and Cold Lake in Northern Alberta are the three main regions where these oil sand deposits are located (ERCB, 2011). Unlike conventional crude oil, oil sands consist of a complex mixture of sand, water, clay and naturally occurring bitumen. Bitumen is heavy, viscous and a thick and sticky form of conventional crude oil which does not flow easily unless heated or diluted with lighter hydrocarbons. Bitumen can be chemically characterized as a complex mixture of hydrocarbons containing lighter fractions rich in naphthenes and heavier fractions rich in asphaltenes, as well as small quantities of heteroatoms such as sulphur, nitrogen and oxygen (Brient et al, 1995). Increase in the global demand for oil and development of new technologies such as open pit mining and other in-situ techniques has enabled the industry to extract profitably and upgrade the oil sand bitumen to usable products despite its viscous and heavy nature. Current studies show that oil sands extraction stands at 236,700 m^3 (1.4 million barrels) per day; by 2015, production is expected to double to about 429 000 m^3 (2.7 million barrels) per day (ERCB 2012; Czarneckia et al, 2005).

2.2 Naphthenic Acids Profiles

Naphthenic acids (NAs) are a complex mixture of organic acid surfactant compounds which naturally occur in crude oils. Naphthenic Acids (NAs), is defined as “acids, chiefly monocarboxylic derived from naphthenes”, while naphthenes was defined as “cycloalkanes especially cyclopentane, cyclohexane, and their alkyl derivatives” (McNaught and Wilkinson, 1997). In petroleum and petrochemical industries, NAs are known as naturally occuring compounds presence in crude oil. NAs mixtures released from bitumen extraction process had concentrations ranged from 40 to 120 mg/L (Holowenko et al., 2002; Clemente et al., 2005). Generally, NAs have the chemical formula of $C_nH_{2n+z}O_2$, where n is the number of carbon atoms and z is number of hydrogen atoms missing in the cyclic NAs. NAs was first analyzed, as an extract of acid fractions from tailings ponds, using Fourier transform infrared (FTIR) spectrum (MacKinnon and Boerger, 1986; Grewer et al., 2010). Presently, NAs are

well-known as non-volatile, chemically stable, toxic compounds, that do not dissolve in water (< 50 mg/L), but soluble in organic solvents and oils (Brient et al., 1995). In addition, NAs are commonly used as fuel additives,wood preservatives, paint driers, aluminium ceramics, anti-wear lubricants, and in the process of manufacturing tires (Brient et al., 1995; Paslawski et al., 2008).

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). Figure 2.1 shows examples of naphthenic acids structures. Besides the carboxylic acid group, cyclic naphthenic acids are believed to be substituted with alkyl groups (R in Figure 2.1). Table 2.1 comprises of the molecular weight of NAs with various Z and carbon number (n) Commercial NA preparations are extracted from petroleum distillates, categorized by acid number, impurity level, and color, and commonly used to produce metal salts such as copper naphthenate, which are largely consumed in the wood preservation industry. Till date, no analytical technique is available for the assessment of this complex mixture of NAs.

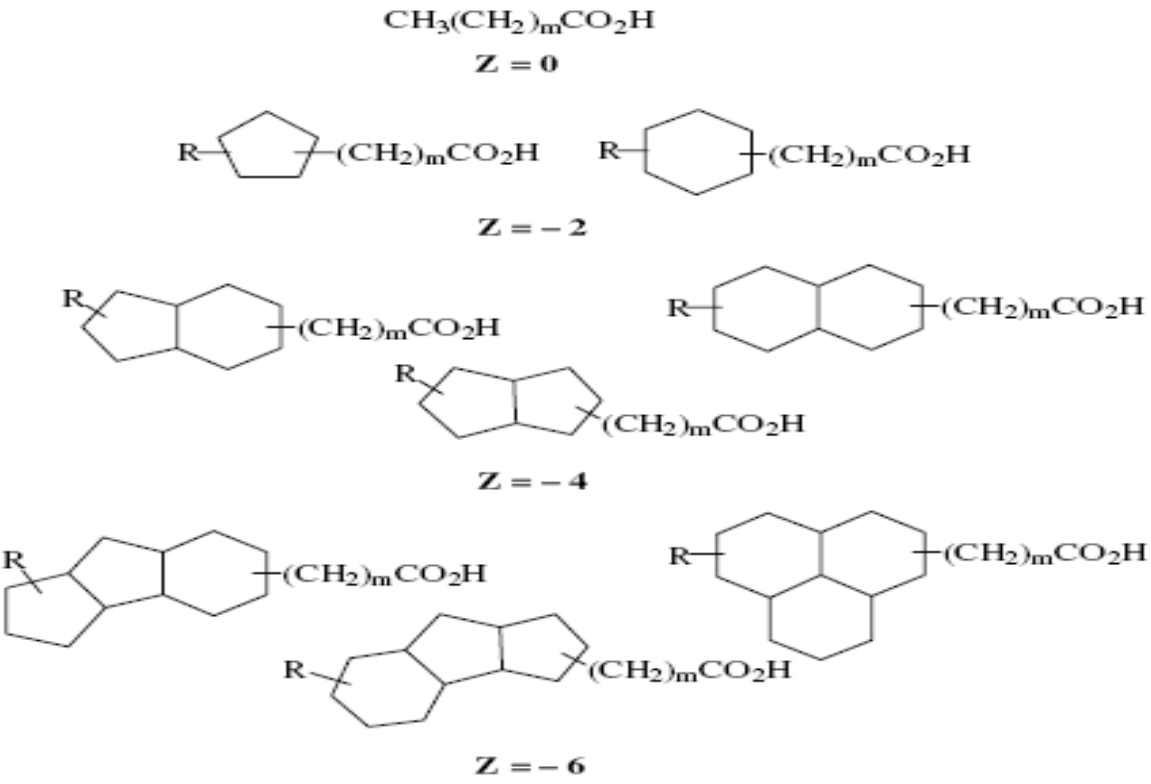


Figure 2.1 General chemical structures of naphthenic acids, where *Z* represents hydrogen deficiency, R is an alkyl chain, and m indicates the number of CH_2 units (Clemente et al., 2005)

Table 2.1 Molecular weight (M.W.) of naphthenic acids with various Z series and carbon number (n) (CEATAG, 1998; McMartin, 2003; Huang, 2011)

Number of Carbon Atoms	M.W. Z=0 (straight chain)	M.W. Z=-2 (1 ring)	M.W. Z=-4 (2 rings)	M.W. Z=-6 (3 rings)
10	172	170	168	166
11	186	184	182	180
12	200	198	196	194
13	214	212	210	208
14	228	226	224	222
15	242	240	238	236
16	256	254	252	250
17	270	268	266	264
18	284	282	280	278
19	298	296	294	292
20	312	310	308	306

2.3 Physical and Chemical Properties of Naphthenic Acids

Naphthenic acids (NAs) physically appear to be either clear or brown viscous liquids and are stable under normal conditions. Their colors range from pale yellow to dark amber. NAs mixture is slightly soluble in water but soluble in certain organic solvents (Brient et al, 1995). They have an offensive odour which is mainly due to the presence of phenolic and sulphur impurities in the mixture. NAs have boiling points at ranging from 250 to 350 ºC (Brient et al., 1995). Naphthenic acids (NAs) are found to be highly toxic in nature.

Chemically, NAs typically have characteristics similar to that of carboxylic acids with acid strength comparable to the higher fatty acids. They are known to be slightly weaker acids when compared to lower molecular weight carboxylic acids such as acetic acid but are stronger acids than phenol and cresylic acid (Brient et al.., 1995). NA dissociation constants are in the range of 10^{-5} to 10^{-6}. Naphthenic acid corrosion is a major problem that has been faced by the petroleum industry since the 1900s. However, NA derivatives are used as corrosion inhibitors for the protection of refining units in the petroleum industry (Brient et al., 1995; Huang 2011).

2.4 Commercial Uses of Naphthenic Acids (NAs)

More than two-thirds of the naphthenic acid produced is used to make metal salts, with the largest volume being used for copper naphthenates, consumed in the wood preservative industry (Brient et al., 1995). Other use of NAs and metal naphthenates includes in textile and

paint driers, emulsifiers, surfactants, and adhesion promoters in tire manufacture (Clemente et al., 2005). Besides metal naphthenates, free naphthenic acids used in concrete additives, motor oil lubricants, asphalt-paving applications and ore flotation for recovery of rare-earth metals (Brient et al., 1995). Various industrial applications of naphthenic acids have been summarized in Table 2.2

Table 2.2 Commercial applications of naphthenic acids (Clemente et al., 2005)

Naphthenic acid metal salt	**Industrial applications**
Ca naphthenates	Additive for lubricating oil
Co naphthenates	Curing agent in rubbers and resins Adhesion promoter of steel cord to rubber
Cu and Zn naphthenates	Wood preservatives
Fe and Mn naphthenates	Fuel additives for improved combustion and reduced corrosion
Mn, Pb, Co, and Ca soaps	Oxidative catalysts
Na salts	Emulsifying agent for agricultural insecticides Additive for cutting oils emulsion breaker in oil industry

2.5 Occurrence and Toxicity of Naphthenic Acids

NAs are oxidative products of petroleum hydrocarbons, which occur naturally in crude oil and/or bituminous oil sands. In Athabasca oil sands, the carboxylic acids (particularly NAs) content is approximately 2% (CEATAG, 1998; Quatrain et al., 2005). The corrosive nature of NAs, especially at high temperature condition (>230 °C),can potentially affect the safety and reliability of oil refining processes.

Currently, the Clarke caustic hot water extraction (79-93 °C) process has been widely adopted in processing of oil sands to separate bitumen from oil sands ore. Through this procedure, the viscosity of bitumen is reduced which favors the subsequent refining and

upgrading processes. However, hot water extraction causes the transfer of NAs into the water fraction as naphthenates (Quagraine et al,2005). Currently, both Syncrude Canada Ltd and Suncor Energy Inc. are involved with surface mining activities in Northern Alberta, use this procedure to extract bitumen from oil sands ore (Rogers et al., 2002; Quagraine et al, 2005). The produced waste slurry from the oil sands extraction process is mainly comprised of sands, clay, water, unrecovered bitumen, and dissolved inorganic and organic compounds (MacKinnon 1989; Schramm et al., 2000). Due to a "zero discharge" policy, these wastes have been contained on-site, primarily in lager settling ponds. In 2003, it was estimated that about 4×10^8 m^3 of process-affected water was retained in the Athabasca region, and the total volume is expected to increase to over 1 billion m^3 by 2020 (Lo et al., 2003; Quagraine et al., 2005). Through the recycling and reuse of tailing waters, the NAs concentrations in the tailings ponds are typically in the range 40 -120 mg/L (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009).

Naphthenic acids are found to be one of the major toxic components present in oil sand process waters affecting fish and aquatic biota. As are considered to be surfactants as they are composed of hydrophobic alkyl groups and a hydrophilic carboxylic group and it is these surfactant properties of NAs that have shown to be the cause of toxicity in the OSPW (Rogers et al, 2002; Mackinnon et al, 1989; Quagraine et al, 2007). This surfactant characteristic makes it possible for the NAs to penetrate the cell wall easily and cause membrane disruption and cytotoxicity (Schramm et al, 2000; Quagraine et al, 2007). Toxicity studies carried out on certain species of freshwater fish using sodium naphthenate also produced 96-h LC50 values of 50 to 75 mg/L (Dokholyan et al, 1983). Previous studies have also reported the toxicity of NAs to fish such as rainbow trout and fathead minnows, invertebrates, mammals, algae and other microorganisms (Rogers et al, 2002; Mackinnon et al, 1986; Brient et al, 1995; Dokholyan et al, 1983; Pinto et al, 1995;Quagraine et al, 2007). Naphthenic acids are also found to contaminate ground and surface water due to run off water from precipitation or direct contact by the river water (Schramm et al, 2000). The acute toxic effects of NAs on aquatic and terrestrial habitat highlights the need for finding means to remove these compounds from the oil sands process water.

As NAs form a very complex mixture and are currently difficult to completely characterize and isolate, the main toxic components present within the NAs mixtures is not clearly identified (Quagraine et al., 2005). Rogers et al. (2002b) reported those lower

molecular weights NAs are the most toxic compounds present in tailing ponds waters. Holowenko et al, (2002) also suggested that the toxicity level decreases as the number of cycloaliphatic rings increase (Holowenko et al., 2002; Mishra, 2009). Hence toxicity levels in tailing ponds decrease with aging as lower molecular weight compounds are more amenable to natural biodegradation over extended periods of time (Holowenko et al., 2002; Frank et al., 2008; Huang 2011).

Another important aspect to be considered when determining the toxicity levels in the tailing ponds is the concentration of naphthenic acids present in the contaminated waters (Holowenko et al., 2002; McMartin, 2003; Paslawski, 2008; Huang 2011). The NA concentrations currently found in the tailing ponds are quite high ranging from 40 to 120 mg/L (Schramm et al., 2000; Holowenko et al., 2002; Mishra, 2009) and are expected to increase even more as oil sands processing operations keep increasing, more water is stored in and recycled from the tailing ponds (Huang 2011).

Currently the toxicity of the tailings wastes is being reduced, partially by natural biodegradation. However, natural biodegradation is a very time consuming process which takes years to complete and cannot keep up with the large amounts of process waters generated. Therefore, present studies have been aimed at improving the biodegradation rate as these oil sand tailings continue to expand over time.

2.6 Treatment Methods for Naphthenic Acids Contaminated Water

Over the past few decades, various methods have been developed to treat NAs contaminated oil sands process water (OSPW). These methods include chemical oxidation (ozonation), adsorption of NAs on activated carbon, membrane filtration (ultrafiltration), and bioremediation (Allen, 2008). Among these methods, bioremediation is considered as one of the most cost-effective and environmental friendly methods (Scott et al., 2008). Chemical oxidation, photocatalysis, and bioremediation as the most studied NAs treatment technologies will be briefly discussed in this section.

2.6.1 Chemical Method of treatment

Chemical treatment typically refers to use of chemical agents to destroy or to convert the contaminants to less toxic compounds (Huang, 2011). Chemical treatment has a short reaction time, but disadvantages in high operating costs and the potential causing " secondary contamination" through producing hazardous by-products. Chemical oxidation

of wastewater has been used for decades, and has been proven to be effective in removing NAs from OSPW.

