FATE AND EFFECT OF NAPHTHENIC ACIDS

IN BIOLOGICAL SYSTEMS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

Naphthenic acids are a group of acyclic, monocyclic and polycyclic carboxylic acids found in crude oil, bitumen and other petroleum and commercial products. NAs are one of the most toxic components of refinery wastewater and oil sands tailings ponds. Numerous studies have found NAs to be acutely toxic to various types of bacteria, yeast, earthworms, fish, mammals, and both aquatic and terrestrial plants. Studies have indicated that biotransformation of NAs under aerobic conditions is possible; however, the more complex NAs with higher cyclization, branching and molecular weight are recalcitrant.

The objectives of the research presented here were to: a) assess the occurrence and fate of NAs in crude oil and refinery wastewater streams; b) evaluate the biotransformation potential and inhibitory effects of NAs under nitrifying, denitrifying and methanogenic/fermentative conditions; c) investigate the factors affecting NA biotransformation under aerobic conditions and the microbes involved; and d) assess the toxicity of individual model NAs using quantitative structure-activity relationships (QSAR) and examine the effect of structure on NA biotransformation potential. NAs are ubiquitous in refinery wastewater streams and the desalter brine was found to be the main source of NAs in refinery wastewater. The biotransformation potential of NAs under nitrifying, nitrate-reducing and methanogenic/fermentative conditions was evaluated using bioenergetics and batch bioassays. A commercial NA mixture was not biodegraded under nitrate-reducing or methanogenic/fermentative conditions; however, NAs were degraded by a mixed aerobic nitrifying culture containing both autotrophic and

heterotrophic bacteria. NAs were degraded under aerobic conditions by an NA-enriched culture; however, a residual fraction was not degraded under all conditions studied. The results indicated that NAs are not inherently recalcitrant and the residual fraction was due to the individual NA concentrations being below the minimum substrate concentrations at which they are no longer degraded. A fraction of the NA mixture was completely mineralized to carbon dioxide, with the remaining portion biotransformed to more oxidized intermediates. The bacterial community involved in the aerobic NA biodegradation was mainly composed of species belonging to the *γ-proteobacteria* class, most closely related to the *Pseudomonas* genus. Overall, the results indicated that NAs were degraded under aerobic conditions; however, biological treatment of NA-bearing wastewater will not completely remove NAs and thus, biological treatment must be combined with physical/chemical treatment to achieve complete NA removal.

This research is the first comprehensive study of NAs from the source (crude oil) to the refinery effluent and contributes to a better understanding of the environmental risks associated with NAs by providing systematic information on physical, chemical and biological processes that determine the fate and effect of NAs in engineered and natural systems. Results of this study can be used to devise effective treatment systems and proper disposal of NA-bearing wastes as well as to provide an acceptable, science-based criterion for environmental regulations. Reducing NA contamination in the environment through improved wastewater treatment and disposal will directly benefit both human health and the environment

CHAPTER 1

INTRODUCTION

1.1 Preface

Naphthenic acids (NAs) are a complex group of carboxylic acids found in crude oil and petroleum products. They are prevalent in refinery process wastewater, which is the main source of NA contamination in the environment. NAs are among the most toxic components of refinery process waters, acutely toxic to both aquatic and terrestrial species including, bacteria, algae, earthworms, rats, fish and plants. Multiple studies have shown that NAs are degradable under aerobic conditions; however, the more complex NAs with higher cyclization, branching and molecular weight are recalcitrant and research is limited as to what chemical, physical and biological factors affect NA biodegradation in aerobic environments. NAs are hydrophobic and partition/adsorb to artificial and organic matter, which in many cases are found in anoxic environments; however, in contrast to studies on the aerobic biodegradation of NAs, very limited information is available on the fate and effect of NAs under anoxic and anaerobic conditions.

NAs consist of many structures with different carbon number, cyclization and branching and are typically found in complex wastewater mixtures. Measurement of the NAs in these complex samples presents many analytical challenges. There is still a need for the development of analytical methods capable of accurately quantifying and identifying NAs without interference from hydrocarbons typically encountered along with the NAs.

NA contamination in the environment creates ecological risks and the lack of available toxicity and biodegradability information makes it difficult to assess and reduce these risks. This research was a study of the fate, toxicity and biodegradation potential of NAs under conditions encountered in both engineered and natural systems. The results of this study provide currently missing information that can be used to improve treatment methods for NA-bearing waste streams as well as provide science-based criteria for the development of environmental regulations.

1.2 Research Objectives

The goal of this research was to investigate the fate, toxicity and biotransformation potential of NAs in biological systems. The specific objectives of this research were to:

1. Assess the occurrence and fate of NAs in crude oil and refinery wastewater streams
2. Evaluate the biotransformation potential and inhibitory effects of NAs under nitrifying, denitrifying and methanogenic/fermentative conditions
3. Determine the factors affecting NA biotransformation under aerobic conditions and the microbes involved
4. Assess the toxicity of individual model NAs using quantitative structure-activity relationships (QSAR) and examine the effect of structure on NA biotransformation potential.

1.3 Significance and Motivation

The concentration and distribution of NAs in refinery process waters is directly correlated to the type and source of crude oil. In order to satisfy the increasing demand for fossil fuels, oil exploration has expanded to extracting petroleum from oil sands in Canada, Venezuela, Mexico and other countries. As the technology improves and increases the capability of extracting and refining the extra heavy crudes and bitumen, the accumulation of toxic NA-bearing wastewaters will continue to increase. In the United States, there is currently no regulated limit for NAs in refinery wastewater discharges; therefore, any NAs that are not degraded or removed in the refinery wastewater treatment systems are being released to the surrounding environments. Studies have shown that NAs have been detected in natural ecosystems such as rivers, wells and groundwater (Clemente and Fedorak, 2005; Holowenko et al., 2002; Quagraine et al., 2005a; Schramm et al., 2000; Scott et al., 2008). Studies have also shown that many of the NAs found in refinery process waters are highly recalcitrant and even after long incubation periods, the high molecular weight NAs persist in the environment (Quagraine et al., 2005b). NAs are emerging contaminants and their fate and effect in both natural and engineered systems is relatively unexplored. Discharge of refinery process waters could potentially lead to an accumulation of NAs, whose biotransformation potential and toxicity is largely unknown.

The results of this study could be used to identify recalcitrant and toxic groups of NAs, to devise effective treatment systems and proper disposal of NA-bearing wastes as well as to provide an acceptable, science-based criterion in environmental regulations.

Reducing NA contamination in the environment through improved wastewater treatment and disposal would directly benefit both human health and the environment.

CHAPTER 2

BACKGROUND

2.1 Source of NAs

Naphthenic acids (NAs) are carboxylic acids found in crude oil, bitumen, refinery wastewater and other petroleum products. NAs are formed from the biooxidation of hydrocarbons in crude oil and thus, the NA content increases with the age of the oil formations (Behar and Albrecht, 1984; Clemente and Fedorak, 2005; Meredith et al., 2000). NAs are found in higher concentrations in bitumen and heavy crudes, and transferred to the extraction water and desalter brine, which is the major source of NAs in refinery wastewater.

The NA content of crude oils can range from 0.1 to 4% (w/w), depending on the source and type of crude. During crude oil and bitumen extraction and desalting (Figure 2.1), NAs become solubilized and concentrated in the process water, which may contain up to 120 mg NA/L (Clemente et al., 2003; Holowenko et al., 2001; Quagraine et al., 2005; Richardson, 2010; Whitby, 2011). Large amounts of water are contaminated with NAs and other petroleum hydrocarbons during bitumen extraction, which uses approximately 2 to 4 barrels of water for every barrel of bitumen extracted (Government of Alberta, 2009). During the extraction process, bitumen, sand, salts, rocks and sediment from the bitumen mine are mixed with hot water, steam or caustic hot water to create a slurry in order to separate bitumen from the acids, salts and solids. The separated bitumen is sent to a recovery unit and then through the refinery where the product is purified and upgraded to different petroleum products. A portion of the used water is recycled and the

remaining extraction water, containing unsettled solids, unrecovered bitumen, NAs and other hydrocarbons, is sent to the refinery wastewater treatment plant and oil sands tailings ponds for treatment, storage and settling until the contaminant and solids levels have decreased to concentrations that are acceptable for discharge (Masliyah et al., 2004). There are currently greater than 900 million cubic meters of contaminated oil sands tailings stored in the Athabasca oil sands region of Canada that cannot be discharged due to Canada's zero discharge policy, which prohibits the release of oil sands process water (OSPW) (Del Rio et al., 2006; Headley and McMartin, 2004).

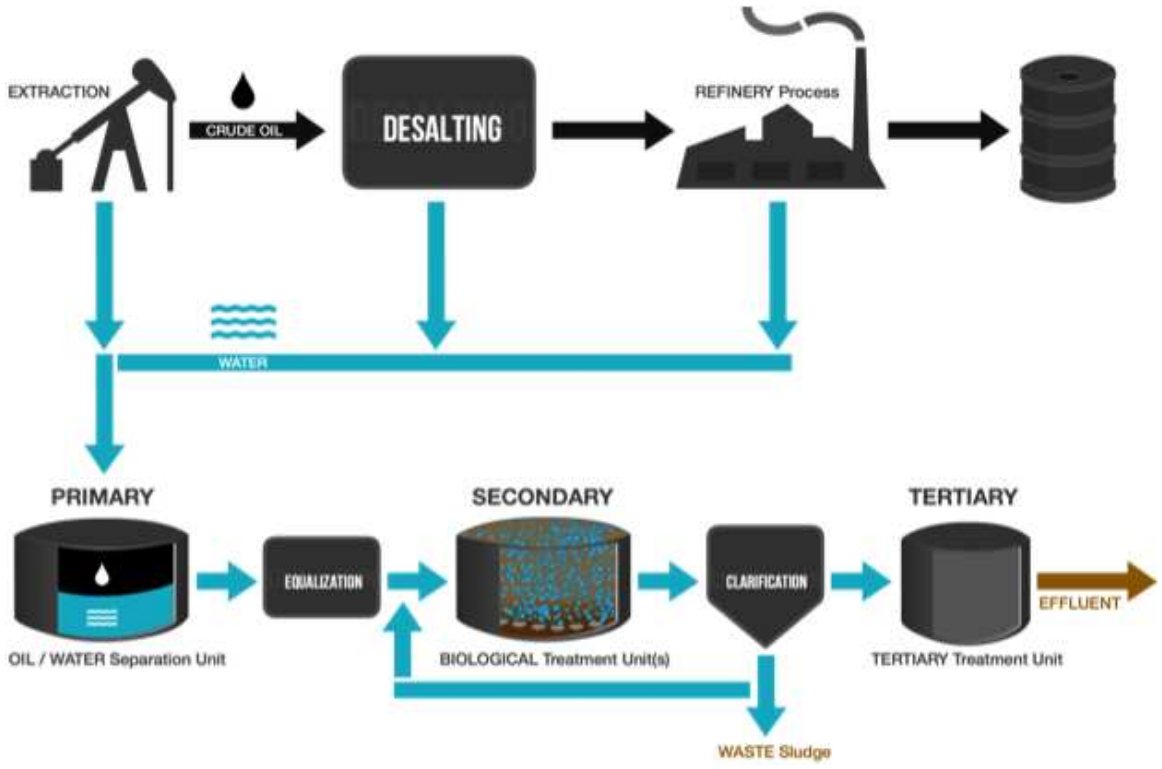


Figure 2.1. Refinery crude desalting and wastewater treatment.

Conventional, lighter crude oils are easier to extract and use less water than bitumen; however, they often require desalting, which also uses large quantities of water

that becomes contaminated with NAs and other petroleum hydrocarbons. The desalting process is used to remove inorganic salts from the crude oil and bitumen, which can range from 4.5 to 113.4 kg per thousand barrels, in order to reduce plugging, fouling and corrosion of the refinery equipment. The desalting process is typically done at neutral pH (6 to 7), at which NAs are deprotonated and less hydrophobic and easily partition to the desalter brine, resulting in large quantities of NA-contaminated wastewater (IPIECA, 2010).