Chemical oxidation processes degrade pollutants through a series of ionic or radical reactions, where the oxidant compound either accepts electrons or donates an electron-accepting group (Allen, 2008). Common oxidants used in wastewater treatment include chlorine (Cl_2), hydrogen peroxide (H_2O_2), ozone (O_3), and permanganate (MnO_4^-) (Singer et al., 1999; Allen, 2008).

Chemical oxidation is normally applied to the more persistent and recalcitrant pollutants that are not amenable to biological treatment. Ozonation (O_3) has been widely investigated for the removal of NAs. Scott et al. (2008) demonstrated that ozonation of sediment-free oil sands process wastewater for 50 minutes led to a non-toxic effluent and decreased the NAs concentration by approximately 70%. After 130 min of ozonation, the NAs concentration was reduced to 2 mg/L from the initial 59 mg/L. These results show ozonation is superior to biodegradation in term of rate of degradation as well the possibility of removing NAs by using powerful chemical agents. However, relatively high costs of generating ozone would be an important factor which should be considered when evaluating whether ozonation could be incorporated as part of a feasible petroleum wastewater management strategy (Scott et al., 2008).

2.6.2 Physical Treatment

Adsorbents are used to remove a wide variety of pollutants associated with oilfield produced waters, especially organic carbon compounds, oil and grease, and heavy metals (Allen, 2008). Adsorbents can be activated carbon, natural organic matter, zeolites, clays and synthetic polymers.

Schramm et al. (2000) reported the removal of 95-100% for naphthenic acids from OSPW using activated carbon as adsorbent. To improve the adsorption capacity, Han et al. (2008) used activated carbon with the acidification for removal of naphthenic acids from OSPW and removal rate was 80-100%. Problems associated with these types of treatment are low adsorption capacity, cleaning and regeneration costs of the adsorbents.

Micro and ultrafiltration are pressure-driven membrane processes that reject particles as small as 0.1 μm and 0.01 μm respectively (Allen, 2008). Del Rio et al. (1985) used synthetic polymer and ceramic membrane, lab and pilot scale studies using membrane to treat produced

waters have shown over 90% oil rejection with permeate concentrations of less than 20 ppm. But on wider scale, problems such as fouling and membrane durability could occur (Allen, 2008).

Nanofiltration has the potential for partial demineralization, softening, and removal of soluble organic compounds from produced water as it can reject divalent ions, dissolved organic matters, pesticides and other macromolecules (Allen, 2008). Peng et al. (2004) reported the removal rate of 95% of NAs in OSPW using nanofiltration (polyamide). Although nanofiltration has proven to be effective, but membrane fouling and membrane replacement costs has limited the use of technology.

2.6.3 Photocatalsis

Photocatalysis is the acceleration process of photoreaction in the presence of catalyst. The process of photocatalysis is based on the redox (reduction oxidation) reactions which take place to form electron-hole pairs and generate free radical for secondary reactions. One example of photocatalysis is microwave treatment, which can be used as another alternative treatment for degradation of NAs. Microwave system was reported to be able to degrade both OSPW NAs and commercial Fluka NAs, with the presence of TiO_2 as catalyst, in short period of time (Mishra et al., 2009). TiO_2 was chosen because it was active, cheap, non-toxic, chemically stable, and did not subject to photo corrosion (Doll and Frimmel, 2005). However, it was noticed that there was a slight increase in toxicity after the treatment in OSPW NAs samples (Mishra et al., 2009).

2.6.4 Ozonation

Ozonation study was conducted for the first time to evaluate the effectiveness of the method to remove NAs and reduce the toxicity level of oil sand processing water (OSPW) (Scott et al., 2008). Scott also reported that ozone treatment had ability to reduce the color of OSPW water and increase the value of IC_{20} (Scott et al., 2008). IC_{20} value was used to show the level of water toxicity; the higher the number indicated the less toxic the water was. However, Martin discovered that ozonation treatment worked best when it was combined with biodegradation (Huang,2011). Ozonation method was suggested to separate highly branched isomers from the more linear forms since the most recent advance technology are not able to perform the isomers separation.

2.6.5 Bioremediation

The term “bioremediation” is a grouping of technologies that uses microbiota (typically, heterotrophic fungi and bacteria) to degrade or transform hazardous contaminants to materials such as carbon dioxide, water, inorganic salts, microbial biomass, and other by-products that may be less hazardous than the parent materials.

Bioremediation is a treatment process relying on the use of microorganisms to remove organic pollutants from the biosphere to minimize the unwanted environmental impacts (Prince, 2009). As a cleanup technology; it has been successfully used to treat contaminated air, water, soils and sediments. Compared to conventional treatment methods, bioremediation is low cost and could be considered as a permanent solution since in most cases the contaminants can be completely destroyed or metabolized by the microorganisms. The bioremediation process can be carried out either *in-situ* or *ex-situ*. *Ex*-situ bioremediation conducted in a bioreactor optimizes the microbial growth and activity by controlling the environmental conditions resulting inefficient conversion of the contaminants to less harmful compounds.

A first study in 1994 reported that toxicity of NAs was reduced over time by the activity of microorganisms (Herman et al., 1993). Later, Han et al. observed the reduction of NAs concentration by biodegradation process from ten different sites of settling basins, where the older OSPW contained less NAs than the active settling basin (Han et al., 2009). In addition, it was observed that the degradation of NAs followed by the decreased of quantity of dissolved organic carbon (DOC) and respired CO_2 (Videla et al., 2009). The commercially available NAs were found to be degraded easily compared to the one extracted from tailings ponds (Scott et al., 2008) with the lower molecular weight NAs were preferentially degraded over the higher molecular weight (Holowenko et al., 2002). These findings suggested that rate of biodegradation depended on molecular structure of NAs (Clemente et al., 2005; Han et al., 2008). Another factor that affects the rate of biodegradation was the variety of microbial cultures. A mixed culture of microorganisms was shown to degrade NAs better than a single culture, indicated that a unique metabolic interaction between microorganisms was happening (Del Rio et al., 2006). Most of the biodegradation studies were successfully done in aerobic condition (Clemente et al., 2005; Han et al., 2008), while in the anoxic condition it was reported that there was no or little biodegradation (Han et al., 2009). All the results concluded that bio-remediation is an effective, low-cost treatment for degrading organics compound in tailing ponds water.

2.6.5.1 Aerobic biodegradation of NAs

The aerobic biodegradation of NAs has been documented in various studies. Predominantly various bacterial *Pseudomonas, Alcaligens, Acninetobacter, Kurthia, and Xanthomonas* species in the tailing ponds were able to readily degrade simple model NAs and NAs from commercial sources, but degradation with NAs from tailing ponds was less efficient (Clemente et al., 2005; Quagraine et al., 2005; Paslawski et al., 2008; Quensel et al., 2011). Scott et al., (2005) reported that microbial degradation of commercially available NAs was complete in 14days, in comparison to NAs in tailing waters which were still remaining after 40days.

Various factors have suggested for the degradation of NAs at slow rate. Based on the studies, there is an influence of chemical structure on biodegrability of NAs.Generally the more persistent NAs have high molecular weight, contain multiple alkyl chains and methyl substituted cycloalkane rings (Paslawski et al., 2008; Johnson et al., 2011). Thus, linear and lower molecular weights NAs in oils sands are removed more rapidly by biodegradation (Quagraine et al., 2005; Paslawski et al., 2008).

Other influencing factors are temperature, DOC, reactor configuration and pH, For example Tanapat (2001) studied three model NAs (*cis*- and *trans*- isomers of 4-MCHAA, *trans* 4-MCHCA, and 3-MHCCA) indicating that temperature had the most significant effect on biodegradation kinetics with an observed ten-fold increase in the first order rate constant between 10 ^{0}C and 30 ^{0}C. Quail et al. (1991) demonstrated that the rate of biodegradation can be greatly improved by treating the pollutants using optimum environmental conditions and a better designed and controlled bioreactor.Paslawski et al.(2008) investigated the enhancement of biodegradation of model NAs (*trans*- 4-methyl-1-cyclohexane carboxylic acid) and reported that the biodegradation rate can be significantly improved by varying the environmental conditions (temperature and pH) and reactor configuration. Another study by Huang & Nemati (2011) involving three model NAs (*cis* and *trans*- isomer 4-MCHAA, *trans*- 4MCHCA) demonstrated the effect of reactor configuration, increasing the maximum specific biodegradation rate of model NAs in circulating packed-bed bioreactor by 4-times than rate reported by Paslawski et al. (2008) in the packed bed bioreactor.

These investigations have led to the identification of number of controlling parameters for biodegradation of NAs and their recalcitrant behaviour in OSPW. Further, these studies have been the references for anaerobic biodegradation.

2.6.5.2 Anaerobic biodegradation

Anaerobic biodegradation had been studied as an alternative to aerobic biodegradation. It is believed that aromatic compounds and hydrocarbons are recalcitrant to anaerobic bio-degradation because of two reasons: (1). the resonance energy of the aromatic ring; (2) the inertness of C-H and C-C bonds in hydrocarbons (Boll et al., 2002). In 1977, it was reported that bacteria were able to degrade aromatic rings under anaerobic condition using electron acceptors such as: nitrate, sulphate, ferric iron, or carbon dioxide (Widdel and Rabus, 2001).

The decomposition of organic compounds by populations of strictly anaerobic bacteria has received a great deal of attention over the past decade and as a result the importance of anaerobic decomposition in the global carbon cycle is becoming apparent. The successful application of anaerobic technology to the treatment of industrial wastewaters is critically dependent on the development and use of high rate anaerobic bioreactors. Various advantages of anaerobic treatment in comparison to aerobic treatment are (Bajpai et al., 1999):

- ***Low space requirements***. When high loading rates are accommodated, the area needed for the reactor is small.
- ***Low energy consumption***. As far as no heating of the influent is needed to reach the working temperature and all plant operations can be done by gravity flow, the energy consumption of the reactor is almost negligible. Moreover, energy is produced during the process in the form of methane.
- ***High efficiency***. Good removal efficiency can be achieved in the system, even at high loading rates and low temperatures.
- ***Simplicity***. The construction and operation of these reactors is relatively simple.
- ***Flexibility***. Anaerobic treatment can easily be applied on either a very large or a very small scale.
- ***Low sludge production***. The sludge production is low, when compared to aerobic methods, due to the slow growth rates of anaerobic bacteria. The sludge is well stabilized for final disposal and has good dewatering characteristics. It can be preserved for long periods of time without a significant reduction of activity, allowing its use as inoculums for the start-up of new reactors.
- ***Low nutrients and chemicals requirement***. Especially in the case of sewage, an adequate and stable pH can be maintained without the addition of chemicals.

Macronutrients (nitrogen and phosphorus) and micronutrients are also available in sewage, while toxic compounds are absent.

Many bacteria are capable of growing under denitrifying condition, some are heterotrophs, autotrophs and one group is photosynthetic. A potential of denitrification exists in every habitats, but from the frequency of isolation *Psuedomonas* and *Alcaligens* groups are of greatest significance. Anaerobic bacteria have been associated with the tailing ponds of oil sands (Holowenko et al., 2001; Quagraine et al., 2005). However, much less is known about the ability and mechanisms by which these can utilize NAs for degradation.

As most of tailing ponds are anaerobic (Clemente et al., 2005), Holowenko et al. (2001) studied the effect of NAs on methanogenesis. Microorganisms from oil sands containing methanogens were grown under anaerobic conditions using commercial NAs, NAs from OSPW and surrogate or model NAs. Neither commercial NAs, nor NAs from OSPW could stimulate the production of methane by microorganisms either from tailing ponds or domestic sewage sludge in the experiments. The methane was produced by surrogate or model NAs (3-cyclohexylpropanoic acid and 4-cyclohexylbutanoic acid) at high concentration (200 mg/L) in another set of experiment using only microbes from tailing ponds. As well, a high concentration of methane was also measured, when surrogates were added to domestic sludge and these model NAs underwent mineralization.

A recent study indicated the existence of anaerobic bacteria in tailing pond waters; these included anaerobic heterotrophic bacteria, sulphate reducing bacteria, and methanogens (Quagraine et al., 2005). The literature suggests, that to stimulate bioremediation various inorganic microbial nutrients are used such as nitrate or nitrogen gas, phosphorous as orthophosphate. Studies have been conducted in the laboratory and in the field showing that the presence of phosphorous enhances bacterial growth and ensuring rapid bacterial degradation of NAs and other inorganic pollutants (Lai et al., 1996). However, it is the macronutrients (N and P) that are of major concern in stimulating bioremediation and thus high concentrations of electron acceptors are required to enhance the anaerobic microbes such as nitrate-reducing bacteria, sulphate-reducing bacteria, and iron-reducing bacteria (Quagraine et al., 2005).

2.7. Knowledge Gap

The (Canada) Alberta's regulation of zero discharged policy causing many researches to be done in order to treat NA. Many studies resulted in successful aerobic biodegradation, however, as it was previously reported, there was no or little biodegradation of NAs under anoxic conditions (Han et al., 2009). Some studies reported that anaerobic biodegradation of aliphatic compounds was observed. With these findings, the biodegradation of NAs became possible. Moreover, one study reported the use of sulfide-reducing bacteria was able to reduce the concentration of NAs, using sulfate as the electron acceptor. Therefore, further investigation of NAs biodegradation, with the help of nitrate, is necessary. This way, we are able to treat two pollutants, NAs and nitrate, using in-situ method at the same time.

Chapter 3
RESEARCH OBJECTIVES

A review of the literature focusing on biodegradation of naphthenic acids highlights the fact that relatively few studies have been carried out on the engineering aspect of the biodegradation of NA compounds, especially biokinetics and bioreactor design (Paslawski, 2008; Huang et al., 2012). Most of these previous studies have been carried out in small scale (mainly serum bottles) batch systems, with the focus being on the impacts of molecular structure on the biodegradation rates of NAs, identification of biodegradation pathways and the bioavailability of naphthenic acids and limited information exists on the engineering aspects especially biokinetics, mass transfer (oxygen and organics) and bioreactor design which are critical in the design and operation of a cost effective bio-treatment process.