The concentration and distribution of NAs is higher and more complex in extra heavy crudes and bitumen than in conventional crude oils (Behar and Albrecht, 1984; Meredith et al., 2000). Based on the current proven world oil reserves, only 30% of the known crude oil is conventional crude, with the remaining 70% consisting of heavy crude, extra heavy crude and bitumen (Figure 2.2) (Alboudwarej et al., 2006). Recently, oil exploration has led to extraction of extra heavy crudes and bitumen from oil sands in Canada, Venezuela, Mexico and other countries. It is estimated that 97% of Canada's oil reserves, are unconventional crude oil, mainly bitumen (US EIA, 2011a). Similarly, Venezuela has the second largest proven oil reserves in the world (Figure 2.3), the majority of which is extra heavy crude oil and bitumen (US EIA, 2011b). In 2011, the United States imported 45% of its crude oil (Figure 2.4), with 13.05%, 4.95% and 3.60% imported from Canada, Venezuela and Mexico, respectively (US EIA, 2012). Such crudes have elevated NA concentrations and result in refinery process waters and wastewaters with relatively high NA levels. Refinery wastewaters vary in NA concentration depending on the crude oil and treatment process (IPEICA, 2010; Whitby, 2010).

Crude oil and refinery process waters are the main source of NAs in the environment; however NAs are also released from other sources. One study indicated that coal is also a source of NAs and it is possible for NAs to leach from the coal into the groundwater (Scott et al., 2009). NAs are also ingredients in many consumer and industrial products such as paint and ink driers, fuel additives, wood preservatives, catalysts, emulsifiers, corrosion inhibitors and surfactants. They are classified by the US EPA as high production volume chemicals (HPVs) and information relative to their biodegradation potential and toxicity is becoming increasingly more important (USEPA, 2006).

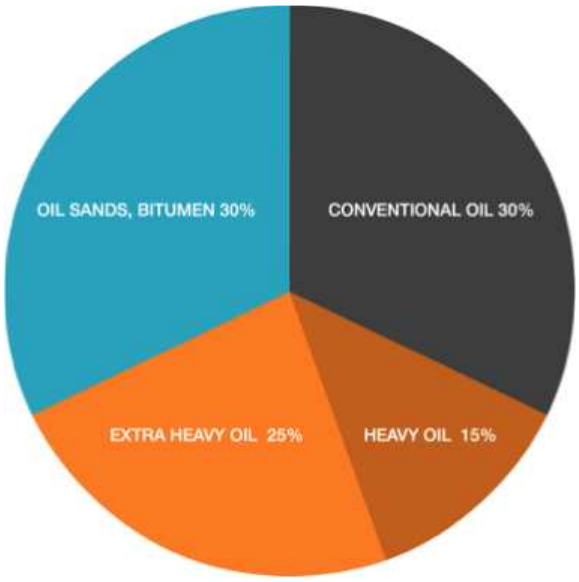


Figure 2.2. Crude oil and bitumen distribution of proven world oil reserves (Alboudwarej et al., 2006).

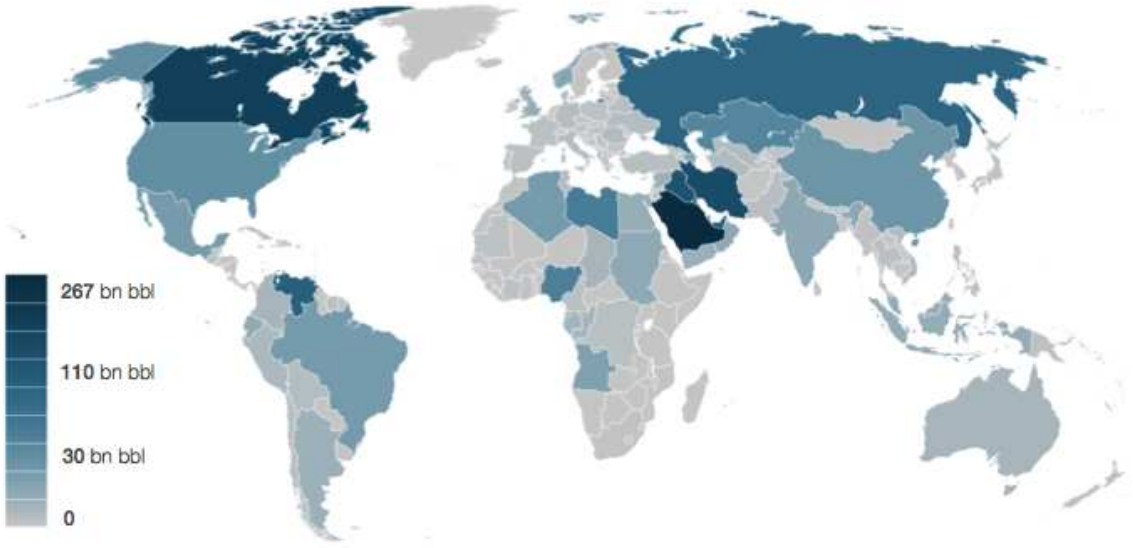


Figure 2.3. Proven world crude oil reserves in 2012 (EIA, 2012; Wikipedia, 2012).

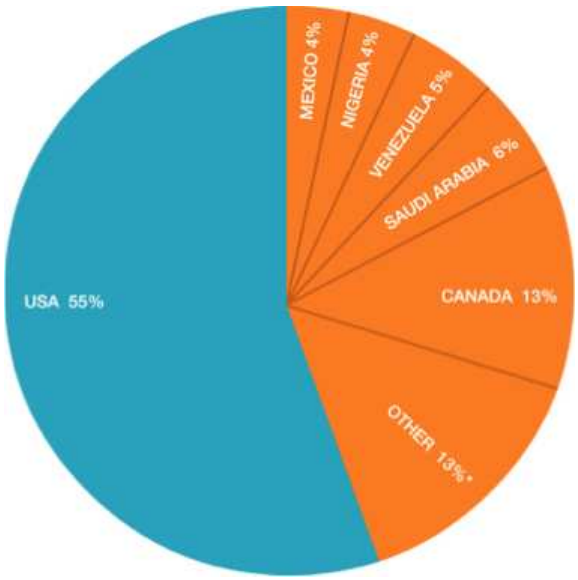


Figure 2.4. US crude oil and imports (*other includes Iraq, Iran, Kuwait, Colombia, Brazil and other OPEC and non-OPEC countries)(US EIA, 2012).

2.2 NAs in the Environment

NAs are released into the environment through refinery wastewater streams, oil sand tailings ponds, oil spills and use and disposal of NA-containing commercial products; however, the occurrence of NAs and their fate in both refinery wastewater streams and the environment are not well studied. NAs have been measured in natural aquatic environments, aquifers and oil sands tailings ponds. NAs concentrations in oil sands tailings ponds are typically between 40 and 120 mg NA/L and concentrations as high as 24 mg NA/L were measured in oil sands storage water runoff (Holowenko et al., 2002; Quagraine et al., 2005). Rainwater and oil sands tailings runoff flows into the Athabasca River, which runs through the Athabasca oil sands mining region in Alberta, Canada. NA concentrations have been reported in the Athabasca River at concentrations of 0.1 to 0.9 mg NA/L (Schramm et al., 2000). NA concentrations from 0.4 to 51 mg NA/L were measured in groundwater samples taken near the oil sands tailing ponds (Clemente and Fedorak, 2005; Scott et al., 2008).

2.3 Chemical and Physical Properties

NAs are a complex group of saturated alkyl-substituted acyclic, monocyclic and polycyclic carboxylic acids (Clemente and Fedorak, 2005; Headley and McMartin, 2004; Lo et al., 2006; Wong et al., 1996). They are monobasic, anionic surfactants with the general formula $C_nH_{2n-Z}O_2$, where n indicates the carbon number and Z indicates the number of hydrogen atoms lost due to ring formation. The hydrogen deficiency, Z, is zero or a negative, even integer such that $Z = 0$ corresponds to no rings; $Z = -2$ corresponds to one ring; $Z = -4$ corresponds to two rings, etc. The pK_a of NAs is similar

to other carboxylic acids in the range of 5 to 6 and the molecular weight (MW) typically varies between 140 and 500 amu (Table 2.1). The aqueous solubility of NAs is approximately 50 mg/L and increases with pH (Headley and McMartin, 2004). Figure 2.5 shows typical NA structures, where *i* is an integer zero or greater and R indicates aliphatic branching from the ring. Some possible combinations for NA structures with varying *Z* numbers, *n* numbers and MW are summarized in Table 2.2.

Table 2.1. Chemical and physical properties of NAs (Headley and McMartin, 2004; Whitby, 2010).

Parameter	General value
Carbon number	5 to 30
Z number	0 to -12
Molecular weight	140 to 450 amu
Solubility	< 50 mg/L at pH 7
pK_a	5 to 6
log K_{ow}	4 at pH 1, 2.5 at pH 7, 2 at pH 10
Boiling point	250 - 350°C

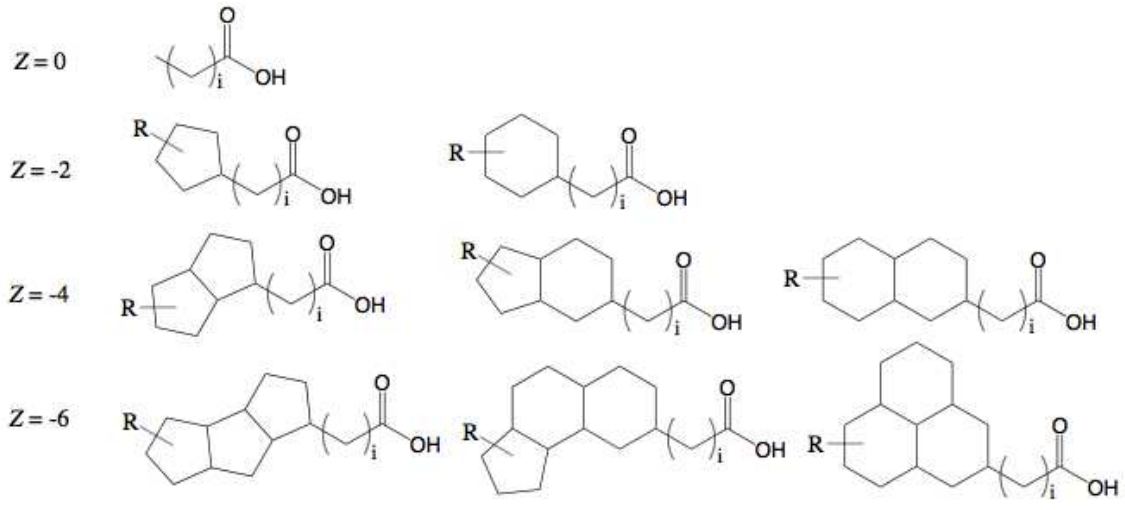


Figure 2.5. General structure of acyclic and cyclic NAs.

Table 2.2. Molecular weight distribution of NAs.

Carbon number	***Z* number**						
n	**0**	**-2**	**-4**	**-6**	**-8**	**-10**	**-12**
5	102						
6	116	114					
7	130	128					
8	144	142					
9	158	156	154				
10	172	170	168				
11	186	184	182				
12	200	198	196	194			
13	214	212	210	208			
14	228	226	224	222			
15	242	240	238	236	234		
16	256	254	252	250	248		
17	270	268	266	264	262		
18	284	282	280	278	276	274	
19	298	296	294	292	290	288	
20	312	310	308	306	304	302	
21	326	324	322	320	318	316	314
22	340	338	336	334	332	330	328
23	354	352	350	348	346	344	342
24	368	366	364	362	360	358	356
25	382	380	378	376	374	372	370
26	396	394	392	390	388	386	384
27	410	408	406	404	402	400	398
28	424	422	420	418	416	414	412
29	438	436	434	432	430	428	426
30	452	450	448	446	444	442	440

2.4 NA Toxicity

NAs are among the most toxic components of refinery wastewater and oil sands tailings ponds. Numerous studies have found NAs to be acutely toxic to various types of bacteria, yeast, earthworms, fish, mammals, and both aquatic and terrestrial plants (Armstrong et al., 2008; Clemente et al., 2003; Frank et al., 2009; Frank et al., 2008; Holowenko et al., 2002; Nero et al., 2006; Peters et al., 2007; Rogers et al., 2002; Sarathy et al., 2002; Scott et al., 2008b; Thomas et al., 2009). Studies have reported NA toxicity using the standard Microtox® assay and found that both commercial NAs and NAs extracted from refinery process waters were toxic to *Vibrio fischeri* and *Daphnia magna* (Frank et al., 2009; Frank et al., 2008; Holowenko et al., 2002; Scott et al., 2008a). The toxicity of both NA mixtures and surrogate NAs were found to have a wide range of EC_{50} values depending on the source and distribution of NA structures. Multiple studies have measured the Microtox® acute toxicity of model NAs and the EC_{50} values ranged from 0.004 to 19.1 mM (Frank et al., 2009; Jones et al., 2011).

There is limited information about the toxicity of NAs to mammals. When rats were exposed to NAs extracted from refinery process waters, the results suggested that the liver is the primary target organ for NA toxicity; however, the brain and kidneys appeared to be affected as well. The results indicated that acute toxicity was unlikely at measured environmental concentrations; however, extended or repeated exposure to NAs would result in chronic toxicity to wild mammals (Rogers et al., 2002).

Similarly, there is very little information available about the effect of NAs on terrestrial and aquatic plants; however, some studies have indicated inhibitory and toxic effects such as increased water uptake, decreased water channel activity, gas exchange

and leaf growth were observed compared to controls (Armstrong et al., 2008; Kamaluddin and Zwiazek, 2002).

NA toxicity has been studied more extensively in fish and aquatic species. Both commercial NA mixtures and NA-bearing refinery settling basin water were found to cause deformities in fish embryos and mortality to adult fish (Peters et al., 2007). Another study showed that exposure of yellow perch to 1 to 2 mg/L of commercial and extracted NAs resulted in high levels of gill proliferative changes and reduced gill surface area; when exposed to 4 to 7 mg NA/L,100% mortality was observed (Nero et al., 2006). Female fathead minnows exposed to 10 mg NA/L for 21 days laid fewer eggs and male minnows had lower concentrations of both testosterone and 11-ketotestosterone (Kavanagh et al., 2012). Dorn (1998) reported that NAs in excess of 2.5 to 5 mg/L in oil refinery effluent may result in effluent toxicity to fish and recommended that the total effluent oil and grease be kept below 10 mg/L. A decrease in effluent pH from 7.8 to 6.6 resulted in a drop of the stickleback (*Gasterosteus aculeatus*) survival rate from 100 to 0%, indicating that acid-base equilibria of the effluent constituents (e.g., NAs) have a profound effect on toxicity (Dorn, 1998). Embryos of amphibian larvae exposed to NA concentrations of 2 to 4 mg/L showed 32 and 25% reductions in growth and development after hatching, respectively, and suffered 100% mortality when exposed to 6 mg NA/L (Melvin and Trudeau, 2012). Hagen et al. (2012) studied the effects of NAs on the immune systems of goldfish and found that exposure to NAs decreased the ability of the fish to fight infection and resulted in drastic increases in mortality. Other studies have indicated that NAs are endocrine disruptors and alter the reproductive development of

fish by interfering with the estrogen and androgen receptors and blocking hormone production (Rowland et al., 2011c; Thomas et al., 2009).

The mode of NA toxicity, especially in higher organisms, is not well studied; however, a recent study by Zhang et al. (2011) investigated which biological pathways conserved in both bacteria and vertebrates are affected by a range of NA concentrations up to 1000 mg/L. The results indicated that the transcriptional activity of two proteins involved in the pentose phosphate pathway, which generates nicotinamide-adenine dinucleotide phosphate (NADPH) and 5-carbon sugars used in biosynthesis reactions, were up-regulated more than 2-fold. On the other hand, NAs resulted in down-regulation of some membrane transporter proteins involved in the ATP–binding cassette (ABC) transporter complex involved in the transport of substrates across the cell membrane. Stress responsive pathways involved in resistance to antibiotics, oxidative stress, organic solvents and heavy metals were altered; however, the results indicated DNA damage was not a direct result of NAs (Zhang et al., 2011).

Biological and physical treatment methods (i.e., ozonation) reduce the toxicity of refinery wastewater; however, it is very difficult to estimate NA toxicity independently in complex wastewater and oil sands tailing water streams (Anderson et al., 2010; Martin et al., 2010; Scott et al., 2008). There are hundreds of types of NAs and it is not currently established which NAs are the most toxic. It has been shown in some NA mixtures that toxicity decreased with decreasing NA concentration; however, it is not solely a function of concentration. The toxicity also depends on the structure and hydrophobicity of the NAs. Frank et al. (2009) found that toxicity increased in NAs with higher molecular

weight and lower carboxylic acid content. Overall, the degree of toxicity depends on the type, concentration and composition of NAs (Headley and McMartin, 2004).

2.5 NA Biotransformation

There is limited information on the biotransformation potential of NAs and the biological, chemical and physical factors that might influence their fate in refinery process waters and natural systems. Refinery wastewater treatment plants typically utilize aerobic suspended or attached growth biological treatment processes; however, NAs are also found in anoxic/anaerobic environments, such as the depths of oil sands tailings ponds and sediments.

2.5.1 Aerobic Biotransformation

Model NAs, commercial NA mixtures and NA-bearing refinery process water samples have been degraded aerobically using inocula obtained from refinery wastewater and oil sands tailings; however, commercial NAs were more readily degradable than the NAs in refinery process waters (Clemente and Fedorak, 2005; Del Rio et al., 2006; Scott et al., 2005). Greater than 90% of two commercial NA mixtures degraded aerobically in a culture developed from refinery process water, while process water extracted NAs typically degraded at a lower rate and to a lesser extent (Clemente et al., 2004; Del Rio et al., 2006; Scott et al., 2005).

Analytical limitations have made it difficult to identify specific structures in complex NA samples; however, commercial surrogate NAs have been used to investigate the effect of structure on NA biodegradation. Under aerobic conditions, the rate and

extent of biodegradation was lower for NAs with higher cyclization (i.e., high *Z* value) and alkyl branching (Han et al., 2008). Similarly, cultures developed from the organisms native to the rhizospheres of plants found in Alberta, Canada preferentially degraded the lower molecular weight NAs in a commercial mixture (Biryukova et al., 2007).
A biodegradation study using synthesized NAs with varying alkyl branching revealed that both the rate and extent of NA biotransformation decreased as the side chain branching increased (Smith et al., 2008). Similarly, carboxylated cycloalkanes exhibited recalcitrance to oil sands tailings microorganisms when methyl groups were added to the rings (Herman et al., 1993). Another study using two surrogate NAs demonstrated a decrease in the rate and extent of biodegradation in the bicyclic NA compared to the acyclic NA (Lai et al., 1996). In general, studies investigating the effect of NA structure on biodegradability have indicated that the more complex NAs, i.e., those with higher cyclization, branching and molecular weight, are the most recalcitrant (Biryukova et al., 2007; Clemente et al., 2004; Han et al., 2008; Herman et al., 1993; Holowenko et al., 2002; Scott et al., 2005; Smith et al., 2008; Watson et al., 2002).

NA degradation is likely to be affected by operational conditions such as temperature, pH and reactor type. Headley et. al. (2002) observed a 10-fold increase in the aerobic biodegradation rate for an increase in temperature from 10 to 30°C. An extended study investigated the biodegradation of one model NA, *trans*-4-methyl-1-cyclohexane carboxylic acid, using a batch reactor and two types of continuous flow systems. The batch study indicated that aerobic biodegradation of the selected NA was possible at temperatures as low as 4°C; however, the biodegradation rate increased significantly with increasing temperature and pH (Paslawski et al., 2009a). The

continuous study found that the biodegradation rates were enhanced when an immobilized cell reactor was used, compared to the traditional continuous flow stirred tank reactor (CSTR) (Paslawski et al., 2009b).

The NA aerobic degradation pathway has been investigated in multiple studies; however, it has not yet been described in detail. Han et al. (2008) concluded that both commercial NAs and those in oil sands process water were more likely degraded via β-oxidation, while α-oxidation and aromatization may also have contributed to some degree (Han et al., 2008). In another study, two different bacterial communities isolated from marine sediments under aerobic and denitrifying conditions were able to completely mineralize (E)-phytol (3,7,11,15-tetramethylhexadec-2(E)-en-1-1ol) by alternating β-decarboxymethylation and β-oxidation (Rontani et al., 1999). Johnson et al. (2010) provided a proposed degradation pathway of four recalcitrant alkyl branched aromatic alkanoic acids that differed in alkyl branching. The study concluded that the extent of biotransformation was reduced as the alkyl branching increased; however, all acids were transformed to their less toxic butylphenylethanoic acid (BPEA) counterparts by β-oxidation of the carboxyl side chain (Johnson et al., 2010). More recently, Johnson et al. (2012) isolated a *Mycobacterium aurum* from sediments, which biodegraded two synthetic NAs, 4'- *n*-butylpheny)-4-butanoic acid and 4'-*t*- butylphenyl-4-butanoic acid; detected metabolites indicated that ω-oxidation and combined ω- and β-oxidation were the primary routes of degradation. The majority of studies have concluded that β-oxidation, possibly combined with α–oxidation or ω-oxidation, is the likely aerobic NA degradation pathway (Del Rio et al., 2006; Han et al., 2008; Johnson et al., 2010;

Quagraine et al., 2005a; Rontani et al., 1999; Smith et al., 2008). The proposed β-oxidation pathway for both acyclic and cyclic NAs is shown in Figure 2.6.

The microbes present in oil sands tailing ponds and refinery wastewater systems responsible for NA degradation are not well understood. Golby et al. (2012) identified the major organisms in biofilms cultured from oil sands tailings ponds to be *Pseudomonas, Thauera, Hydrogenophaga, Rhodoferax* and *Acidovorax.* Other studies found that *Pseudomonas, Burkholderia* and *Sphingomonas* were the most abundant organisms in enrichment cultures developed from oil sands tailings and bitumen contaminated sediments on a feed of different model NAs with different structures and alkyl side chain branching (Del Rio et al., 2006; Johnson et al., 2010).

Activated sludge wastewater systems are common biological treatment processes used to treat refinery process waters; however, no reports have been made to whether activated sludge units are capable of reducing the NA concentration and toxicity of the effluent. Although NA degradation of both commercial and refinery samples has been demonstrated in many laboratory studies, the higher molecular weight NAs with increased ring number and alkyl branching have shown to be more recalcitrant. The NAs in refinery systems have been found to resemble recalcitrant NAs identified in biodegradation studies of commercial NA mixtures and are likely to include very complex structures with very high molecular weights, cyclization and branching (Del Rio et al., 2006). Based on degradation studies, these NA structures will not be degraded in the refinery activated sludge units (ASU) and will be discharged into natural systems where their fate and effect is unknown.

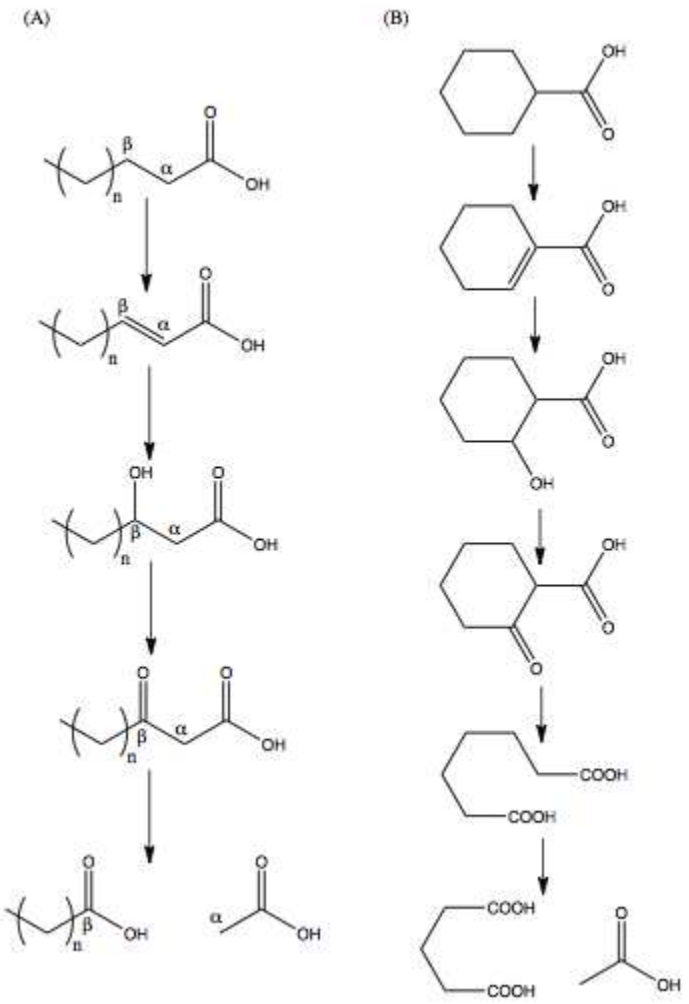


Figure 2.6. Proposed β-oxidation aerobic degradation pathway for acyclic and cyclic NAs (Quagraine et al., 2005a; Whitby, 2010).