Biodegradation of individual surrogate naphthenic acids in a circulating packed-bed bioreactor has been studied in our research group as part of an earlier work (Paslawski 2008; Huang 2011). However, naphthenic acids in tailing ponds consist of a complex mixture of NAs of different molecular structures. Based on the available literature there is a belief that in a mixture, NAs with simpler molecular structure undergo biodegradation at a faster rate and the more complex molecules represent the untreated portion. There is also a general consensus that biodegradation rate of an easily biodegradable compound could be influenced by the presence of a recalcitrant compound and vice versa, and in some cases co-biodegradation of easily biodegradable and recalcitrant NAs might result in enhancement of biodegradation for the latter. Thus co-biodegradation of NAs with different structures (linear and cyclic) would be worthy of investigation.

This research is aimed for model naphthenic acids (NAs) in CSTR and batch.The two model NAs that were selected for this study include trans-4- methyl-1-cyclohexane carboxylic acid (trans-4MCHCA), and octanoic acid as the linear candidate. The specific objectives of this research are listed below:

1. Batch biodegradation and co-biodegradation of individual NAs and various combinations of NAs.

- To study the effect of initial concentration of NAs under varying concentration of nitrate 10 mM (620 mg/L) on biodegradation kinetics and nitrate reduction rate.

- To study the effect of temperature (10, 15, 20, 25, 30, 35 °C) on co-biodegradation rate of octanoic acid and trans-4MCHCA with the nitrate concentration 10 mM (620 mg/L).

2. To study co-biodegradation of octanoic acid and trans-4MCHCA under denitrifying conditions in a continuous bioreactor at nitrate concentration of 10mM (620 mg/L). It involves:
   - Effect of residence time.
   - Effect of loading rate and removal rate.
3. Analysis of kinetic data obtained from the above investigations.

Chapter 4
MATERIAL AND METHODS

4.1 Selection of Candidate Compounds

Based on the existing literature, various substrates (NAs) have been used for research and investigation for studying biodegradation and these falls into three categories: surrogate or model naphthenic acids which are individual surrogate (pure) naphthenic acid fitting the formula $C_nH_{2n+Z}O_2$, commercially available mixture of NAs (from Fluka or Kodak), and NAs extracted from the oil sands tailing ponds water (Clemente et al., 2005).

Based on the literature review, oil sand tailing ponds water has been reported to consist of toxic naphthenic acids of varying composition and structure. Hence for this study, two model naphthenic acids, *trans*-4-methyl-1-cyclohexane carboxylic acid (referred to as *trans*-4MCHCA, CAS NO. 13064-83-0), and octanoic acid ($C_8H_{16}O_2$, CAS NO. 124-07-2) were chosen based on difference in molecular structure, biodegradability and commercial availability.

Octanoic acid is an oily colorless liquid at room temperature, while trans-4MCHCA appear as white crystalline solids under similar conditions (Huang, 2011). Octanoic acid (CAS NO. 124-07-2) purchased from Sigma-Aldrich Co.(~97 % purity). At the room temperature, octanoic acid appears physically yellow oily liquid. The molecular formula for this compound is $CH_3(CH_2)_6COOH$ with molecular weight of 144.21 Daltons and fits the formula $C_nH_{2n+Z}O_2$ with Z=0, represents linear NA. Trans-4MCHCA was known for its commercial availability and biodegradation. Trans-4MCHCA was prepared in modified McKinney's medium to inoculate the culture. The molecular structures of trans-4MCHCAand octanoic acid are shown in the Figures 4.1 and 4.2.

Figure 4.1 Molecular structure of octanoic acid (adapted from Sigma Aldrich Co., 2009)

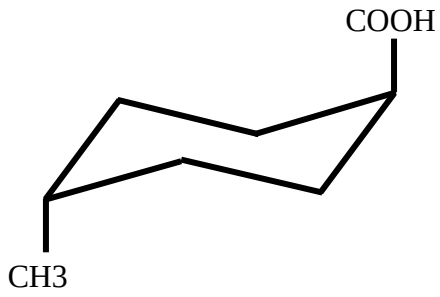


Figure 4.2 Molecular structure of *trans*-4-methyl-1-cyclohexane carboxylic acid, *trans*-4MCHCA (adapted from Sigma Aldrich Co., 2009)

4.2 Microbial Cultures and Medium

The microbial culture utilized during this study was isolated from one of the tailing ponds contaminated with oil sands process water (OSPW) and developed using commercially prepared NAs (Fluka, Sigma-Aldrich, CAS No. 1338-24-5) as substrate in Mc-Kinney's medium (Paslawski et al., 2008). The culture was capable of degrading trans-4MHCA and studies demonstrated consortium comprises as mixed culture, dominant bacterial species *Pseudomonas aerugonisa* and *Variovax paradoxus* were identified (Paslawski et al., 2008). The same culture was used as inoculums for biodegradation of

octanoic acid and trans-4MCHCA under denitrifying conditions and nitrate as electron acceptor.

4.2.1 Medium

Modified McKinney's medium was used to provide the necessary nutrients and to maintain the microbial culture and in all experiments carried out in this study. The medium was prepared in 6L batches of reverse osmosis (RO) water and had the following composition: KH_2PO_4 (840 mg/L); K_2HPO_4 (750 mg/L); $(NH_4)_2SO_4$ (474 mg/L); NaCl (60 mg/L); $CaCl_2$ (60 mg/L); $MgSO_4 \cdot 7H_2O$ (60 mg/L); $Fe(NH_4)_2(SO4)_2$ $6H_2O$ (20 mg/L).Trace mineral solution was added to the prepared medium at a level of 0.1% on a volumetric basis. The trace mineral medium was comprised of: H_3BO_3 (600 mg/L); $CoCl_3$ (400 mg/L); $ZnSO_4 \cdot 7H_2O$ (200 mg/L); $MnCl_2$ (60 mg/L); $NaMoO_4$ $\cdot 2H_2O$ (60 mg/L); $NiCl_2$ (40 mg/L); and $CuCl_2$ (20 mg/L). The modified McKinney's medium was selected based on the previous studies with naphthenic acids (Paslawski et al, 2008; Huang, 2011).

4.2.2 Acclimatization of the culture

The culture isolated above during the earlier work was acclimatized to the anaerobic conditions. The initial concentration of octanoic acid (100 mg/L) and trans-4MCHCA (50 mg/L) with nitrate 10 mM (620 mg/L) was used and culture was grown in a serum bottle containing 100 ml of Mc-Kinney's media and purged with nitrogen gas to create anaerobic conditions at room temperature 23±1.5 ^{0}C. Measurement of optical density, octanoic acid and trans-4MCHCA concentration and nitrate was done at regular intervals. Following the complete biodegradation of the octanoic acid and trans-4MCHCA this culture was used as inoculums for further experiments. Subculturing was carried out in the same way described above after 7-8 days.

4.3 Experimental Systems of Batch Reactor for Biodegradation Studies

Following the establishment of a suitable microbial culture for biodegradation of Octanoic acid and trans-4MCHCA, batch experiments were conducted to study the microbial growth kinetics and kinetic rate of biodegradation in anaerobic condition. The effects of concentration of octanoic acid, trans-4MCHCA and temperature were investigated.

Batch experiments were carried out in 150 mL serum bottles, where each contained 100 mL of sterilized medium (described previously) and organic substrate trans-4MCHCAand

octanoic acid.). The various initial concentrations of trans-4MCHCA (25, 50, 100 mg/L) and octanoic acid (100 mg/L) were tested separately. Following these co-biodegradation of octanoic acid (100 mg/L.) and trans-4MCHCA (25, 50,100 mg/L), were conducted to verify the impact of co-biodegradation of an easily degradable linear compound with a more recalcitrant one. The initial concentration of nitrate was adjusted to 10 mM (620 mg/L).

Each concentration experiment was done in duplicate and every set of experiment was done consecutively. Each flask was inoculated by a 7-day old culture (10% on a volumetric basis). Flasks were maintained at room temperature (24 ± 2^0C) and were placed at rest on the table (so that there will be no or little diffused oxygen). Samples were taken on daily basis, started from the first day of inoculation.

The samples were analyzed for trans-4MCHCA, octanoic acid concentration, nitrate ion concentration, and optical density. The sampling frequency was increased during exponential phase and sampling was carried out until the stationary growth was achieved. The progressive experiments were carried out using the preceding batch culture, as an inoculum, in order to permit adaptation of the consortium microbial to the higher substrate concentration.

The temperature controlled environmental chamber was utilized to assess the effect of temperature on biodegradation. The temperature, initially, was set to 20^0C. Then, it was decreased slowly to temperature of 15 and 10. Similarly, the initial temperature was incrementally increased to 25, 30, and 35 degree Celsius. All the sampling procedures were preceded similarly as described earlier.

4.4. Experimental System of Continuous Stirred Tank Reactor

The continuous stirred tank bioreactor (CSTR) was set up for study co-biodegradation of octanoic acid and trans-4MCHCA under denitrifying conditions. The reactor vessel was constructed of glass, with a working volume of 200ml as shown in Figure 4.3. Initially, bioreactor was operated batch wise, with an initial concentration of 100 mg/L (octanoic acid), 50 mg/L (trans-4MCHCA) and 10 mM nitrate and purged with nitrogen gas to create anaerobic conditions at room temperature 23±1.5 °C. The reactor was inoculated with 10% (v/v) from 7-day old batch culture and magnetic stirrer was used to achieve mixing and maintain biomass and substrate in suspension. The bioreactor was switched to continuous mode when octanoic acid, trans-4MCHCA and nitrate were completely utilized. To feed the reactor with continuous supply of medium with octanoic acid (100 mg/L), trans-4MCHCA (50 mg/L) and nitrate 10 mM (620 mg/L) peristaltic pump was used.

Prior to pumping the feed, it was purged with nitrogen gas and even at regular intervals the reactor was also purged to create anaerobic conditions. The initial flow rate was 10 mL/h and effluent was removed by the overflow tube. After the running of the reactor, samples were taken from the reactor and nitrate concentration came to be limiting and was increased to 15 mM (930 mg/L). At each flow rate, sufficient time was given to establish the steady state which was assumed to be established when residual concentrations of octanoic acid ,trans-4MCHCA and nitrate (change by 10%) were constant in bioreactor. The samples were taken from rubber septum located in bioreactor, for determining biomass (OD), residual octanoic acid, trans-4MCHCA and nitrite concentrations. The flow rate was increased incrementally from 10 mL/h to 150 mL/h till washout conditions were achieved. The peristaltic pump was calibrated before use for various flow rates and verified by measuring the effluent volume after some interval. Applied dilution rates were in the range of 0.05 to 0.75 h^{-1}.

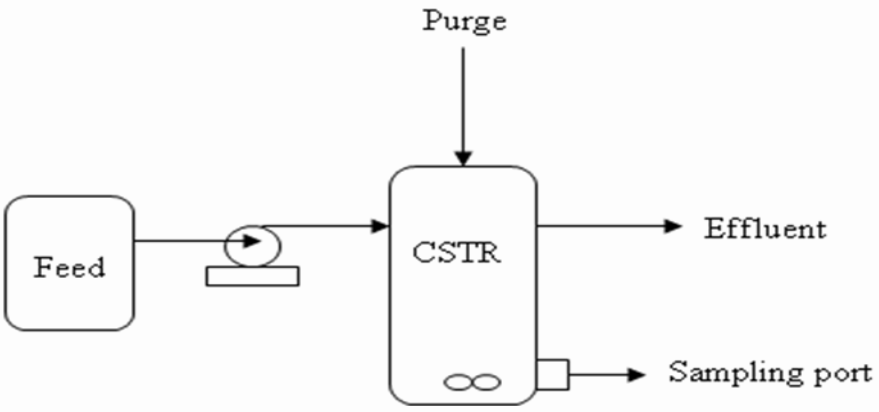


Figure 4.3 Continuous stirred tank bioreactor set-up for degradation of Octanoic acid and trans-4MCHCA under denitrifying conditions

4.5 Measurement of Naphthenic Acids Concentration

The assessment of naphthenic acids is limited by analytical techniques because of the concentrations and types of acids present in these complex oil sands mixtures. There is currently no method or technique that quantifies or separates individual acids of the mixture. Thus, the most of techniques rely on the treating them as group or sub-group, based on the carbon or Z numbers (Clemente et al., 2005). Various techniques have been used and these includes, high-performance liquid chromatography (HPLC), fourier transform infrared (FTIR) spectroscopy, negative ion electrospray ionization-mass spectrometry (ESI-MS), gas chromatography with a flame ionization detector (GC-FID),

gas chromatograph-mass spectrometry (GC-MS), liquid secondary ion mass spectrometry (LSI-MS), electrospray ionization (ESI), and quantitative quadrupole time of flight –MS (QTOF_MS) (Paslawski et al., 2008; Bataineh et al., 2006; Clemente et al., 2005; Barrow et al., 2004). All of the above methods have unique strengths as well limitations. However, in previous works in our laboratory, gas chromatography with a flame ionization detector (GC-FID) has been successfully used as a relatively simple and speedy approach to determine NA concentrations in aqueous solutions (Paslawski, 2008; Huang, 2011).

In this work a Varian- 430 gas chromatograph with a flame ionization detector (FID) and HP-INNOWAX high resolution gas chromatography column (19091N-133) was used for the determination of naphthenic acid concentration.Helium at a flow rate of 29 mL/min served as the carrier gas, while hydrogen and air at flow rates of 30 ml/min and 300 ml/min were used for combustion in the FID. The specifications of the gas chromatography column were as follows: length: 30 m, diameter: 0.250 mm and film thickness: 0.25 µm. The column temperature at the start up was around 90 $^{\circ}$C and the detector and injector temperature was maintained at 250 $^{\circ}$C and 220 $^{\circ}$C, respectively. The column oven temperature was then increased up to about 210 $^{\circ}$C at a rate of 40 $^{\circ}$C/min from the initial 90 $^{\circ}$C during the 7 minute run.

A linear calibration curve was developed to convert gas chromatography readings to the actual NAs concentrations (mg/L) in the sample. The standard concentrations used were 4, 10, 20, 40, 80 and 100 mg/L for trans-4MCHCA and 20, 50, 100, 200 ,400 and 500 mg/L for the octanoic acid. A calibration curve was then developed for each tested compound and used for analysis of the samples taken during the experiments. The standard solutions were prepared by first dissolving the model NA into McKinney's medium at the highest concentration and then diluting this solution into five different concentrations as indicated above. Calibration curves for *trans*-4MCHCA and octanoic acid are shown in Figures B1 to C1 in the appendix B and C. The elution time for the studied NAs was as follows: octanoic acid at 3.23 min, *trans*-4MCHCA 3.5min.