2.5.2 Anaerobic/Anoxic Biotransformation

NAs are hydrophobic and partition and adsorb to artificial and organic matter, which in many cases are found in anoxic environments; however, in contrast to studies on the aerobic biodegradation of NAs, very limited information is available on the fate and effect of NAs under anoxic and anaerobic conditions. Methane production in oil sands tailings ponds is significant; however the substrates utilized for methane production are not completely understood (Holowenko et al., 2000; Siddique et al., 2011; Whitby, 2010).

One single study found that NAs from oil sands process waters caused short-term inhibition of methanogenesis from H_2 or acetate and methane yields measured in microcosms amended with two surrogate NAs suggested complete mineralization under anaerobic conditions (Holowenko et al., 2001).

2.6 Ozonation of NAs

Since microbial biodegradation strategies have sometimes shown to be ineffective or slow at treating recalcitrant NAs, multiple studies have investigated the possible chemical and physical treatment options for NA-bearing wastewater streams. Scott et al. (2008) demonstrated that ozonation of NA-containing oil sands process water (59 mg/L NAs) for 50 minutes resulted in a non-toxic effluent based on the Microtox bioassay and decreased the NAs concentration to about 20 mg/L, whereas prolonged ozonation (130 minutes) resulted in an effluent with a NAs concentration of 2 mg/L, not measurable decrease in dissolved organic carbon (DOC) and a 50% decrease in chemical oxygen demand (COD). Ozonation resulted in a decrease in the relative proportion of high molecular weight NAs (carbon number, $n \geq 22$). Perez-Estrada et al (2011) found that commercial NAs and

OSPW NAs with higher cyclization and carbon numbers were ozonated more rapidly than the smaller, less complex NAs. Similarly, Martin et al. (2010) reported that ozonation of NA-bearing OSPW oxidized recalcitrant, high MW NAs, making them more susceptible to microbial biodegradation. The results indicate that ozonation may be complementary to microbial biodegradation and a combination of physico-chemical and biological processes may be necessary to achieve the complete removal of NAs from the petroleum refineries effluents.

2.7 NA Partitioning

There is very little information available about the partitioning behavior of NAs to both natural (i.e., sediments, soil, sand) and artificial (i.e., organoclays, powder and granular activated carbon, resins) media. Janfada et al. (2006) investigated the phase distribution of oil sands NAs to soils taken from an oil sands mining site in Alberta, Canada and found that the NAs rapidly adsorbed and the soils with higher organic content displayed higher levels of NA adsorption. All isotherms were linear and NAs with carbon number between 13 and 17 were preferentially adsorbed. Peng et al. (2002) also reported linear isotherms for two monocyclic model NAs in the concentration range of 0 to 100 mg/L to the same soils. The estimated adsorption coefficients (K_d) were very low and ranged from 0.1 to 0.22 mL/g, indicating that the model NAs did not adsorb strongly to the soils. Similarly to natural adsorptive media, and despite the fact that activated carbon is commonly added to refinery activated sludge or tertiary treatment units, there are no reports of the partitioning behavior of NAs or the effectiveness of artificial media in removing NAs from refinery wastewater streams. Understanding the partitioning

behavior of NAs to media used in refinery wastewater treatment plants is required to ensure proper treatment of NA-bearing wastewater streams and in order to decrease NA contamination in the environment.

2.8 Analysis of NAs

NAs in NA-bearing refinery process wastewaters vary in concentration depending on the crude oil and treatment process. NAs are extremely difficult to analyze because they consist of hundreds of structures with different cyclization, branching and carbon chain lengths (Headley et al., 2009; Richardson, 2010; Whitby, 2010). Additionally, NAs are typically found in complex wastewater samples and oil sands process water, which are complex mixtures containing petroleum hydrocarbons and other contaminants The complex wastewater matrix makes measurement of NAs even more challenging and thus, there is limited research on the occurrence, concentration and composition of NA-bearing refinery wastewaters (Clemente et al., 2003; Richardson, 2010).

Multiple analytical methods have been developed using electrospray ionization (ESI), mass spectrometry (MS) combined with liquid (LC) or gas chromatography (GC), Fourier Transform Infrared Resonance (FTIR) spectroscopy, and Fourier Transform Ion Cyclotron Resonance mass spectrometry (FT-ICR/MS) (Headley et al., 2009; Headley et al., 2007; Hemmingsen et al., 2006; Hsu et al., 2000; Kim et al., 2005; Qian et al., 2001; Teravainen et al., 2007).The most common method used by the oil sands industry for NA quantification is a FTIR method involving acidification of the liquid sample followed by methylene chloride extraction, concentration and measurement of the absorbance by FTIR spectroscopy (Holowenko et al., 2001; Scott et al., 2008a). GC/MS methods have

also been used extensively to analyze NAs in OSPW and typically involve derivatization of the NAs to esters prior to analysis (Headley et al., 2009). Comparison of FTIR and GC/MS methods indicated that the GC/MS method is more selective for NAs and the FTIR method overestimates NA concentrations in OSPW (Scott et al, 2008).

Electrospray ionization FT-ICR/MS is a more recent, high resolution method that has been used to selectively identify NAs and has been used for characterizing heavy petroleum samples and crude oil (Barrow et al., 2004; Headley et al., 2011; Miyabayashi et al., 2009; Qian et al., 2001). Scott et al., (2009) used FT-ICR/MS to measure low concentrations of NAs in groundwater samples at various distances from oil sands mining sites in Alberta, Canada. Recent advancements have utilized FT-ICR/MS to analyze both commercial NAs and NAs in oil sands process waters and found that less than 50% of the total acids in OSPW could be classified as 'classic NAs' (i.e., NAs having 2 oxygen atoms), with a large fraction being more oxygenated and containing 3, 4 and 5 oxygen atoms, referred to as oxy-NAs (Barrow et al., 2010; Han et al., 2009; Grewer et al., 2010).

One of the greatest challenges of characterizing NA-bearing wastewater is to identify individual NA structures in the complex matrix of other petroleum contaminants and hydrocarbons. Several major NA structures were identified in commercial NA mixtures and NAs extracted from crude oils by derivatizing the NAs to amides and analyzing with HPLC/ESI-MS/MS. The NA-amide derivatives were separated using liquid chromatography and individually applied to a tandem mass spectrometry after positive electrospray ionization. The fragmentation pattern of the NA-derivatives was identified using standards and unknown structures were identified by interpretation of the

MS fragmentation patterns. The study indicated that bi- to polycyclic acids containing alkyl and ethanoate side chains were abundant in the oil sands-extracted NAs (Smith and Rowland, 2008). More recently, Rowland et al. (2011a, 2011b, 2011c) used two-dimensional comprehensive GC/GC/MS to identify individual NAs in a commercial NA mixture refined from petroleum and OSPW. The commercial NA mixture was found to consist of mainly 8 to 18 carbon straight chain, methyl branched, acyclic isoprenoid, cyclohexyl and isomeric octahydropentalene, perhydroindane and perhydronaphthalene (decalin) acids (Rowland et al., 2011a). Analysis of oil sands NAs suggested for the first time that tri-, tetra- and pentacyclic, diamondoid NAs are present in OSPW and may be a result of advanced biodegradation of some of the oil in the oil sands (Rowland et al., 2011b, 2011c). Although these methods have made significant advancements in identification of NAs in complex samples, they are difficult to use routinely for NA analysis and significant developments are still required to help identify the most toxic and recalcitrant NA structures.

As mentioned above, NAs in refinery process waters consist of hundreds of different structures and there is currently not one universal method that allows for their complete analysis. Advancements in the analysis of naphthenic acids calls for a method that can accurately quantify NA concentrations, NA congener distributions (i.e., ring number, carbon number) and more specifically, NA structures, in complex natural and process water samples.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Analytical Methods

3.1.1 pH

All pH measurements were performed using the potentiometric method with a ATI Orion Model 370 digital pH meter (Orion Research Inc., Boston, MA) and a gel-filled combination pH electrode (VWR International, West Chester, PA). The meter was calibrated monthly with pH 4.0, 7.0, and 10.0 standard buffer solutions (Fisher Scientific, Pittsburg, PA).

3.1.2 Ammonia

Ammonia was measured using the distillation method described in *Standard Methods* (APHA, 2012). The samples were centrifuged at 10,000 rpm for 15 minutes and filtered through a 0.2 μm nitrocellulose membrane filter (Fisher Scientific, Pittsburgh, PA). Ammonia distillation was performed using a distillation apparatus (Labconco Corp., Kansas City, MO). The distillate was then titrated with 0.2 N H_2SO_4 and the ammonia was quantified.

3.1.3 Total and Soluble Chemical Oxygen Demand

COD was measured using the closed reflux, colorimetric method as described in *Standard Methods* (APHA, 2012). An aliquot of 3 mL digestion solution composed of 4.9 g $K_2Cr_2O_7$, 6 g $HgSO_4$, 6 g Ag_2SO_4 and 500 mL H_2SO_4 was transferred to COD digestion

vials and then 2 mL of sample was added to the vial. After tumbling the vial 4 to 8 times, the vial content was digested at 150^{o}C for 2 hours and then cooled down to room temperature. The absorbance was measured at 620 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. Samples were centrifuged and filtered through a 0.2 μm nitrocellulose membrane filter if the soluble COD was measured; otherwise, well-mixed samples were used after appropriate dilution for total COD measurements. All samples were prepared in triplicate and a calibration curve was prepared using 1 g/L standard solution of potassium hydrogen phthalate (KHP).

3.1.4 Total and Volatile Suspended Solids (TSS and VSS)

TSS and VSS were determined according to procedures described in *Standard Methods* (APHA, 2012). Whatman GF/C glass fiber filters (47 mm diameter and 1.2 μm nominal pore size; Whatman, Florham Park, NJ) were washed with de-ionized (DI) water and ignited at 550^{o}C for 20 minutes in a Fisher Isotemp Model 550-126 muffle furnace before use. The filters were then cooled in a desiccator and weighed. Samples of known volume were filtered through the glass fiber filters. The filters were then rinsed with 10 mL DI water to remove dissolved organics and inorganic salts. The filters containing the samples were dried at 105^{o}C for 90 minutes. After cooling in a desiccator, the dry weight was recorded and the filters containing the dry samples were ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled down in a desiccator and the weight was measured. TSS and VSS concentrations were then calculated using the equations below.

$$\text{TSS (mg/L)} = \frac{\text{Filter weight after } 105^{o}\text{C (mg)-Filter tare weight (mg)}}{\text{Sample volume (L)}}$$

$$\text{VSS (mg/L)} = \frac{\text{Filter weight after } 105^{o}\text{C (mg)-Filter weight after } 550^{o}\text{C (mg)}}{\text{Sample volume (L)}}$$

3.1.5 Total and Volatile Solids (TS and VS)

TS and VS of samples were determined according to procedures outlined in *Standard Methods* (APHA, 2012). Samples were weighed in pre-ignited (550^{o}C) and cooled ceramic crucibles using an Ohaus AP250D Analytical Balance (precise to ±0.02 mg up to 52 g, and to ±0.1 mg between 52 and 210 g). The samples were then dried at 105^{o}C for 24 hours in a Fisher Isotemp Model 750G oven. After drying, the crucibles were transferred to a desiccator until cooled, and then the dry weight was measured. If VS were to be determined, the crucibles were transferred to a Fisher Isotemp Model 550-126 muffle furnace and ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled in a desiccator and the remaining solids weight was measured. TS and VS were then calculated using the equations below.

$$\text{TS (mg/L)} = \frac{\text{Crucible weight after } 105^{o}\text{C (mg)- Crucible tare weight (mg)}}{\text{Sample volume (L)}}$$

$$\text{VS (mg/L)} = \frac{\text{Crucible weight after } 105^{o}\text{C (mg)- Crucible weight after } 550^{o}\text{C (mg)}}{\text{Sample volume (L)}}$$

3.1.6 Total Gas Production

Total gas production in closed assay bottles and large volume reactors was measured by either the gas-water displacement method or with a VWR Pressure/Vacuum transducer (resolution –1 atm to 1.974 atm with an accuracy of 0.002 atm).

3.1.7 Gas Composition

The gas composition was determined by a gas chromatography (GC) unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane (CH_4), oxygen (O_2) and dinitrogen (N_2) were separated with a 15 m HP-Molesieve fused silica, 0.53 mm i.d. column (Agilent Technologies, Inc.). Carbon dioxide (CO_2), nitric oxide (NO) and nitrous oxide (N_2O) were separated with a 25 m Chrompac PoraPLOT Q fused silica, 0.53 mm i.d. column (Varian, Inc., Palo Alto, CA). Helium was used as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150^{o}C, the oven was set at 40^{o}C and the detector temperature was set at 150^{o}C. All gas analyses were performed by injecting a 100 μL gas sample. The minimum detection limit for CH_4, CO_2, NO, N_2O, O_2 and N_2 was, 500, 800, 500, 7, 50 and 50 ppmv, respectively.