4.6 Measurement of Nitrate and Nitrite Ion Concentrations

The concentrations of nitrate and nitrite ions were determined using a Dionex ion chromatograph (ICS-2500) with a conductivity detector (CD25A) equipped with an IonPac CG5A guard column and an IonPac CS5A analytical column. The eluent was 1.0 mM KOH and the flow rate of the eluent was set at 1.0 mL/h. The system was calibrated using standard solutions of nitrite and nitrate with concentrations of 5, 10, 20, 30, and 50 mg/L. To establish the calibration curves, standard solutions with each concentration were injected three times (injection volume = 25.0 μL). The calibration curves were quadratic for all the ions with standard deviation associated with measurements of nitrate, and nitrite are 1.49% and 0.90%. To prepare the samples for IC analysis, liquid samples (0.1 mL), was transferred into a 1.5 mL micro-centrifuge tube filled with 0.9 mL Millipore water (10 times dilution). Samples were further diluted (overall dilution ratio of 40 folds) to ensure the concentrations of ions fell in the concentration ranges of the developed calibration curves. Diluted samples were then analyzed by IC, measuring the ion concentrations of nitrate and nitrite simultaneously.

4.7 Measurement of Biomass Concentration

The concentration of free biomass was determined by direct measurement of the optical density (OD) of samples collected from batch and continuous bioreactors using the glass needles. The OD was measured at wavelength of 620 nm using spectrophotometer (Shuler, 2002). An UV-visible spectrophotometer (Mini Shimazdu, Model 1240) was used for determining the OD. The OD was then related to dry weight using the calibration curve developed in these experiments. The standard curve is shown in Appendix as Figure A.1

4.8 Statistical Analysis of Results

During batch and continuous experiments, samples were periodically taken in duplicate. Each sample was analyzed three times to determine the biomass and substrate concentrations. The mean values with one standard deviation were reported throughout this thesis. The standard deviations were calculated using Microsoft Excel and presented as error bars. For experiments conducted in the continuous bioreactors, following the establishment of steady state at each applied conditions, three additional samplings were done and the average value of these data was used as the final result. The average value of the data and associated standard deviation were used to present the results. For the purpose assessing the reproducibility a number of experiments were repeated as stated previously.

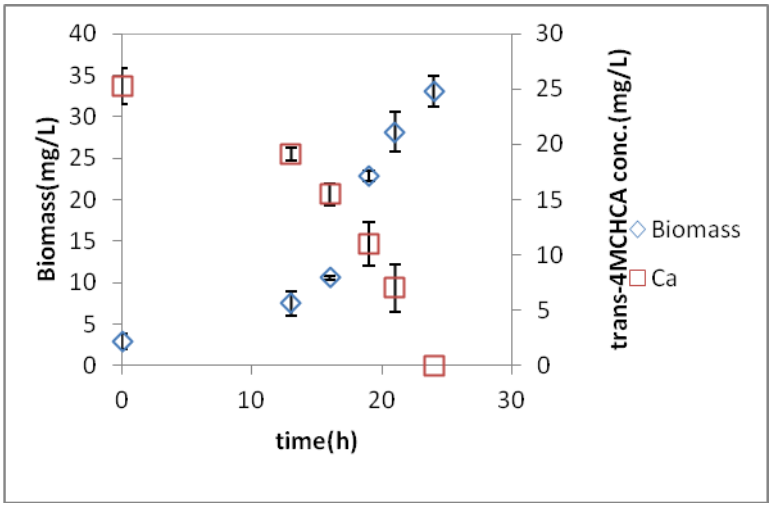
Chapter 5
RESULTS AND DISCUSSION

5.1 Batch Biodegradation

5.1.1 Batch biodegradation of individual NA compounds

Biodegradation of *trans*-4-methyl-1-cyclohexane carboxylic acid (*trans*-4MCHCA) and Octanoic acid under denitrifying conditions with different initial concentrations of substrate and nitrate was observed.

The results of microbial growth, substrate removal and nitrate reduction as function of time in batch reactors containing different initial concentrations of *trans*-4MCHCA (25, 50,100 mg/L) and octanoic acid (100 mg/L) and nitrate at room temperature 23±1.5 ˚C is shown in figure 5.1



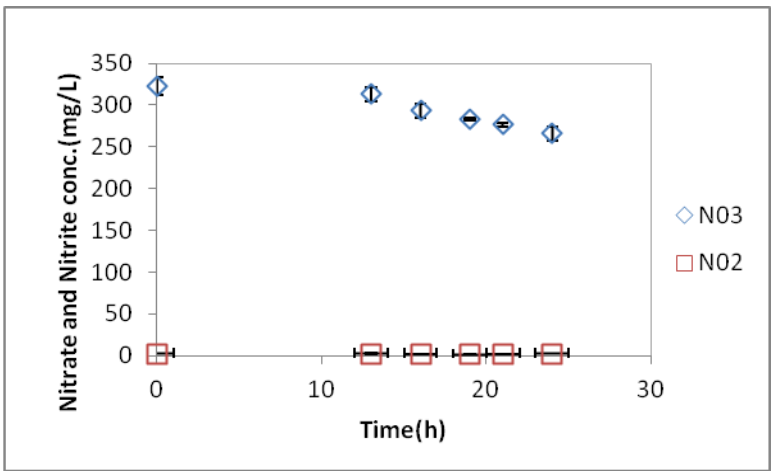
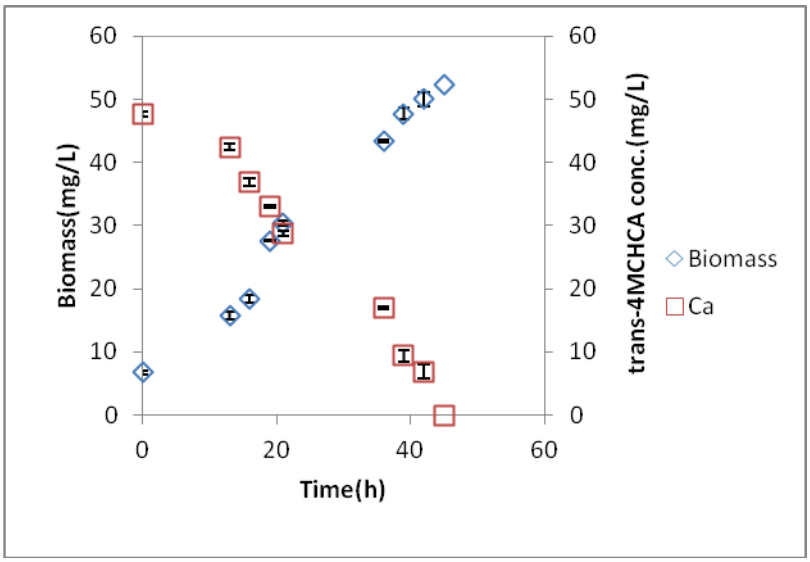


Figure 5.1 Substrate biodegradation, nitrate reduction and biomass growth as a function of time in batch reactors 25mg/L trans-4MCHCA and nitrate at temperature 23±1.5 ˚C. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



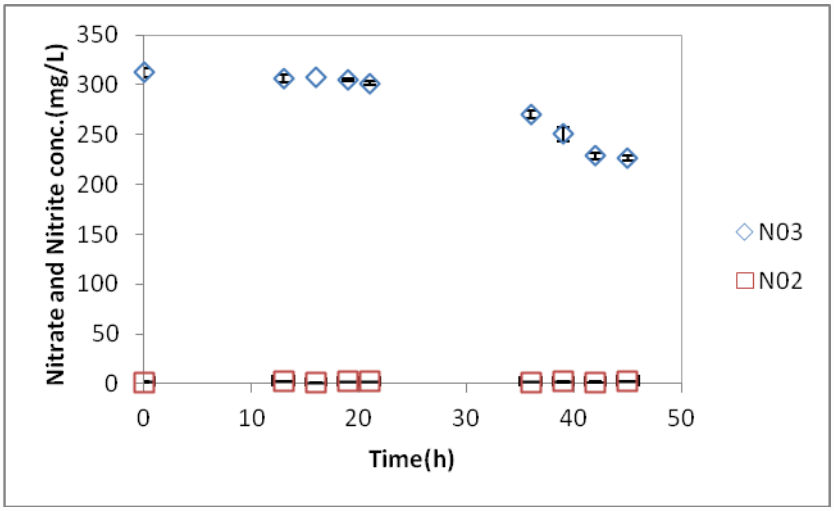
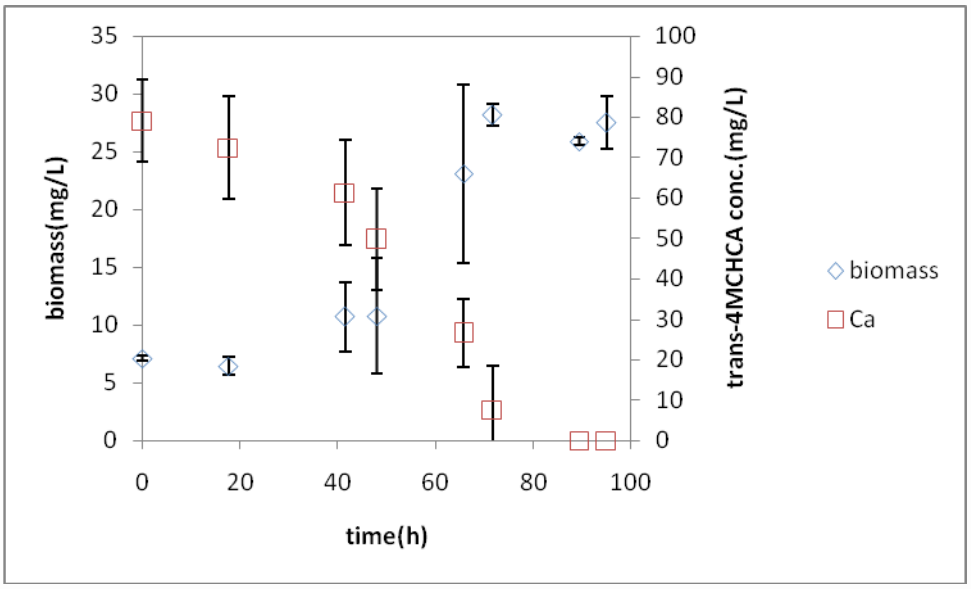


Figure 5.2 Substrate biodegradation, nitrate reduction and biomass growth as a function of time in batch reactors 50mg/L trans-4MCHCA and nitrate at temperature 23±1.5 ˚C. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



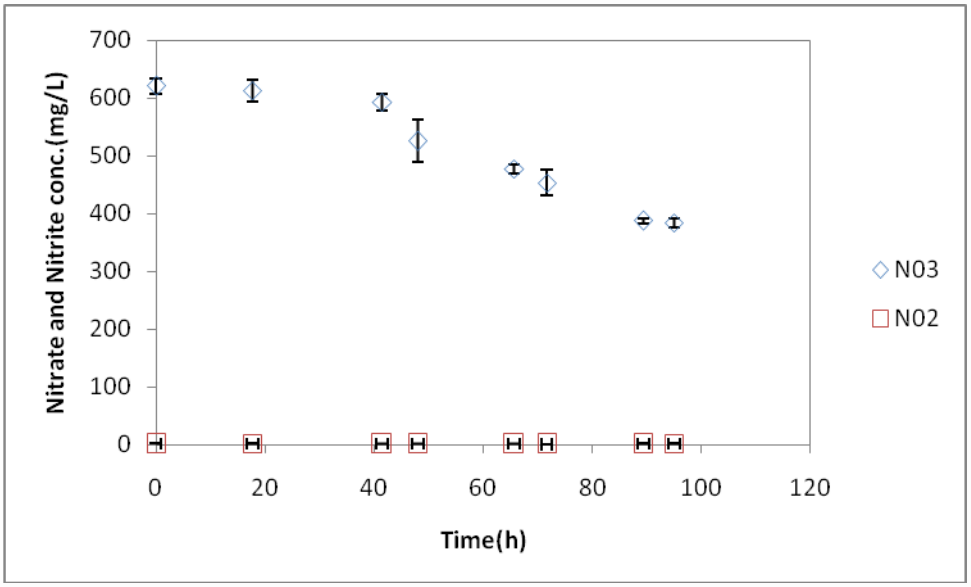


Figure 5.3 Substrate biodegradation, nitrate reduction and biomass growth as a function of time in batch reactors 100mg/L trans-4MCHCA and nitrate at temperature 23±1.5 ˚C. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