3.1.8 Volatile Fatty Acids (VFAs)

VFAs (C_2 to C_7, i.e., acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, iso-caproic, n-caproic and heptanoic acids) were measured after acidification of filtered samples with a 2.5% H_3PO_4 solution containing 1.5 g/L acetoin as the internal standard (sample:acid, 2:1 volume ratio) using an Agilent 6890 Series GC unit equipped with a

flame ionization detector and a 35-m Stabilwax-DA, 0.53-mm I.D. column (Restek, Bellefonte, PA). Samples used for the measurement of VFAs were prepared by centrifugation at 10,000 rpm for 30 minutes and filtration through 0.22-μm PVDF membrane filters before acidification. The minimum detection limit for each acid mentioned above was 0.25, 0.10, 0.03, 0.02, 0.10, 0.08, 0.02, 0.02, 0.05 mM, respectively.

3.1.9 Organic Acids

Non-flame ionizable organic acids (formic, oxalic, citric, malic, pyruvic, lactic, succinic and fumaric acids) were measured with a HP 1100 Series HPLC (Hewlett Packard, Palo Alto, CA) unit equipped with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm)(Bio-Rad, Richmond, CA) and an Agilent 1100 Series UV/visible diode array and refractive index detectors (Agilent Technologies, New Castle, DE). A 0.01 N H_2SO_4 solution was used as the mobile phase with a flow rate of 0.6 mL/min and the column was maintained at 65°C. The samples were centrifuged and the supernatant was acidified with 0.2 N H_2SO_4 in a 1:1 volume ratio, and filtered through 0.2 μm membrane filters before the analysis. Organic acids were detected by the UV detector at 210 nm wavelength.

3.1.10 Anions

Chloride (Cl^-), nitrite (NO_2^-), bromide (Br^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) anion concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Corporation, Sunnyvale, CA) equipped with a suppressed

conductivity detector, a Dionex IonPac AG14A (4x50mm) precolumn, and a Dionex IonPac AS14A (4x250 mm) analytical column. The unit was operated in autosupression mode with 1mM $NaHCO_3$/8mM Na_2CO_3 eluent and a flow rate of 1 mL/min. All samples were filtered through 0.2 μm membrane filters prior to injection. The minimum detection limit for each anion listed above was 0.03, 0.02, 0.03, 0.04, 0.02 and 0.05 mM, respectively.

3.1.11 Dissolved Oxygen (DO)

The DO concentration of the cultures used in this study was measured using a HACH HQ40d portable dissolved oxygen meter in conjunction with a IntelliCAL LDO101 Standard Luminescent Dissolved Oxygen (LDO) Probe (HACH Company, Loveland, CO). The instrument was periodically calibrated to water-saturated air, at a given temperature, before using.

3.2 Microtox® Acute Toxicity

The acute toxicity of NA-bearing samples was assessed using the standard Microtox® test. All samples were adjusted to 2% NaCl before analysis in order to maintain the proper osmotic pressure (ionic strength of 342 mM) for *Vibrio fischeri*. Sample dilutions ranging from 1 to 2^7, corresponding up to 128-fold dilution, were tested for 5 and 15 minutes exposure time. The luminescence emitted at different sample dilutions was compared to that of the control, which consisted of 2% NaCl in DI water (pH 6.5-7.0) having no NAs. The effective concentration of a toxicant (%, v/v of sample strength or

NA concentration, mg/L) that causes the bacteria to emit light at 50% of the control (EC_{50}) was used as the descriptor of toxicity.

3.3 Total Acid Number (TAN)

TAN, which is also referred to as neutralization number, is the amount of alkalinity consumed to neutralize the total acidity in crude oil due to several acidic constituents. The TAN of crude oil samples was measured according to the ASTM D664-09 method (ASTM, 2010) as follows. A 20 g sample of crude oil was diluted with 125 mL of solvent composed of toluene, i-propanol and wMMater (500:495:5 mL). The crude oil/solvent mixture was then titrated with a standard alcoholic 0.1 N KOH solution potentiometrically to an inflection point. The TAN was expressed as the amount of KOH used for the titration of one gram of crude (mg KOH/g crude).

3.4 Naphthenic Acids

NA concentrations and congener distributions were determined using a pair-ion extraction method (PIX), followed by liquid chromatography/mass spectrometry (Tezel et al., 2010). In the case of wastewater samples, the extraction was performed as follows. The pH of a 10 mL sample was increased to 10 with 1N NaOH. Then, 60 µL of a 100 mM *p*-toluene sulfonate (*p*TS) surrogate standard and 1 mL of a 10 mM benzyl tributyl ammonium (BTBA) counter-ion solution were added and the sample extracted with 10 mL methylene chloride overnight (Figure 3.1). For the quantification of the total NAs (TNA) in crude oil samples, the extraction was performed as follows. A 0.2 g crude oil sample was mixed with 3 mL toluene, 17 mL methanol and 0.1 mL 5M NH_4OH. The

mixture was amended with 0.03 mM *p*TS and filtered through 0.2 µm PTFE filters. For the quantification of total extractable NAs (TENA) in crude oil samples, the extraction was performed as follows. A 0.2 g crude oil sample was mixed with 20 mL 1N NaOH solution and agitated for 3 hours. After storage for one day to allow phase distribution, the mixture was centrifuged at 10,000 rpm for 15 min. The aqueous phase was amended with 1 mL of 10 mM BTBA and 60 µL of 100 mM *p*TS and then extracted with methylene chloride overnight. The extracts of wastewater and crude oil samples were transferred into 2-mL amber glass vials and used in the LC/MS analysis described below.

The NA concentration and congener distribution in PIX extracts of NA-bearing samples, prepared as described above, were determined using an HP 1100 Series LC unit and an HP 1100 Series LC/MSD mass spectrometry detector unit. A 10 µL standard or PIX extract sample was introduced into the MS by direct infusion using a 60:40 (v/v) mixture of 10 mM NH_4OH in acetonitrile and 10 mM NH_4OH in water (pH 10) as the mobile phase at a flow rate of 0.7 mL/min. MS analysis was conducted by electron spray ionization in negative mode (ESI-) at 70 eV fragmentation voltage with a mass scan range of *m/z* 100-500. The drying gas (nitrogen) flow rate was 13 L/min at 350ºC, the nebulizer pressure was 50 psig, and the capillary voltage was 3500 V. Then, the NA concentration was determined relative to the standard (*p*TS) and a calibration curve prepared with a commercial NA mixture (TCI NA mixture; see Chapter 6). The congener distribution was determined as follows: the *m/z* of each ion was compared to the deprotonated *m/z* value of a NA based on the general formula of $C_nH_{2n+Z}O_2$ and if there was agreement, the corresponding *n* and *Z* numbers were recorded and plotted to display the relative abundance of each corresponding NA structure (Tezel et al., 2010)

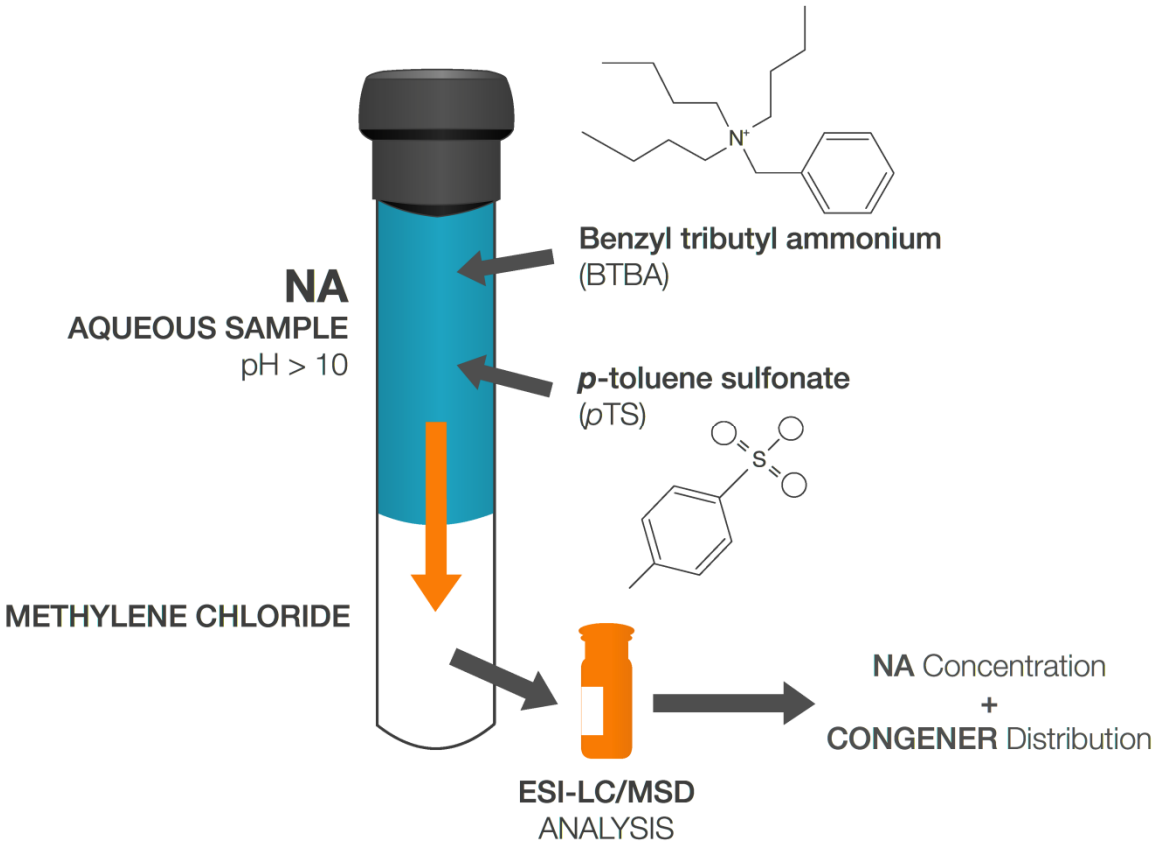


Figure 3.1. Pair ion extraction (PIX) method for the quantification of naphthenic acids.

3.5 General Procedures

3.5.1 Aerobic Culture Media

Aerobic cultures used in this study were sustained in growth media shown in Table 3.1. The media were utilized without autoclaving.

Table 3.1. Composition of media for the mixed aerobic culture used in this study.

Compound/Solution	**Concentration**
K_2HPO_4	1.07 g/L
KH_2PO_4	0.524 g/L
$CaCl_2 \cdot 2H_2O$	0.068 g/L
$MgCl_2 \cdot 6H_2O$	0.135 g/L
$MgSO_4 \cdot 7H_2O$	0.268 g/L
$FeCl_2 \cdot 4H_2O$	0.068 g/L
Trace metal stock solution	0.67 mL/L

Trace metal stock solution	**Concentration**
$ZnCl_2$	0.5 g/L
$MnCl_2 \cdot 4H_2O$	0.3 g/L
H_3BO_3	3.0 g/L
$CoCl_2 \cdot 6H_2O$	2.0 g/L
$CuCl_2 \cdot 2H_2O$	0.1 g/L
$NiSO_4 \cdot 6H_2O$	0.2 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.3 g/L

3.5.2 Methanogenic Culture Media

A mixed methanogenic culture used in this study was sustained in medium which supplied necessary nutrients, trace metals, and vitamins. The composition of the culture media is shown in Table 3.2. Resazurin was used as a redox indicator (ORP < -110 mV).

Culture media were prepared by adding the first six ingredients in Table 3.2 to 8 L DI water in 9-L Pyrex serum bottles. The bottles were then autoclaved at 250^{o}F (121^{o}C) and 21 psi (1.43 atm) for 45 minutes. After autoclaving, the bottles contents were purged with helium for 1.5 hours in order to strip oxygen from the media. After purging, and while the media were still warm, the rest of the ingredients listed in Table 3.2 were added.

Table 3.2. Composition of media for the mixed methanogenic culture used in this study.