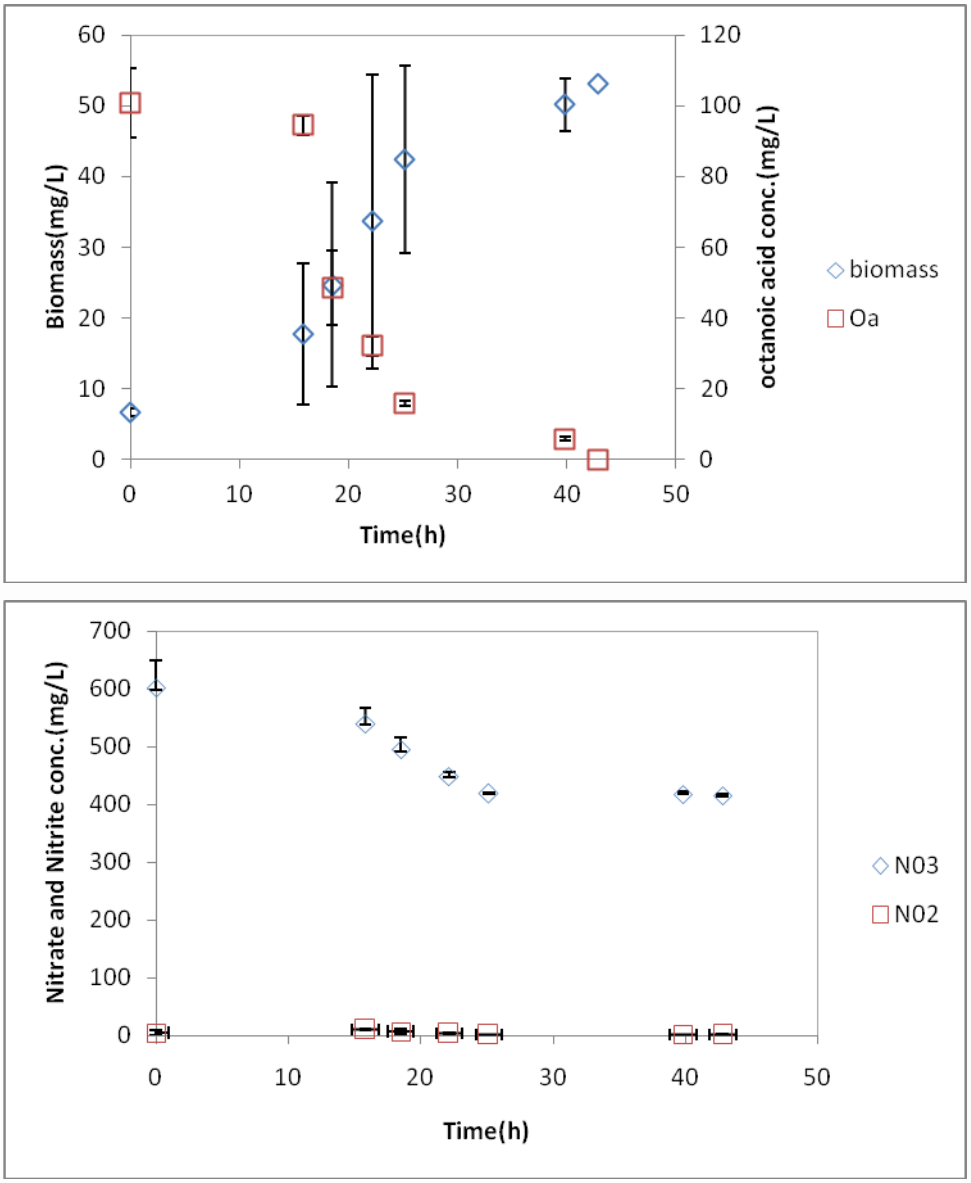


Figure 5.4 Substrate biodegradation, nitrate reduction and biomass growth as a function of time in batch reactors 100 mg/L Octanoic acid and nitrate at temperature 23±1.5 ˚C. Each point represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

In all cases, it was observed that there was a direct relationship between the microbial growth and substrate utilization. As the initial trans-4MCHCA concentration was increased, there was an increase in final biomass concentration in the system. Adaptation period of microorganisms was also noticed, shown by the present of lag phase the system, when the initial concentration of trans-4MCHCA was increased. Table 5.1 presented the calculated

values of biodegradation rate of substrates (trans-4MCHCA,octanoic acid and nitrate). These rates were calculated using the concentration profiles during the exponential phase of microbial activity as it is customary for biological systems. The observed biodegradation rate ranged from 1.2 mg/ L .h, at the concentration of trans-4MCHCA (100 mg/L), to 1.7 mg/ L. h at the concentration of trans-4MCHCA (25 mg/L). The biodegradation rate was observed 2.6 mg/L. h for octanoic acid(100 mg/L).

Table 5.1 Summary of specific growth rate, biodegradation rate and nitrate reduction rate at various initial concentrations at 23±1.5 °C (batch reactors).

Initial Substrate(trans-4MCHCA) Concentration (mg/L)	**Initial Nitrate Concentration (mg/L)**	**Biodegradation Rate (mg/L.h)**	**Nitrate Reduction Rate (mg/L.h)**
25	310	1.72 (R^2=0.97)	4.1 (R^2=0.90)
50	310	1.19 (R^2=0.98)	2.5 (R^2=0.91)
100	620	1.2 (R^2=0.92)	4.0 (R^2=0.94)
100(octanoic acid)	620	2.6 (R^2=0.7)	12.8 (R^2=0.98)

5.1.2 Batch co-biodegradation of mixture octanoic acid and 4MCHCA

The microbial consortium was able to degrade the two compounds simultaneously but with varying removal rates.

The results of co-biodegradation of the two model NA compounds are shown in figure 5.5 to 5.7. Although biodegradation of all compounds occurred simultaneously, the rates of

biodegradation were different for the different compounds depending on the complexity of the molecular structure (Table 5.2).

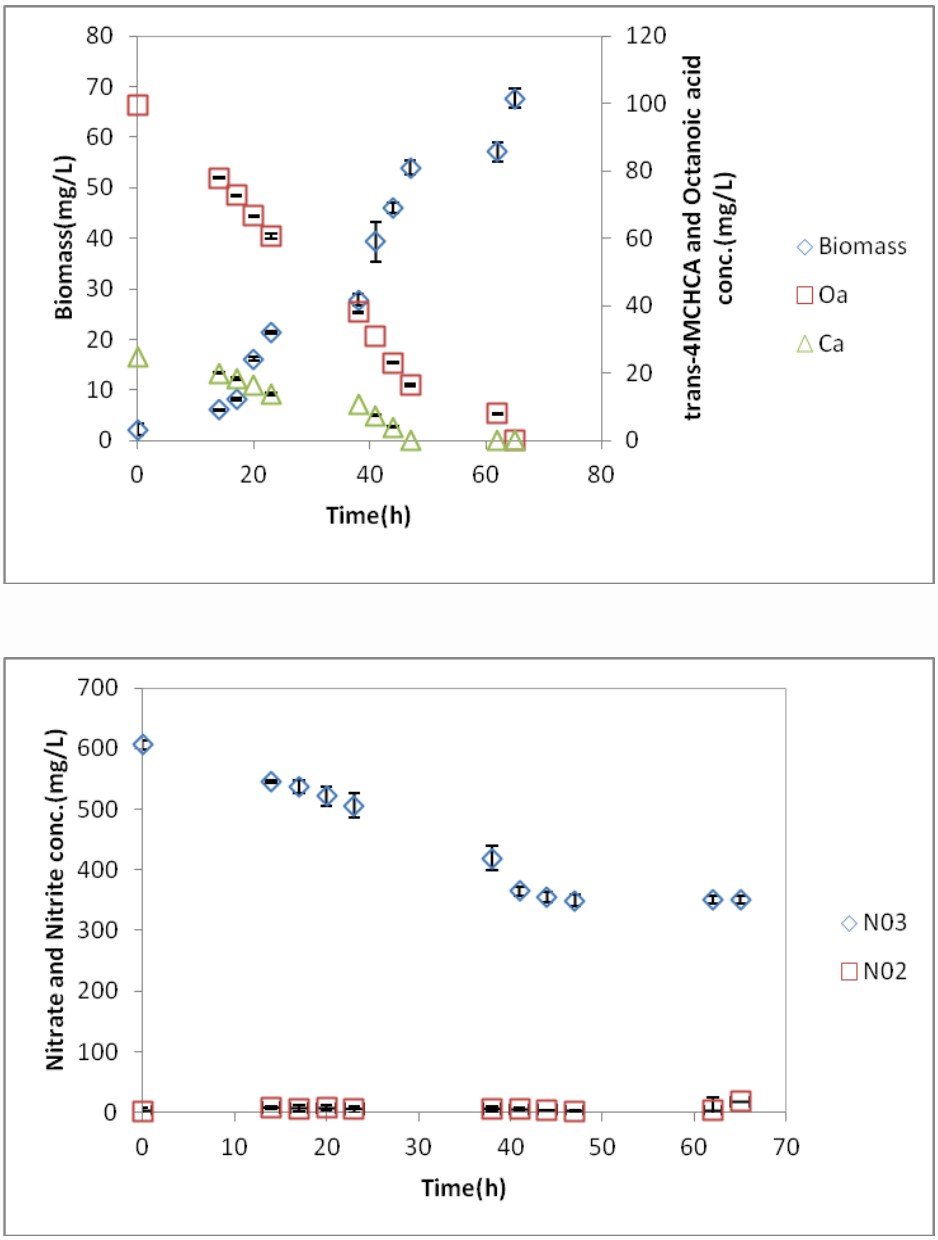
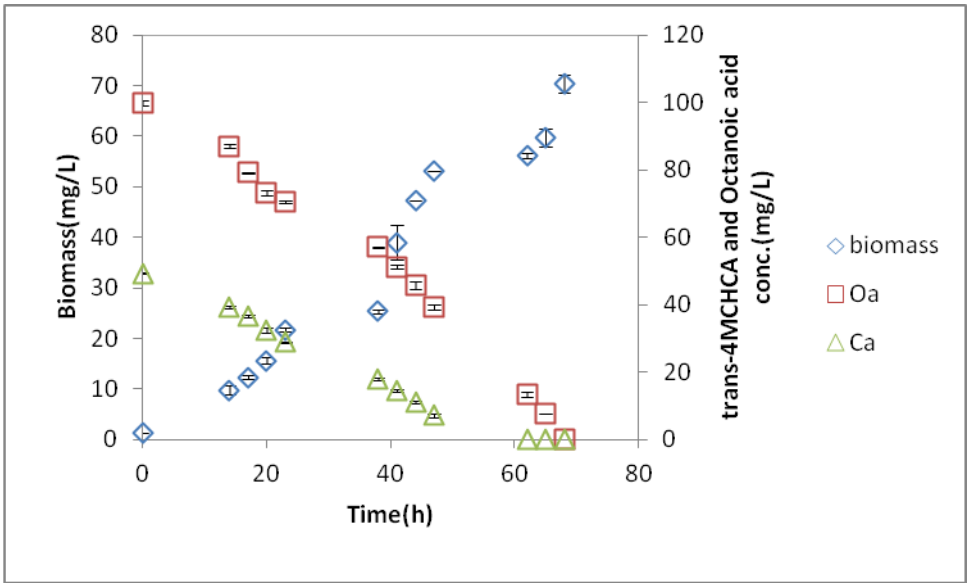


Figure 5.5: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (25±10 mg/L). Data represent the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



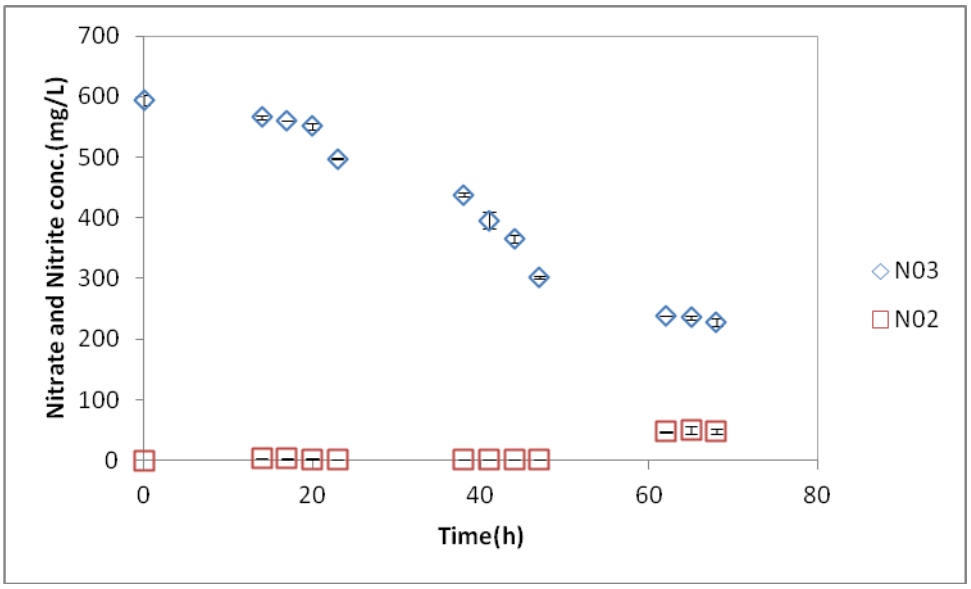


Figure 5.6: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50± 10 mg/L). Data represent the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

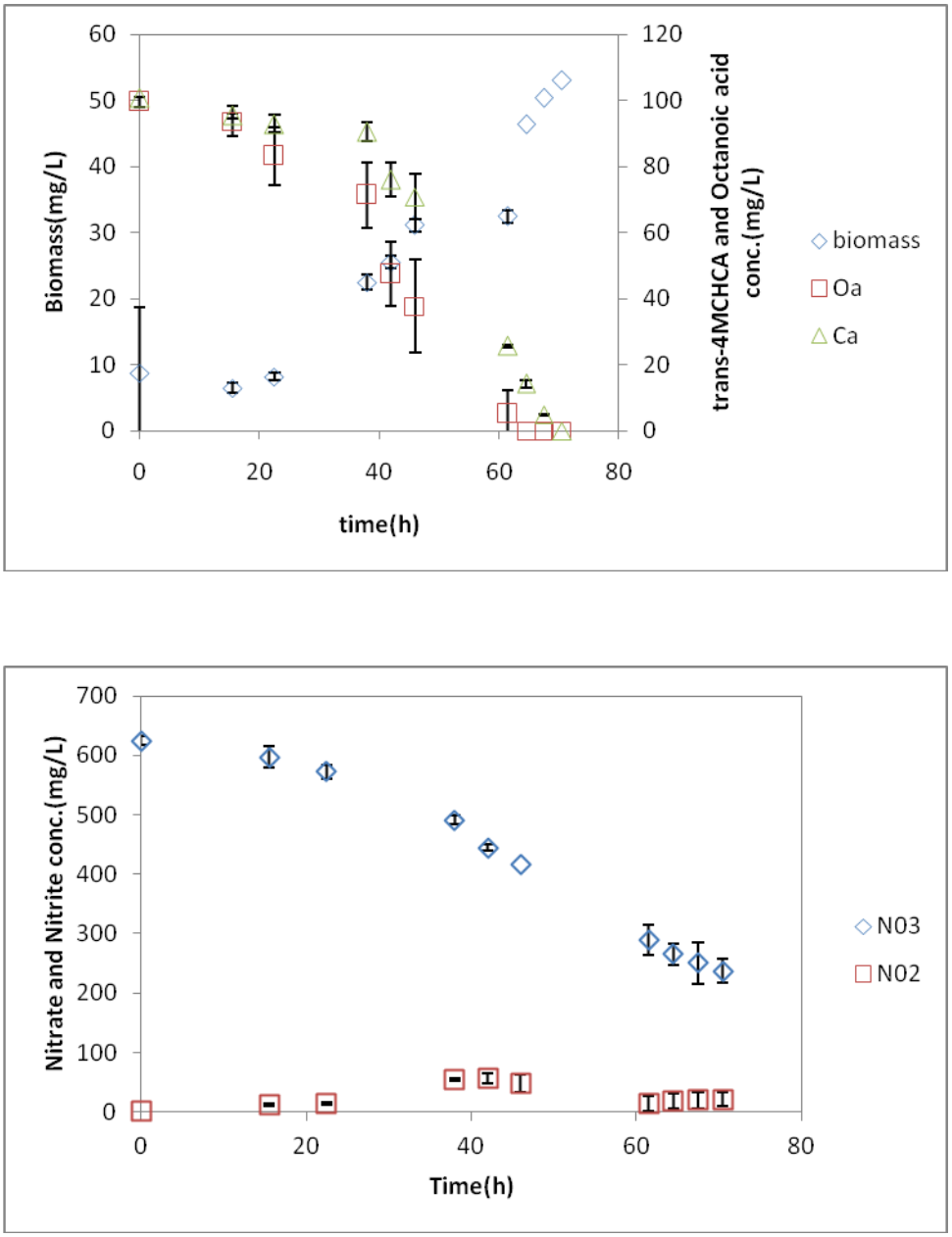


Figure 5.7: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (100± 10 mg/L). Data represent the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The mixed microbial culture used in this study was capable of degrading all the selected model NA compounds both the linear and cyclic ring structures at varying removal rates. In case of other organic compounds that substrates that are recalcitrant can be degraded more effectively when an easily biodegradable compounds is present (Veeresh et al, 2004). In the present experiment, the effect of co-biodegradation was studied using octanoic acid

with trans-4MCHCA. It was observed that the biodegradation rate of octanoic acid was ranged from 1.56 mg/.L.h to 1.94 mg/L.h and for trans-4MCHCA biodegradation rate ranged from 0.49 mg/L.h to 2.41 mg/L.h., Table 5.2 presented the calculated values of biodegradation rate of substrates (trans-4MCHCA,octanoic acid and nitrate).

Table 5.2: Summary of co-biodegradation rates of mixture of Octanoic acid and trans-4MCHCA.

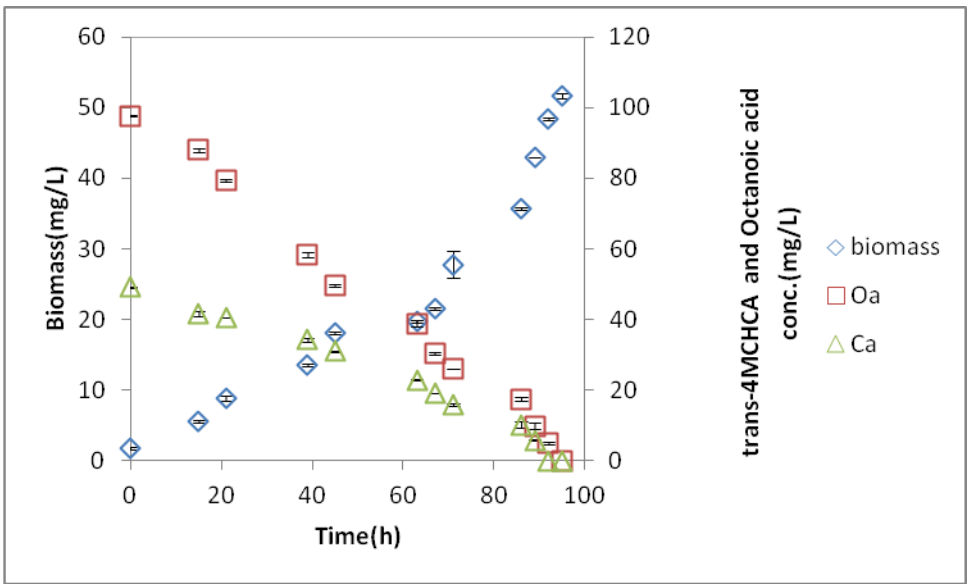
Mixture of Naphthenic Acids	**Biodegradation Rate of Octanoic Acid (mg/L.h)**	**Biodegradation Rate of trans-4MCHCA (mg/L.h)**	**Nitrate Reduction Rate (mg/L.h)**
Octanoic Acid (100 mg/L) and trans-4MCHCA (25 mg/L)	1.56 (R^2=0.98)	0.49 (R^2=0.95)	5.89 (R^2=0.97)
Octanoic Acid (100 mg/L) and trans-4MCHCA (50 mg/L)	1.46 (R^2=0.98)	0.84 (R^2=0.90)	7.2 (0.97)
Octanoic Acid (100 mg/L) and trans-4MCHCA (100 mg/L)	1.94 (R^2=0.93)	2.41 (R^2=0.95)	7.01 (R^2=0.98)

5.1.3 Temperature Effect on Co-biodegradation of Octanoic acid and trans-4MCHCA

To evaluate the temperature effect on biodegradation rate of NAs, a series batch experiments were conducted in a temperature controlled environmental chamber where the temperatures were increased from 10 °C to 35 °C in 5 °C increments.

Figure 5.8 to 5.12 illustrated the profile of temperature effect in biodegradation performance at initial concentration of Octanoic acid (100 mg/L) and trans-4MCHCA (50 mg/L) and nitrate concentration of 620 mg /L under various temperatures. Similar to the concentration profile, in all cases, when the concentration of NAs started to decline, a noticeable decline in nitrate concentration was noticed, accompanied by an increase in biomass concentration. Temperature is considered to be one of the important parameter in biodegradation as it was

noticed that temperature change resulted in different period of lag phase. For example, a lag phase of approximately 73 and 24 hours was observed from batch at temperature of 10 and 15°C, while at temperature of 20 and 24±2°C, lag phase was not detected. However, when temperature was further increased (temperature of 30° and 35°C), lag phase of 20 hours were observed again. The length of biodegradation period was also affected by change in temperature. Longer period of time was required to completely biodegrade trans-4MCHCA and reduce nitrate at low temperature (10° and 15°C).



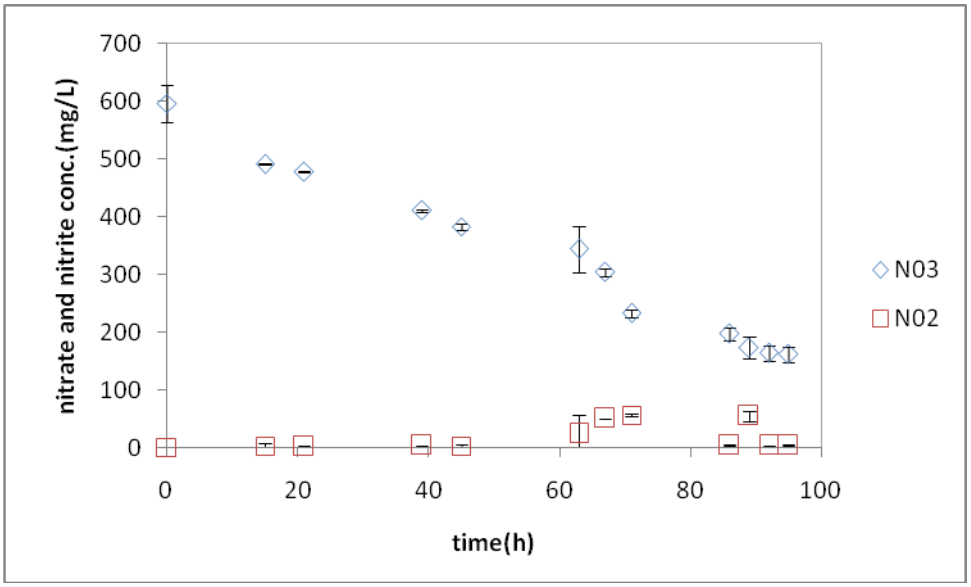
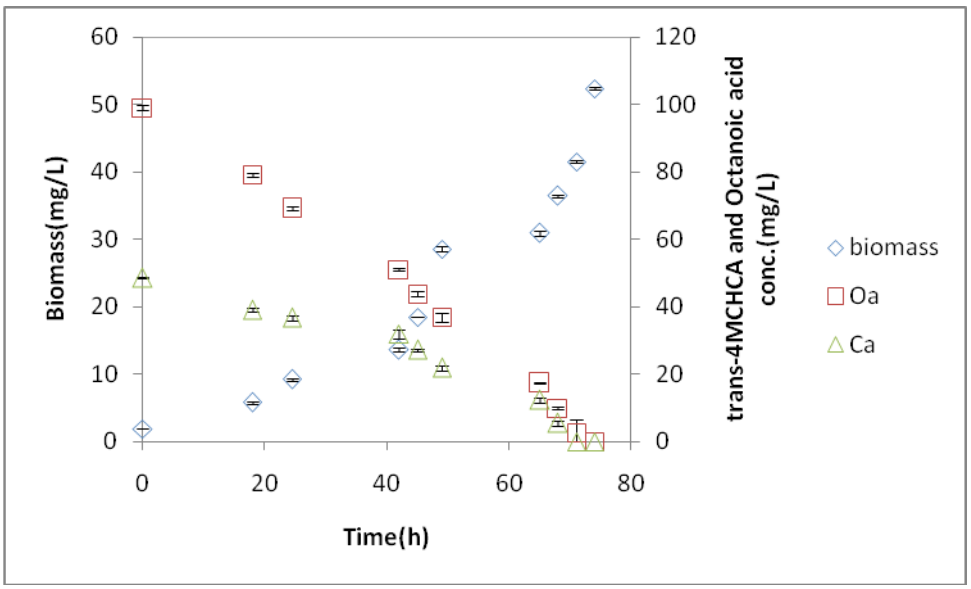


Figure 5.8: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50±10 mg/L) at 10°C. Data represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



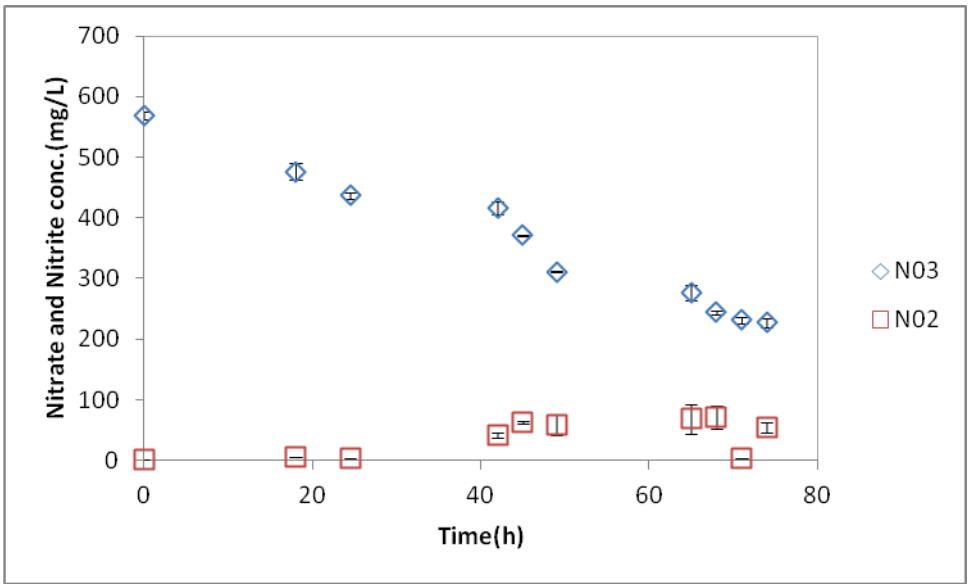
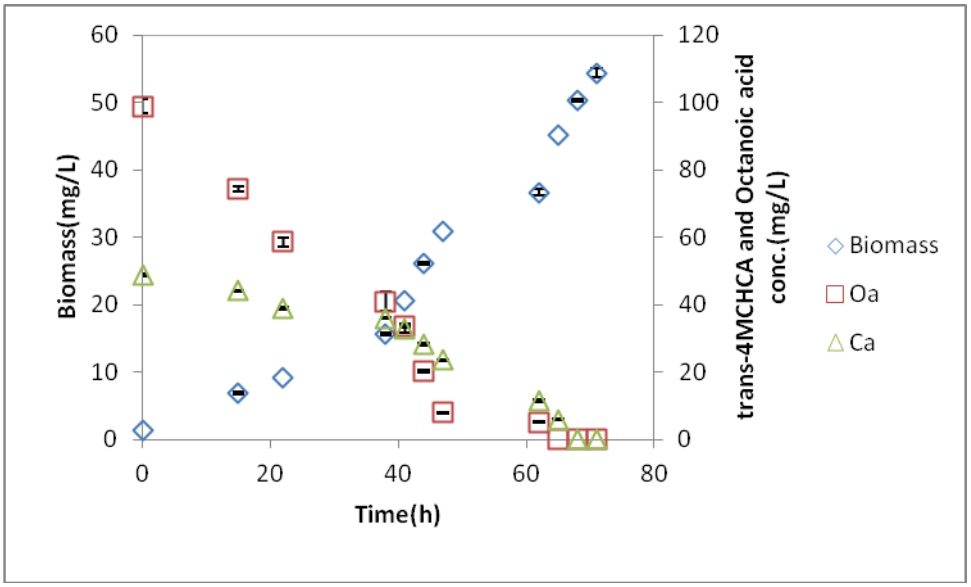


Figure 5.9: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50±10 mg/L) at 15°C Data represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