Compound/Solution	**Concentration**
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	0.5 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
Trace metal stock solution	1 mL/L
1 g/L resazurin stock	2 mL/L
Vitamin stock solution	1 mL/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
$NaHCO_3$	6.7 g/L
$Na_2S \cdot 9H_2O$	0.5 g/L

Trace metal stock solution	**Concentration**
$ZnCl_2$	0.5 g/L
$MnCl_2 \cdot 4H_2O$	0.3 g/L
H_3BO_3	3.0 g/L
$CoCl_2 \cdot 6H_2O$	2.0 g/L
$CuCl_2 \cdot 2H_2O$	0.1 g/L
$NiSO_4 \cdot 6H_2O$	0.2 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.3 g/L

Vitamin stock solution	**Concentration**
Biotin	0.2 g/L
Folic Acid	0.2 g/L
Pyridoxine hydrochloride	1.0 g/L
Riboflavin	0.5 g/L
Thiamine	0.5 g/L
Nicotinic Acid	0.5 g/L
Pantothenic Acid	0.5 g/L
Vitamin B12	0.01 g/L
p-Aminobenzoic Acid	0.5 g/L
Thioctic Acid	0.5 g/L

CHAPTER 4

FATE AND EFFECT OF NAPHTHENIC ACIDS ON OIL REFINERY ACTIVATED SLUDGE WASTEWATER TREATMENT SYSTEMS

4.1 Introduction

Petroleum refineries are process plants where crude oil is transformed to refined products, such as gasoline, diesel and kerosene. Refining processes use large quantities of water, primarily for extraction, desalting, and cooling, generating waste streams, which are combined and carried to the refinery wastewater treatment plant (Dorn, 1998; IPEICA, 2010). Many wastewater components are easily removed by the refinery wastewater treatment plant; however, some compounds found in process waters, such as naphthenic acids (NAs), are more difficult to treat and create operational problems, such as corrosion and toxicity (Dorn, 1998; IPEICA, 2010; Whitby, 2010). Activated sludge systems are common biological treatment processes used to treat refinery wastewaters; however, very limited information exists relative to the fate and effect of NAs in refinery activated sludge units and whether such treatment processes are capable of reducing the NA concentration and toxicity of refinery wastewater.

NAs are present in crude oil and bitumen from reservoirs and oil sands. Recently, oil exploration has led to extraction of extra heavy crudes and bitumen from oil sands, which have elevated NA concentrations and result in refinery process waters and wastewaters with relatively high NA levels. Refinery wastewaters vary in NA concentration depending on the crude oil source and treatment process (IPEICA, 2010;

Whitby, 2010).

Detailed information about the occurrence of NAs in refinery wastewater streams and what factors affect the fate and biodegradation of NAs in refinery wastewater treatment plants is very limited. The objectives of this research were to: a) characterize crude oil and wastewater streams from six oil refineries; b) determine the occurrence and fate of NAs in various refinery wastewater treatment systems; and c) assess the inhibitory and biotransformation potential of NAs on refinery activated sludge microcosms.

4.2 Materials and Methods

4.2.1 Crude Oil, Refinery Wastewater Samples and Chemicals

Crude oil and wastewater samples taken from the desalter brine, influent, mixed liquor and effluent steams of activated sludge units were received from six United States refineries, referred to as refinery A through F. Refinery A uses powder activated carbon (PAC) in the activated sludge mixed liquor. Crude oil samples were analyzed for total acid number (TAN), total NAs (TNA), and total extractable NAs (TENA). All wastewater samples were analyzed for pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), ammonia, nitrite, nitrate and NAs. It is important to note that although the crude oil and wastewater samples were not received at exactly the same time from each refinery, they are considered to be representative of each refinery.

A commercial NA sodium salt was purchased from TCI Chemicals (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The mixture contains approximately 8.6% (w/w) sodium. All NA concentrations mentioned in this work refer to NAs and not the

NA salt. The characteristics of the NA salt are described in detail in Chapter 5, Section 5.3.1.

4.2.2 Micro Dilution Susceptibility Test

A batch test was performed to assess the inhibitory effect and biotransformation potential of TCI NAs of the native refinery activated sludge microbial communities. The inhibition test was performed as follows: a 200 µL sample of well mixed and aerated mixed liquor was transferred to a 1800 µL sterile nutrient broth composed of 3 g/L peptone, 0.5 g/L casein, 0.2 g/L K_2HPO_4, 0.05 g/L $MgSO_4$ and 0.002 g/L $FeCl_3 \cdot 6H_2O$. The culture was incubated overnight at room temperature (22 to 24^oC), diluted 10000 times with the same broth and re-incubated overnight. The mixed heterotrophic culture obtained was diluted to about 0.005 McFarland (approximately 10^6 cells/mL) and 1 mL of the diluted culture was transferred to 10 mL culture tubes containing 1 mL nutrient broth and a range of TCI NA concentrations from 0.5 to 500 mg/L. The tubes were incubated at room temperature (22 to 24^oC) overnight and the growth was measured with a UV/Vis spectrometer at 600 nm wavelength. Tubes containing nutrient broth having the same NA concentrations but without culture were used as blanks. Control tubes contained sterile broth, dextrin and casein, culture, but no NAs.

A similar test was performed to assess the NA biotransformation potential of the activated sludge microorganisms, using a nutrient broth composed of inorganic salts as mentioned above and NA salt as the sole carbon source at a range of NA concentrations from 0.5 to 500 mg/L. The sterile broth was composed of 0.2 g/L K_2HPO_4, 0.05 g/L $MgSO_4$ and 0.002 g/L $FeCl_3 \cdot 6H_2O$. Tubes containing the sterile broth having the same

NA concentrations but without culture were used as blanks. Tubes containing sterile broth and culture but without NAs were used as controls.

4.2.3 NA Desorption Assay

The desorption assay was performed with triplicate 20-mL samples of two NA-bearing activated sludge mixed liquors from refineries A and B, with and without PAC, respectively. The mixed liquor samples were added to 40-mL Teflon centrifuge tubes and centrifuged at 10,000 rpm for 15 minutes. Approximately 75% of the supernatant volume was removed and replaced with 10 mM phosphate buffer (pH 7) that contained 200 mg/L sodium azide to inhibit any microbial activity. The tubes were agitated on an orbital shaker by continuous mixing at 190 rpm. On days 1, 2, 4, 7 and 10 the tubes were centrifuged and 75% of the supernatant volume was removed and analyzed for NAs. The removed supernatant was replaced with 200 mg/L sodium azide phosphate buffer solution and the tubes were returned to the orbital shaker. The interstitial water in the solids pellet after centrifugation was determined gravimetrically and the mass of NAs in the interstitial water was determined as the product of the measured NA concentration in the supernatant and the mass (volume) of the remaining interstitial water at each successive desorption step. Over the successive desorption period, the solid-phase NA concentration was determined by difference of the measured total and liquid-phase NA concentrations.

4.2.4 Biotransformation Assay of Chronically-sorbed NAs

Samples of activated sludge mixed liquors, received from refinery A (PAC-containing) and B (PAC-free) and used in the desorption assay (Section 4.2.3, above), were also used in this biotransformation assay. The two mixed liquor samples were aerated at room temperature (22 to 24^{o}C) without any exogenous substrate addition for 5 days. During this incubation period, the soluble COD concentration did not decrease in both mixed liquor samples, indicating very low biodegradability of the mixed liquor organic components, which resulted in very low microbial activity. Then, an organic mixture of 700 mg COD/L was added to both mixed liquors to increase their microbial activity. The organic mixture was composed of (mg COD/L in the mixed liquor): sodium acetate, 180; sodium propionate, 180; sodium butyrate, 90; and sodium benzoate, 250. Incubation was carried at room temperature (22 to 24^{o}C) with continuous aeration using pre-humidified, compressed air. Throughout the incubation period, the two microcosms were fed three times with the same organic mixture as mentioned above. The pH was maintained between 7 and 7.5 in both microcosms throughout the incubation period. Soluble COD, pH, and total and liquid-phase NAs were monitored throughout the incubation period.

4.3 Results and Discussion

4.3.1 Characteristics of Crude Oil and Refinery Wastewater

Refinery processes depend on the amount and type of crude oil as well as the target petroleum products. The refinery processes produce large amounts of wastewater, including desalter brine, spent caustic, sour water and water used in other refining processes such as cooling and steam. The desalter brine and other refinery process waters

are combined and sent through the wastewater treatment plant before reuse or discharge into storage ponds or the environment. A typical refinery wastewater treatment plant consists of primary treatment (i.e., oil/water separation), equalization, secondary biological treatment (i.e., activated sludge with or without PAC, sequencing batch reactors, membrane bioreactors) and in some cases, tertiary treatment (i.e., sand filtration, activated carbon, chemical oxidation) (Figure 2.1) (IPIECA, 2010).

The six refineries considered in this study process different types of crude oil from various sources (Table 4.1). Each refinery processed different volumes of crude oil in the range of 105 to 306 thousand barrels per day (KBD). The measured TAN values ranged from 0.12 to 1.50 mg KOH/g crude oil. The TAN potentiometric titrations for all six refinery crude oils are shown in Figure 4.1. For comparison of TNA and TENA values (see below) to the crude oil TAN values, calculations were based on measured TNA, TENA and TAN values, their average MW and KOH MW, performed as follows:

$$\text{TNA/TAN ratio (\%)} = \frac{\text{TNA}}{\text{MW}_{\text{NA}}} \text{x} \frac{\text{MW}_{\text{KOH}}}{\text{TAN}} \text{x} \frac{\text{mg}}{1000\ \mu\text{g}} \text{x} 100\%$$

$$\text{TENA/TAN ratio (\%)} = \frac{\text{TENA}}{\text{MW}_{\text{NA}}} \text{x} \frac{\text{MW}_{\text{KOH}}}{\text{TAN}} \text{x} \frac{\text{mg}}{1000\ \mu\text{g}} \text{x} 100\%$$

where TNA is the total NA concentration in the crude oil (µg NA/g crude oil), TENA is the total extractable NA concentration (µg NA/g crude oil), MW_{NA} is the weighted average MW of the NAs in that specific sample (g/mol), MW_{KOH} is the molecular weight of KOH (g/mol) and TAN is the total acid number (mg KOH/g crude oil)

Table 4.1. Crude oil source and type for six refineries included in this study.

Refinery	Crude Oil Source	Crude Oil Type
A	Venezuelan	Intermediate/Heavy
B	Midcontinent	Intermediate
C	Sea	Light
D	Midcontinent	Intermediate
E	Midcontinent	Intermediate
F	Alaskan	Light

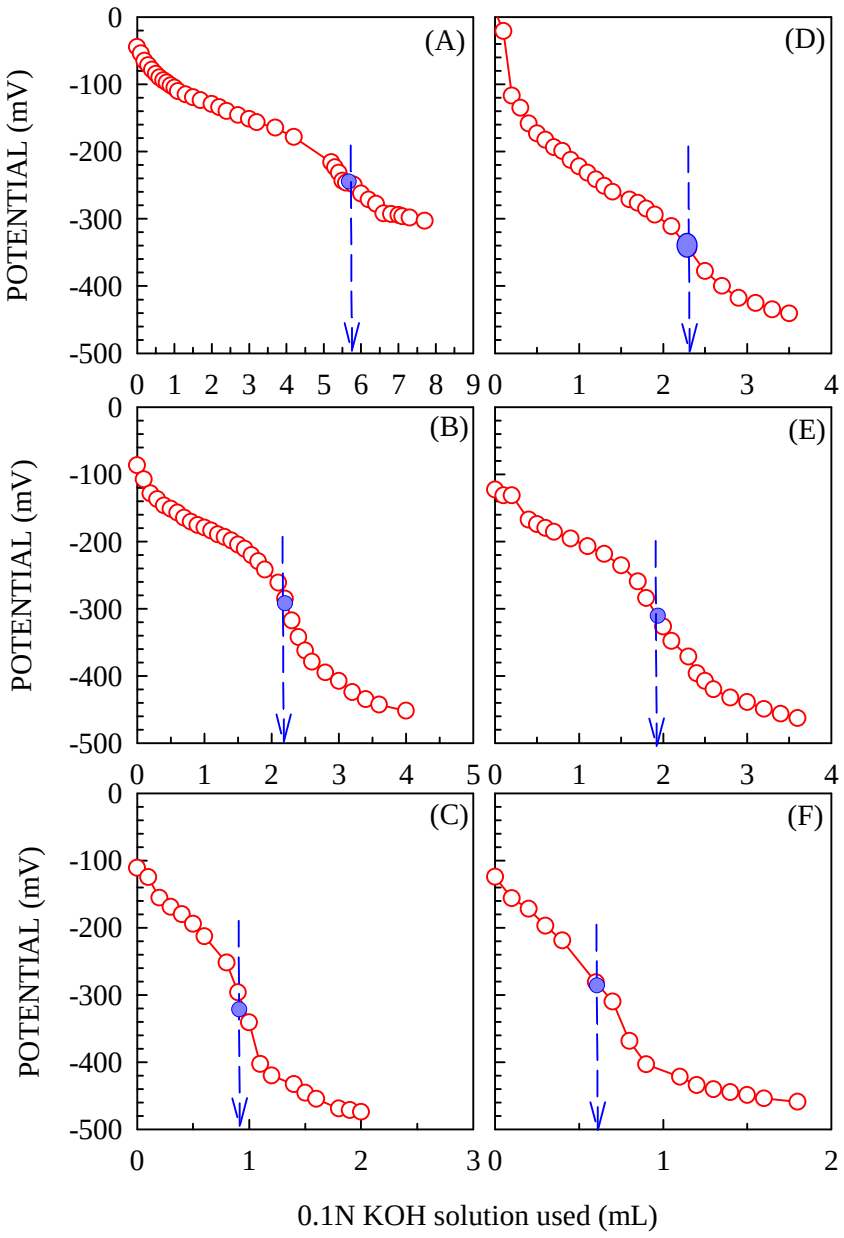


Figure 4.1. Potentiometric titration for TAN measurement of crude oils from refinery A to F (blue dot and arrow indicate inflection point).

The TNAs in crude oil samples ranged between 900 to 4000 µg/g crude (Table 4.2). The highest TNA value was measured for the crudes having the highest TAN values. The Venezuelan crude oil had the highest total NA concentration followed by the Midcontinent, Sea and Alaskan crude oils. All crudes were dominated with TNAs having Z = 0, -2, -4, -6, -8, and -10 and n between 10 and 45 carbons. The weighted average MW of NAs in the crude oil samples ranged between 370 and 430 Da. The TNA concentration correlates to the TAN (r^2 = 0.88) (Figure 4.2) and represents 30 to 100% of the TAN values (Table 4.2).

Table 4.2. Total acid number (TAN) and total NAs (TNA) weighted average molecular parameters and formulae in crude oil samples from six refineries.

Refinery	TAN mg KOH/ g oil	Total NAs					TNA/TAN[b] %
		Z	*n*	MW	Formula	Conc.[a] µg/g oil	
A	1.50	-4.9	28.8	430.3	$C_{28.8}H_{52.7}O_2$	3629.3	31.5
B	0.58	-5.2	28.2	421.6	$C_{28.2}H_{51.2}O_2$	1868.8	42.9
C	0.22	-4.5	25.0	377.5	$C_{25}H_{45.5}O_2$	1265.9	85.5
D	0.60	-5.4	26.6	399.0	$C_{26.6}H_{47.8}O_2$	1898.2	44.5
E	0.52	-5.2	26.7	400.6	$C_{26.7}H_{48.2}O_2$	957.8	25.8
F	0.12	-6.0	26.1	391.4	$C_{26.1}H_{46.2}O_2$	1007.3	120.3

[a] NA concentration expressed using average TNA MW in each crude oil sample

[b] Calculated based on measured TNA and TAN values, their average MW and KOH MW

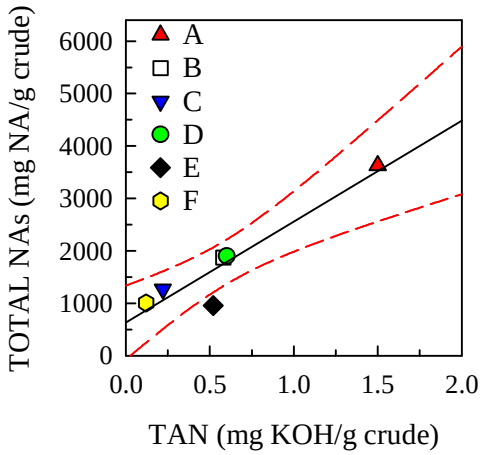


Figure 4.2. Relationship between total NAs (TNAs) and TAN of crude oil samples obtained from six refineries (A to F)(Broken lines are 95% confidence bands).

The total NA values, as well as the total NA contribution to TAN were consistent with previously reported values (Meredith et al., 2000). These researchers analyzed 33 different crude oils with TAN values ranging from 0.1 to 2.67 mg KOH/g oil and reported total NA concentrations from 33 to 8697 µg/g, contributing up to 96% of the TAN. The results of the present study indicate that the total NA contribution to the total crude oil acidity is pronounced and about 40% on average for crudes having TAN above 0.5 mg KOH/g oil (Table 4.2). As an example, the total NA distribution in the crude oil sample from refinery A is given in Figure 4.3A. All six crude oil TNA distributions are summarized in Table 4.2. The total extractable NAs (TENA) in crude oil samples ranged between 100 to 175 µg/g crude (Table 4.3). The highest TENA value was measured for the Venezuelan crude oil (refinery A) which had the highest TAN value. All crudes were dominated with TENAs having Z = -2, -4 and -6 and n between 10 and 20 carbons. The

TENA represents 2.5 to 20.6% of the TAN and 4.8 to 12.5% of the total NA (Table 4.3). Generally speaking, the ratio TENA/TNA decreased with increasing TNA MW. The weighted average MW of the TENAs in the crude oil extracts ranged between 256 and 286 Da, which is 14 and 44 Da lower than the TNA weighted average MW range. The TENA distribution in the extract of the crude oil sample from refinery A is given in Figure 4.3B.

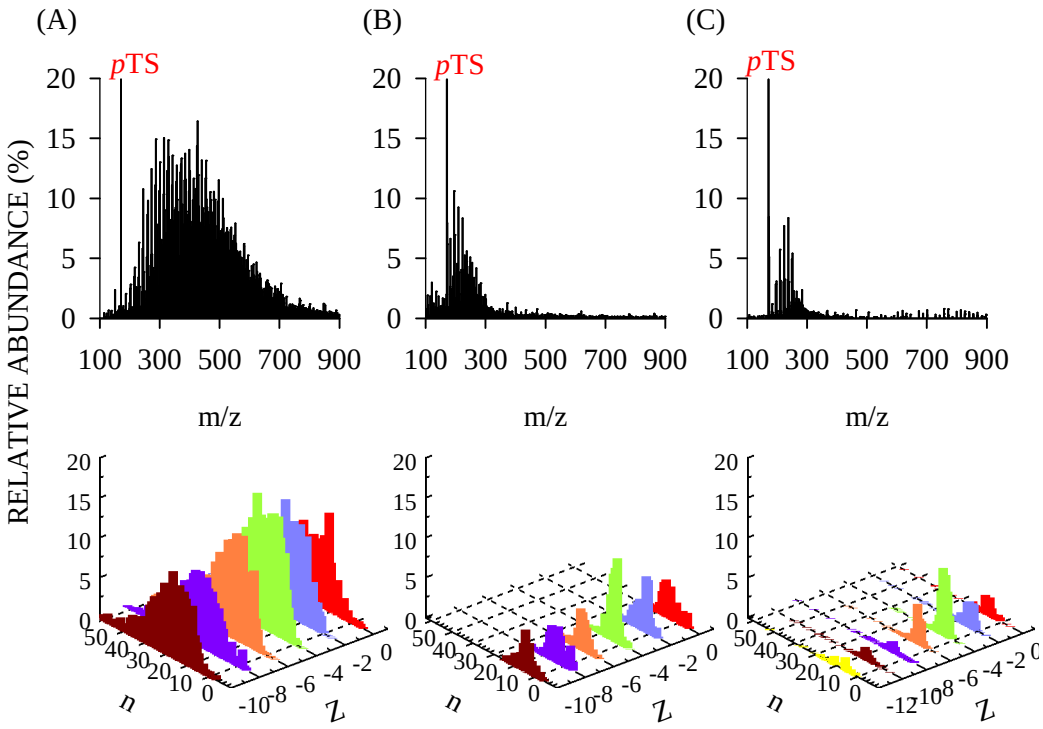


Figure 4.3. NA congener distribution in refinery A crude oil total NA extract (A), crude oil total extractable NA extract (B), and desalter brine (C) samples.

The characteristics of wastewater samples collected at the six refineries, including mean and range values for pH, total and liquid-phase NAs, total and soluble COD, TS, VS, ammonia, nitrate, and Microtox® EC_{50}, are summarized in Table 4.4. Nitrite was not detected in any of the samples. NAs were detected in all wastewater samples analyzed and accounted for less than 16% of the total COD (Figure 4.4). The theoretical oxygen demand (ThOD) of NAs present in each sample was calculated using the average NA molecular formula, molecular weight and NA concentration. The NA oxidation reaction is:

$$C_nH_{2n+Z}O_2 + \frac{6n+Z-1}{4} O_2 \rightarrow n\ CO_2 + \frac{2n+Z}{2} H_2O$$

The ThOD was calculated using the following equation:

$$ThOD = 32 \times \frac{6n+Z-1}{4*MW_{NA}}$$

The TCI NA mixture (MW = 272.1 g/mol) was used as a standard to determine NA-COD and the ThOD was calculated as follows:

$$C_{17.4}H_{30.8}O_2 + 24.1\ O_2 \rightarrow 17.4\ CO_2 + 15.4\ H_2O$$

$$ThOD = 32 \times 24.1/272.1 = 2.83\ mg\ O_2/mg\ NA$$

As shown above, the theoretical TCI NA-COD was estimated to be 2.83 mg O_2/mg NA. The total NAs concentration in the desalter brine samples ranged from 4 to 40 mg/L. The desalter brine of the Midwestern crude oils had the highest NA concentration followed by the brines of Venezuelan, Sea and Alaskan crude oil. The NA congener distributions indicate that the lower molecular weight NAs in the crude oil are transferred to the desalter brine and the higher MW, more hydrophobic NAs, are not removed from

the crude oil during the desalting process (Table 4.2 and 4.3). NAs contribute 1 to 7% of the total COD of the desalter brines which indicates that many other organic compounds are present in the desalter brine and NAs are a minor component (Figure 4.4). On average, the liquid-phase NAs accounted for about 73% of the total NAs in the desalter brine samples analyzed (Figure 4.5A). The NA congener distribution in the desalter brine sample from refinery A is shown in Figure 4.3C; the total and liquid-phase weighted average *Z*, *n*, MW, formula and NA concentration in all desalter brine samples from the six refineries are summarized in Table 4.5. The NA concentration and weighted average NA MW of the liquid-phase NAs were slightly lower than for the total NAs. In addition, NAs with higher cyclization and lower carbon number are the predominant NAs in the aqueous portion of the desalter brines. These results indicate that a portion of NAs having high MW, lower cyclization and higher carbon number are sorbed to the desalter brine solids. All desalter brine samples had NAs with MW ranging between 210 and 265 Da and carbon numbers ranging from 13 to 17. It should be noted that due to refinery process variability over time, along with changes in removal efficiencies and other operational parameters along the process water/wastewater train, a direct comparison of NA profiles and abundance among the various waste streams, even at the same refinery, cannot be drawn.

The total NA concentration in the influent and mixed liquor samples ranged from 5 to 17 and 10 to 140 mg NA/L, respectively (Figure 4.5B and 4.5C). The ratio of liquid/total NAs in the mixed liquor samples ranged from 0.03 to 0.3 (Figure 4.5C). Between 70 and 97% of NAs in the mixed liquor samples were associated with the solid phase (i.e., biomass or biomass plus PAC in refinery A) (Figure 4.6). Effluent NAs

ranged from 3 to 12 mg/L (Figure 4.5D), and on a COD basis, calculated based on a mean NA theoretical oxygen demand of 2.83 mg O_2/mg NA, accounted for less than 16% of the total effluent COD (Figure 4.4). Thus, on a COD basis, NAs are a minor organic component of the effluents from the refinery biological treatment units.

Table 4.3. Total extractable NAs (TENA) weighted average molecular parameters and formulae in crude oil samples from six refineries.

Refinery	Total Extractable NAs					TENA/TAN[b]	TENA/TNA
	Z	***n***	**MW**	**Formula**	**Conc.[a]** **µg/g oil**	**%**	**%**
A	-4.8	17.0	265.2	$C_{17}H_{29.2}O_2$	174.6	2.5	4.8
B	-4.4	18.0	279.6	$C_{18}H_{31.6}O_2$	160.9	5.6	8.6
C	-3.9	16.3	256.3	$C_{16.3}H_{28.7}O_2$	124.0	12.3	9.8
D	-4.7	18.1	280.7	$C_{18.1}H_{31.5}O_2$	101.8	3.4	5.4
E	-4.6	18.5	286.4	$C_{18.5}H_{32.4}O_2$	119.6	4.5	12.5
F	-4.7	17.9	277.9	$C_{17.9}H_{31.1}O_2$	122.5	20.6	12.2

[a] NA concentration expressed using average TENA MW in each crude oil sample

[b] Calculated based on measured TENA and TAN values, their average MW and KOH MW

Table 4.4. Characteristics of desalter brine, influent, activated sludge mixed liquor and effluent samples from six refineries.