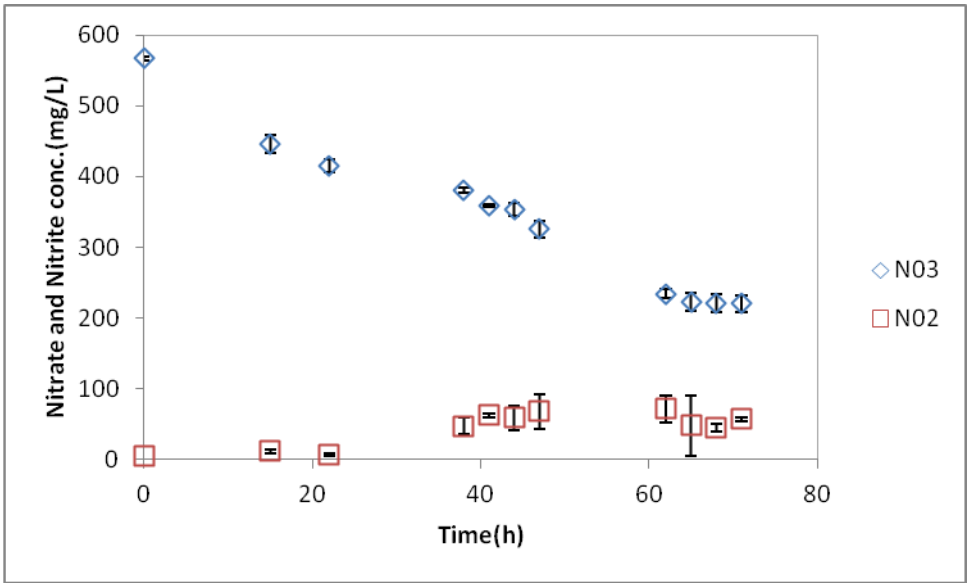
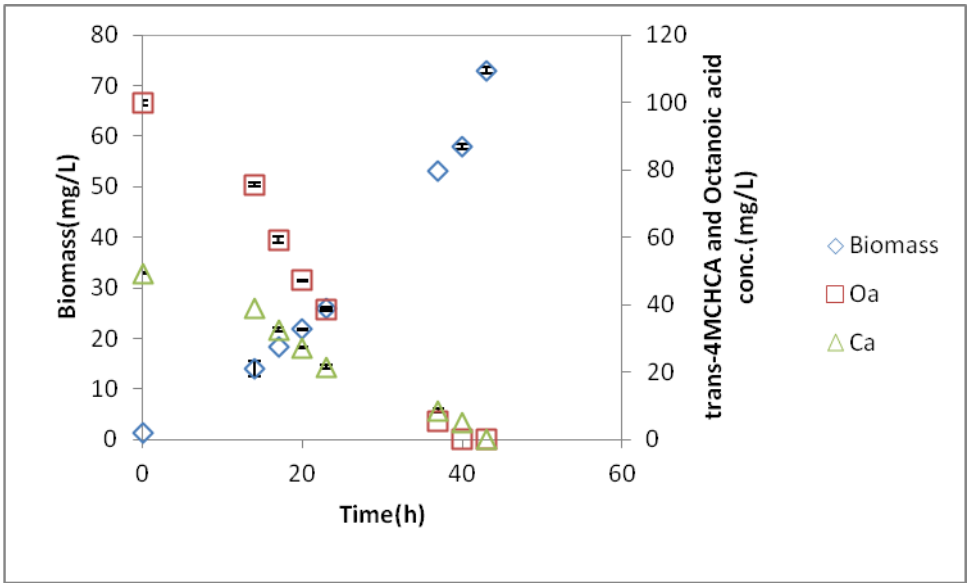


Figure 5.10: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50±10 mg/L) at 20°C Data represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



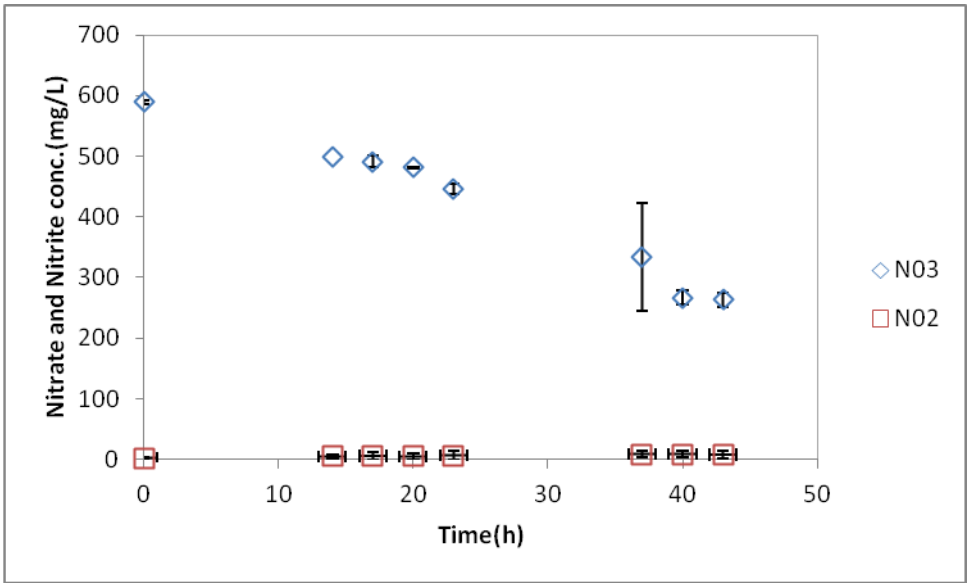
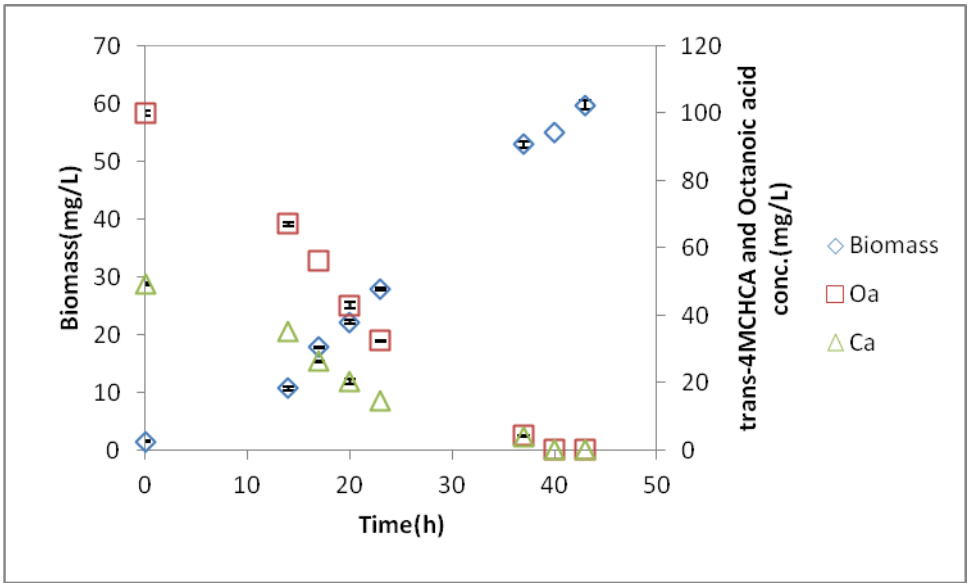


Figure 5.11: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50±10 mg/L) at 30°C. Data represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.



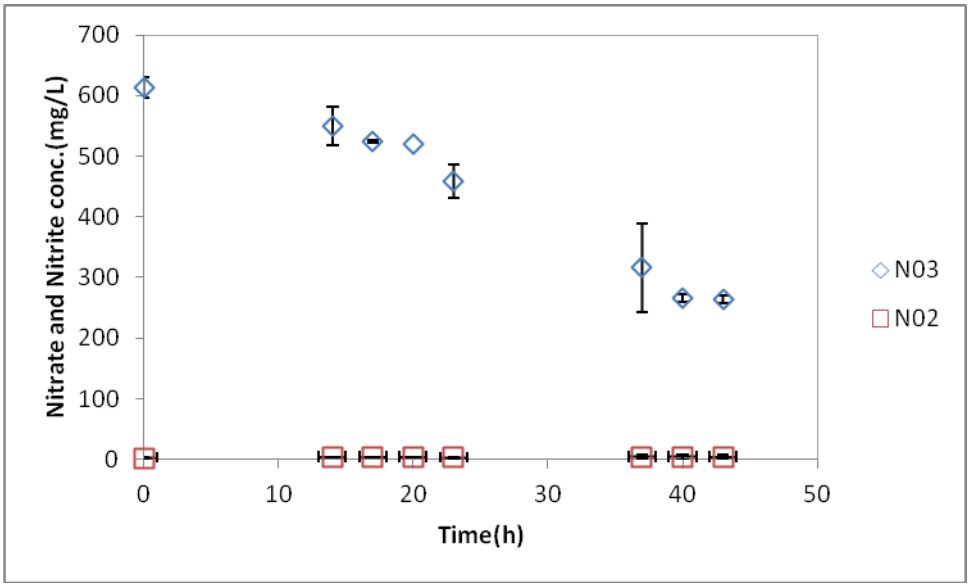


Figure 5.12: Co-biodegradation of octanoic acid (100±10 mg/L), trans-4MCHCA (50±10 mg/L) at 35°C Data represents the average value of the data obtained by multiple sampling and error bars represent standard deviations. Error bars may not be visible at all points due to small value of SD.

The biodegradation rate and nitrate reduction rate at various temperatures for octanoic acid trans-4MCHCA) are shown in Table 5.3. At higher temperature, there was little production of nitrite, which indicated that microorganisms might utilize nitrite at the same time as nitrate in short period of time.

Table 5.3: Summary of co-biodegradation rates of mixture of Octanoic acid and trans-4MCHCA at various temperatures

Temperature, (°C)	Biodegradation Rate of Octanoic Acid (mg/L.h)	Biodegradation Rate of trans-4MCHCA (mg/L.h)	Nitrate Reduction Rate (mg/L.h)
10	1.02 (R^2 =0.99)	0.48 (R^2 =0.98)	4.4 (R^2= 0.96)
15	1.35 (R^2 =0.99)	0.65 (R^2=0.93)	4.5 (R^2=0.96)
20	1.49 (R^2 =0.93)	0.81 (R^2=0.99)	4.7 (R^2=0.96)
23±1.5	1.46 (R^2=0.98)	0.84 (R^2=0.90)	7.2 (0.97)
30	2.73 (R^2 =0.97)	1.20 (R^2=0.92)	8.9 (R^2=0.90)
35	2.49 (R^2 =0.97)	1.19 (R^2=0.93)	11.0 (R^2= 0.76)

The maximum co-biodegradation rate for octanoic acid and trans-4MCHCA was observed at 30 °C with a value of 2.73 mg/L.h and 1.20 mg/L.h respectively and lowest at 10 °C with a value of 1.02 mg/L.h and 0.48 respectively.

5.2 Co-biodegradation of Octanoic Acid and Trans-4MCHCA in a Continuous Stirred Tank Bioreactor under Denitrifying Conditions

The effect of volumetric loading rate of octanoic acid and trans-4MCHCA under anaerobic condition on biodegradation rate was examined in the CSTR. The performance of the CSTR was assessed in terms of biodegradation rates of octanoic acid and trans-4MCHCA, since nitrate was not the limiting substrate in the reaction The biodegradation profile for trans-4MCHCA were obtained by measuring residual concentration of octanoic acid and trans-4MCHCA and residual concentration of nitrate from the outlet port at three

residence times during steady state condition. Figure 5.13 shows the steady state profiles of biomass and substrate obtained at different dilution rates ranging from 0.01 to 0.65 h^{-1}.

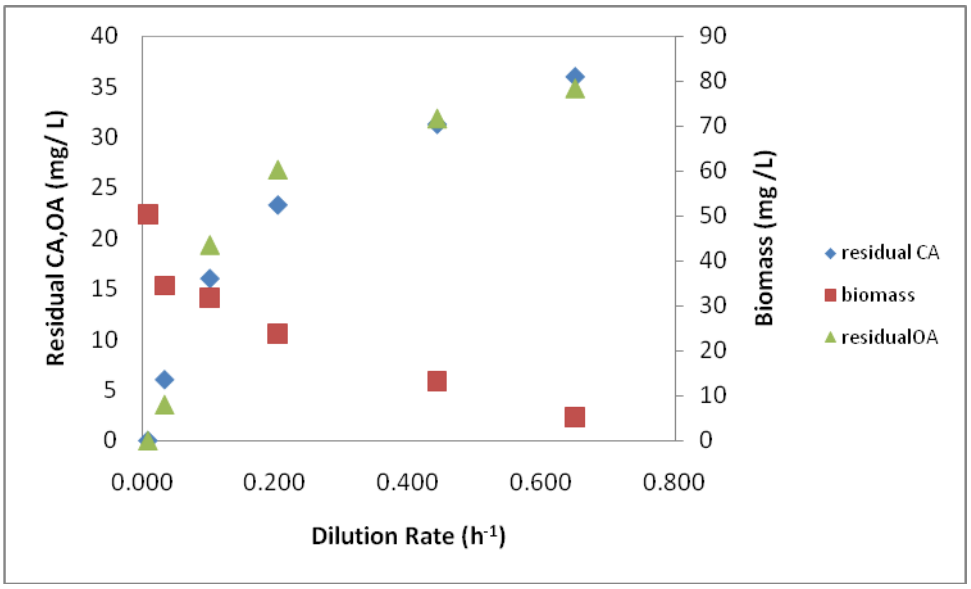


Figure 5.13: Steady-state profiles of Octanoic acid,trans-4MCHCA and biomass concentrations observed in continuously stirred tank reactor (CSTR) as a function of dilution rate at 100 mg/L octanoic acid,50 mg/L trans-4MCHCA and 10 mM (620 mg/L) nitrate.

As dilution rate was increased, there was a decrease in biomass concentration (which was shown by decrease in turbidity of the solution in bioreactor and eventually the liquid became clear), led to an increase in residual concentration of octanoic acid, trans-4MCHCA and nitrate, because there was less micro-organisms available in CSTR to utilize octanoic acid, trans-4MCHCA and nitrate .The wash out in form of decrease in concentration of biomass was observed.

The rate of removal of substrate and nitrate was obtained as function of loading rate in the CSTR operated at initial concentration of 100 mg/L octanoic acid, 50 mg/L trans-4MCHCA and 10 mM (620 mg/L) nitrate as shown in Figure 5.14 to 5.16

The maximum removal rate of octanoic acid was observed in the range of 10-12 mg/L·h at loading rate ranging from 25 to 45 mg/L.h and the percentage removal was in the range of 70-80%. In the case of trans-4MCHCA maximum removal rate was observed in the range of 6-7 mg/L·h at loading rate ranging from 20 to 22 mg/L.h and the percentage removal was in the range of 85-90%.

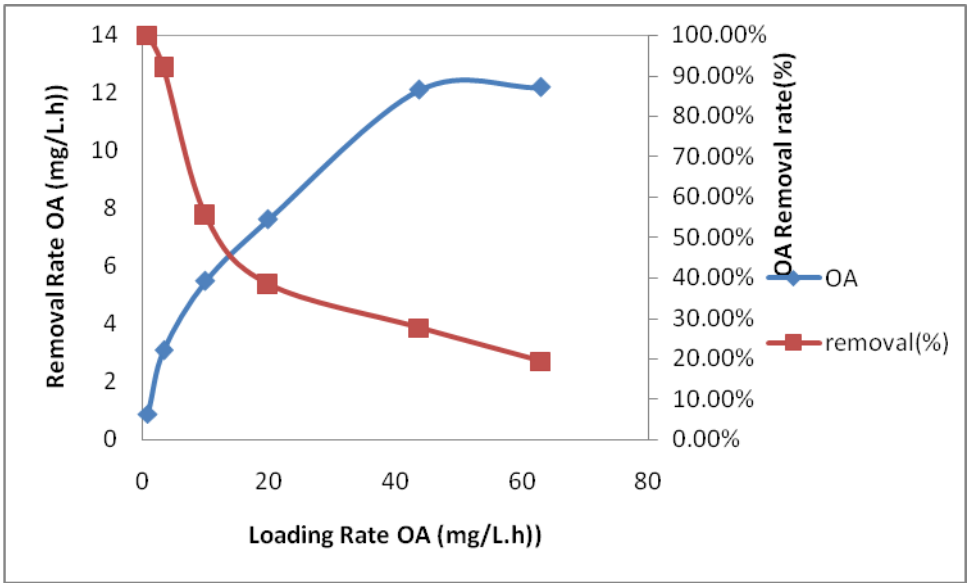


Figure 5.14 Removal rate OA as function of loading rate OA in CSTR operated at an initial substrate of 100 mg/L Octanoic acid,50mg/L trans-4MCHCA and 10 mM (620 mg/L) nitrate at 23±1.5 ˚C.

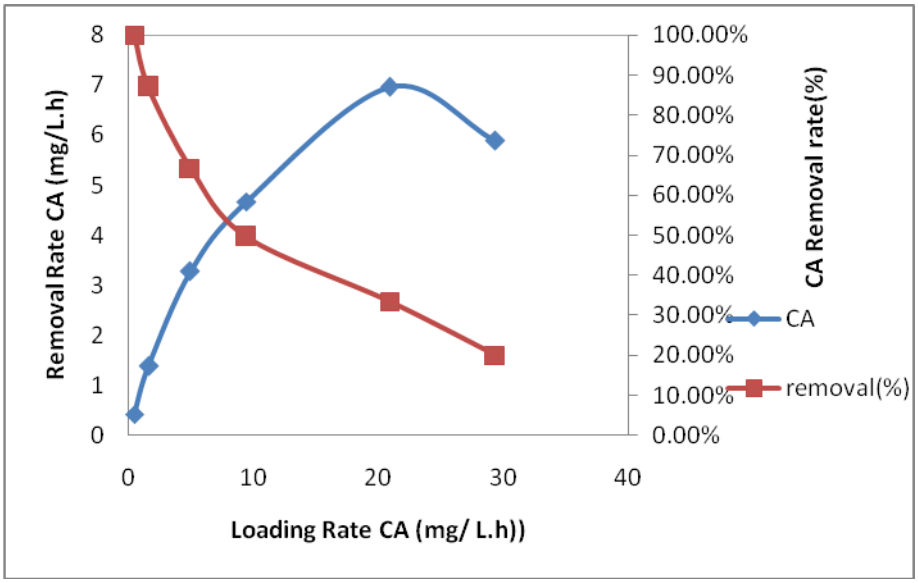


Figure 5.15 Removal rate CA as function of loading rate CA in CSTR operated at an initial substrate of 100 mg/L Octanoic acid,50mg/L trans-4MCHCA and 10 mM (620 mg/L) nitrate at 23±1.5 ˚C.

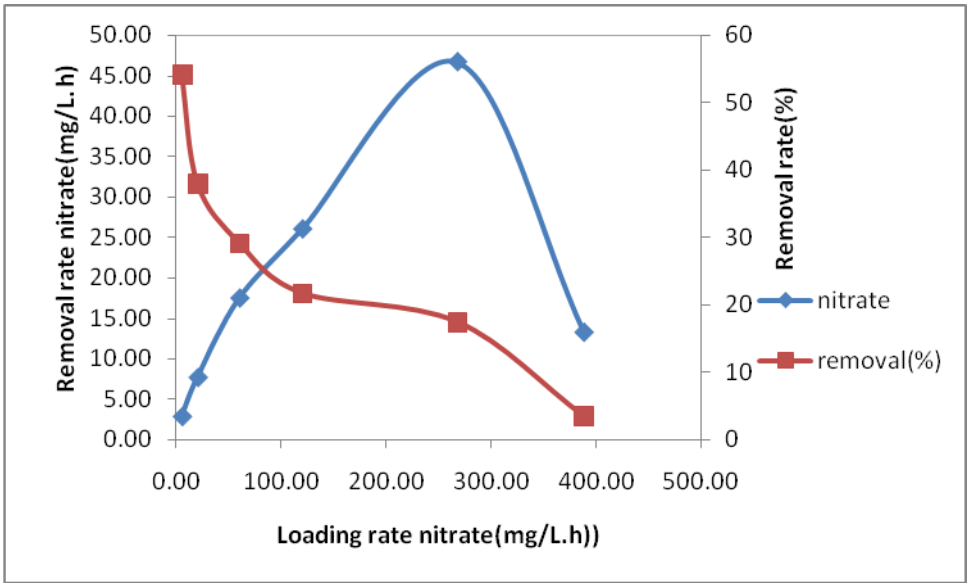


Figure 5.16 Removal rate of nitrate as function of loading rate nitrate in CSTR operated at an initial substrate of 100 mg/L Octanoic acid,50mg/L trans-4MCHCA and 10 mM (620 mg/L) nitrate at 23±1.5 ˚C.

The maximum removal rate of nitrate was 46.7 mg/L.h observed at loading rate of 267.81 mg/L.h with a percentage removal of 17.4%. The removal percentages for both substrate and nitrate decreased, when loading rates increased slightly from the critical value. These maximum removal rates were achieved at the condition prior to wash out. Washout condition was likely caused by short residence time as the loading rate was increase, and hampered the microorganism's growth, thus affecting their biological activity.

Chapter 6
CONCLUSIONS

The naphthenic acid mixtures present in oil sand tailing ponds are very complex; the present work was focused primarily at studying the batch and continuous biodegradation of different combinations of model NAs to get a better understanding of the engineering aspects specially the biokinetics of biodegradation in mixtures made up of different combinations of linear and cyclic NAs.

Earlier works in involved the study of individual model naphthenic acids biodegradation and the results obtained in those works have been used in the present study as a basis for comparison. It was observed that in a mixture biodegradation of octanoic acid (linear compound) proceeded at a rate faster than the other cyclic model compounds (trans-4MCHCA) used in this study. In a mixture octanoic acid (linear compound) degradation was not influenced by the presence of the other cyclic compounds, even the most recalcitrant one (4MCHAA) with the removal rate in the mixture being close to that of octanoic acid as the sole substrate.

It appears that when both linear and cyclic NAs with comparable biodegradability are present in the mixture, microbial culture preferentially uses the linear NA. The findings of the current work for the mixture of NAs also further reinstate past findings obtained with the individual model NAs that the biodegradability of the naphthenic acids is influenced by linearity vs cyclicity, carbon number (presence of additional methyl groups), as well as the spatial arrangement of the alkyl side branch. The biodegradation results thus obtained in the present work would certainly help in further studies aiming at bioremediation of naphthenic acids in oil sand process water.

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APPENDIX

A. Biomass Calibration

The concentration of biomass was determined by direct measurement of theoptical density (OD) of the samples taken from the flasks at a wavelength of 620 nm (Shuler and Kargi, 1992). 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.

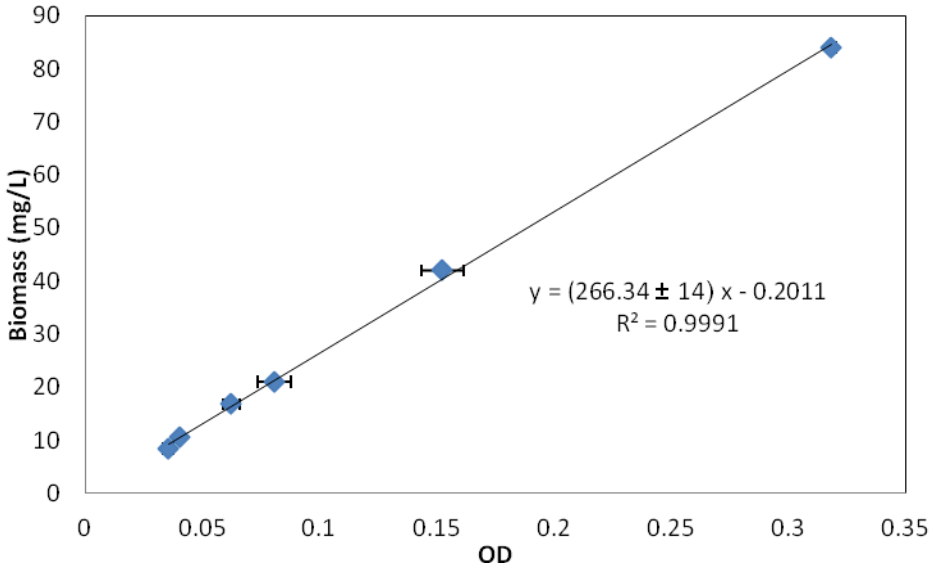

Figure A.1 Biomass calibration. Error bars represent standard deviation in optical density readings

B. Calibration Curves for Octanoic Acid

Utilization of GC-FID for direct analysis of model NAs in water and biological media requires a liner calibration curve to convert GC reading (uv.min) into actual concentration (mg/L). During the research standard solutions were prepared for octanoic acid. The generated calibration curves were updated regularly to ensure the accuracy of experimental results. The representative calibration curves for the model NA are presented through Figure B.1

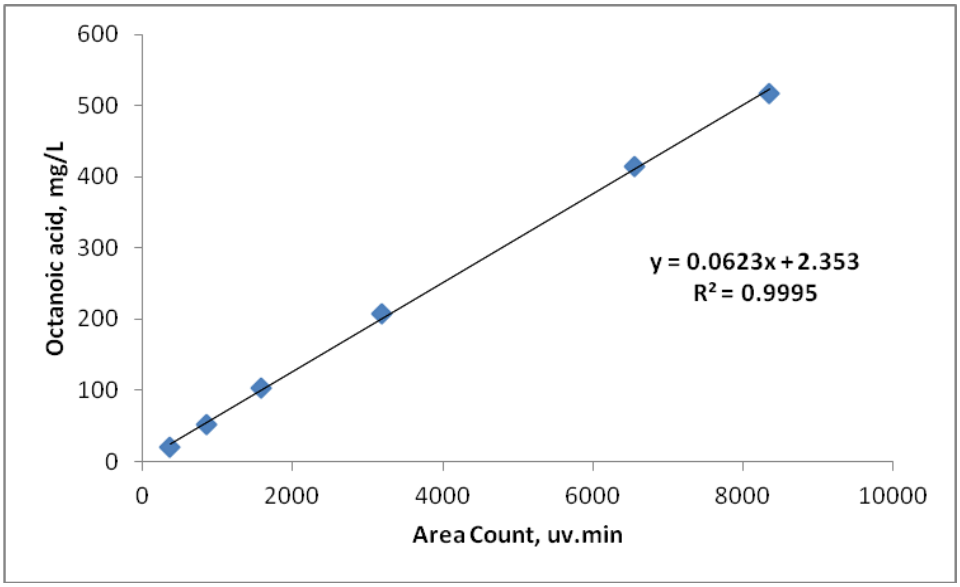

Figure B.1 The representative calibration curves for octanoic acid concentration measurement. Error bars represent standard deviation in GC readings and may not visible as the associated error is small.

C. Calibration Curve for *trans*-4MCHCA

The calibration curve for the measurement of trans-4MCHCA is shown in the Figure C.1.

The equation of the best fit line was the following:

$$C_{biomass} = 0.0347 \times \text{Reading} - 1.2032 \quad (R^2=0.997)$$

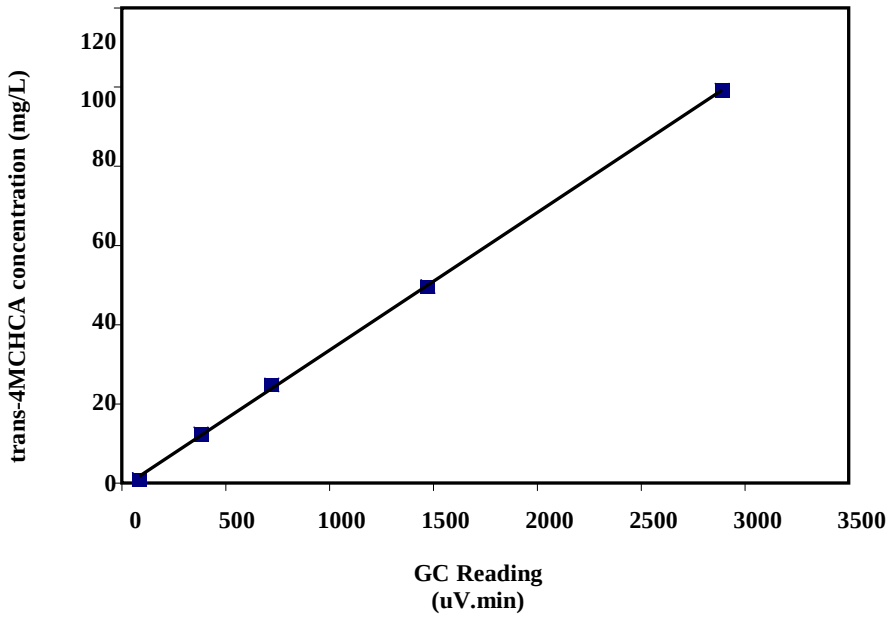

Figure C.1: The representative calibration curve for trans-4MCHCA concentration measurement. Error bars represent standard deviation in GC readings and may not visible as the associated error is small.