Parameter	Desalter Brine	Influent	Mixed Liquor	Effluent
pH	7.4 (5.2 – 8.5)[a]	7.5 (6.9 – 8.3)	7.3 (6.4 – 7.7)	7.6 (7.3 – 8.0)
Total NA (mg/L)	22.7 (4.2 – 40.4)	10.2 (4.5 – 16.6)	70.6 (9.6 – 140.3)	6.8 (2.8 – 11.6)
Liquid NA (mg/L)	15.6 (3.6 – 28.2)	6.9 (3.4 – 12.2)	5.5 (2.9 – 8.5)	5.5 (2.9 – 9.5)
Total COD (mg/L)	1280 (415 – 1942)	704 (467 – 1117)	4641 (609 – 9100)	370 (114 – 847)
Soluble COD (mg/L)	1139 (391 – 1805)	544 (211 – 966)	375 (69 – 878)	317 (40 – 831)
Total Solids (g/L)	2.9 (0.5 – 8.4)	3.8 (1.1 – 12.0)	8.3 (1.6 – 21.7)	4.2 (1.4 – 12.8)
Volatile Solids (g/L)	0.7 (0.2 – 1.6)	0.45 (0.22 – 1.23)	3.1 (0.38 – 6.2)	0.32 (0.13 – 0.91)
Ammonia (mg N/L)	8.1 (0 – 50.5)	11.4 (0 – 28)	9.6 (0 – 30.8)	ND
Nitrate (mg N/L)	ND[b]	238 (0 – 440)	737 (0 – 2126)	447 (0 – 1314)
EC_{50} % (v/v)	6.1 (0.03 – 12.8)	69.1 (5.9 – 160)	116.7 (16.2 – 271)	262 (107 – 540)
EC_{50} (mg NA/L)	1.3 (0.001 – 2.9)	3.4 (0.4 – 9.9)	5.9 (0.7 – 13.3)	11.2 (7.5 – 15.7)

[a] Mean value and range (i.e., minimum – maximum)($n \geq 18$)

[b]ND, not detected

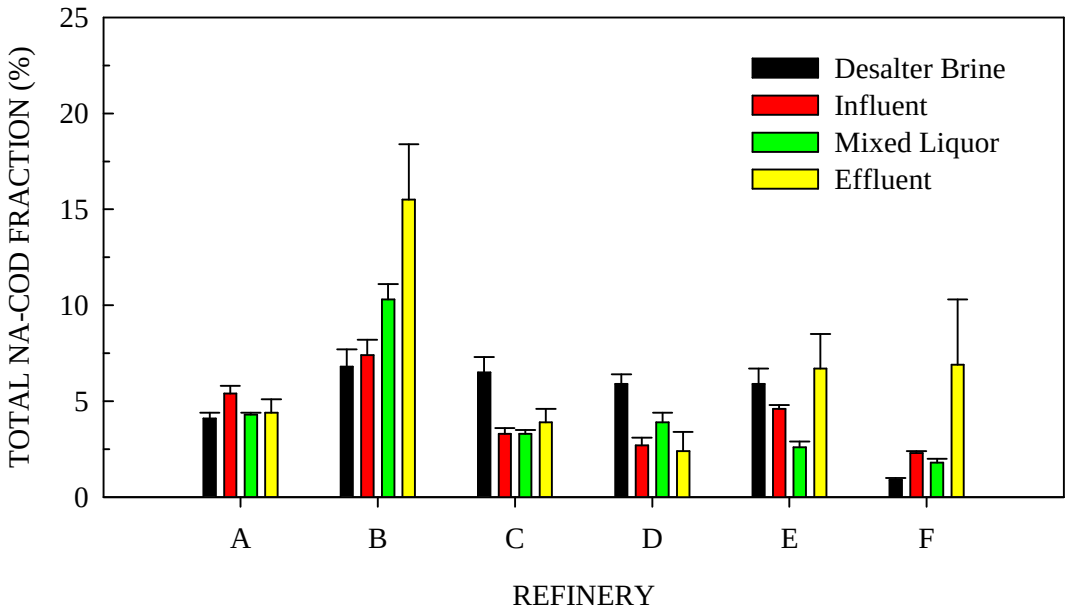


Figure 4.4. Mean NA-COD fraction of total COD in desalter brine, influent, mixed liquor and effluent wastewater streams obtained from six refineries (A to F; error bars represent mean values ± one standard deviation, *n* = 3).

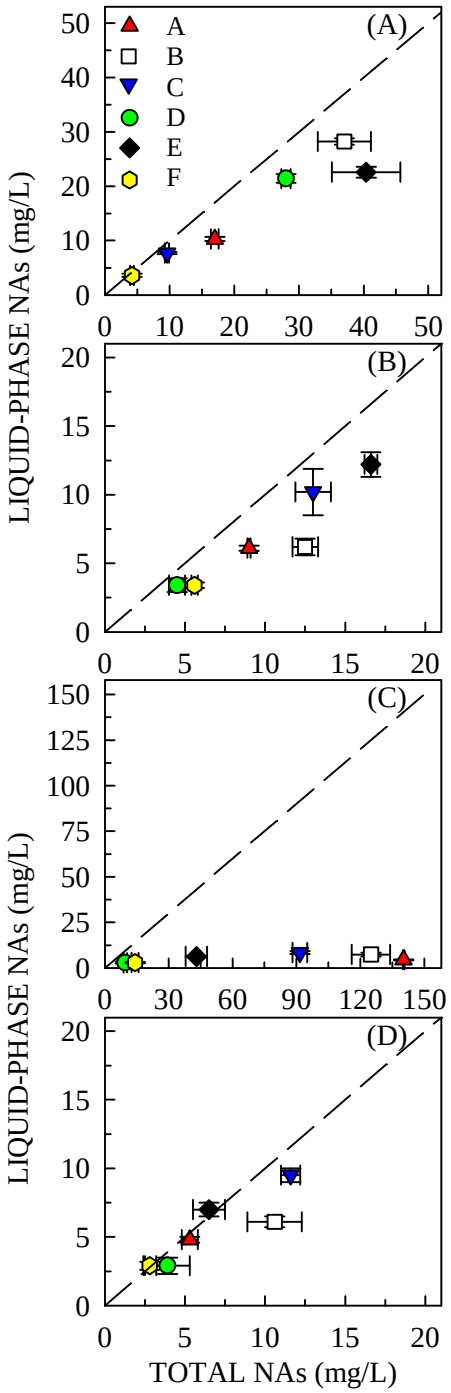


Figure 4.5. NA phase distribution in desalter brine (A), influent (B), mixed liquor (C) and effluent (D) samples obtained from six refineries (A to F; error bars represent mean values ± one standard deviation, *n* = 3).

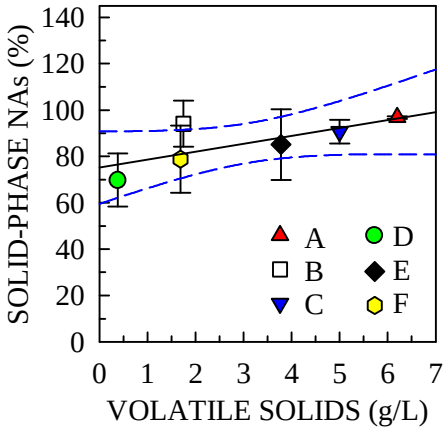


Figure 4.6. Relationship between the solid-phase fraction total NAs and volatile solids in activated sludge mixed liquor samples obtained from six refineries (A to F; error bars represent mean values ± one standard deviation, *n* = 3; broken lines are 95% confidence bands).

Table 4.5. NAs weighted average molecular parameters and formulae in desalter brine samples from six refineries.

Refinery	Phase	*Z*	*n*	MW	Formula	NA (mg/L)
A	Total	-5.5	14.6	231.3	$C_{14.6}H_{23.8}O_2$	17.0±0.6[a]
	Liquid	-5.6	14.0	222.3	$C_{14}H_{22.3}O_2$	10.3±0.4
B	Total	-4.4	17.0	265.4	$C_{17}H_{29.6}O_2$	37.0±4.1
	Liquid	-4.6	16.2	254.2	$C_{16.2}H_{27.8}O_2$	28.2±0.6
C	Total	-4.6	13.4	220.3	$C_{13.4}H_{22.3}O_2$	9.6±0.3
	Liquid	-4.8	13.1	210.6	$C_{13.1}H_{22.4}O_2$	7.7±0.2
D	Total	-4.9	15.4	242.6	$C_{15.4}H_{25.9}O_2$	28.0±0.7
	Liquid	-5.3	14.8	234.0	$C_{14.8}H_{24.4}O_2$	21.4±0.8
E	Total	-4.2	16.9	264.4	$C_{16.9}H_{29.6}O_2$	40.4±5.3
	Liquid	-4.6	15.8	248.6	$C_{15.8}H_{27}O_2$	22.6±1.0
F	Total	-5.5	13.7	218.8	$C_{13.7}H_{22}O_2$	4.2±0.3
	Liquid	-5.0	14.3	227.2	$C_{14.3}H_{23.6}O_2$	3.6±0.3

[a] Mean ± standard deviation (n = 3)

4.3.2 NA Toxicity

The toxicity of desalter brine, influent, mixed liquor and effluent sample filtrates was measured by the standard acute Microtox® toxicity assay and the 5-min exposure EC_{50} values are reported in Table 4.4 as sample strength (% v/v) and the corresponding equivalent NA concentration (mg/L) based on the measured NA concentrations. There was negligible difference between the 5- and 15-min exposures. The desalter brine EC_{50} values in terms of liquid-phase NA concentration ranged from 0.001 to 2.9 mg NA/L (0.03 to 12.8% v/v), which accounted for less than 3% of the soluble COD. Thus, only a fraction of the desalter brine toxicity measured by the standard acute Microtox® toxicity assay is attributed to the liquid-phase NAs. The influent, mixed liquor and effluent EC_{50} values ranged from 5.9 to 160%, 16.2 to 271%, and from 107% to 540% (0.4 to 9.9, 0.7 to 13.3, and 7.5 to 15.7 mg NA/L), respectively (Table 4.4). Previous studies have reported NA toxicity using the standard Microtox® assay and found that both commercial NAs and NAs extracted from refinery process waters were toxic to *Vibrio fischeri;* however, the toxicity of both refinery and commercial NAs were found to have a wide range of EC_{50} values depending on the NAs source and distribution of NA structures (Frank et al., 2009; Frank et al., 2008; Holowenko et al., 2002; Scott et al., 2008). Similarly in this study, a wide range of EC_{50} values were measured and no correlation between EC_{50} and liquid-phase NA concentrations could be made for the six refineries tested; however, it is noteworthy that a significant toxicity reduction is achieved by the biological treatment units in all six refineries tested, indicated by the increasing mean EC_{50} values from desalter to effluent wastewater streams (Figure 4.7).

The TCI NA salt mixture was used to determine if the Microtox® acute toxicity can be correlated to NA concentrations, despite NAs representing only a small fraction of the soluble COD. The TCI NA mixture consists of mostly 0, 1 and 2 ring NA structures with carbon numbers ranging between 10 and 25 and a weighted average MW of 271.4 Da. When prepared in deionized (DI) water, the TCI NA mixture has an EC_{50} value between 8 and 10 mg NA/L. Figure 4.8A and 4.8B shows the measured Microtox® acute toxicity and predicted toxicity effect (broken red line) based on the TCI NA Microtox® response and measured NA concentration for the influent and effluent wastewater streams from refinery A. The influent and effluent streams have measured EC_{50} values of 5.9% (0.4 mg NA/L) and 218% (10.5 mg NA/L), respectively. The influent predicted EC_{50} value based on the measured NA concentration is very low and much lower than the above-reported, measured EC_{50} value for the TCI NA mixture in DI water. In contrast, the effluent predicted EC_{50} value based on measured NA concentration is very close to the EC_{50} value of the TCI NA mixture in DI water. The difference between the measured and predicted EC_{50} values in terms of NA concentration for the influent and effluent samples is attributed to compositional differences between these two wastewater samples, brought about by the biological treatment process, leading to a lower total effluent toxicity as compared to the influent. The effluent toxicity is very low, with the undiluted effluent resulting in only 20% effect on *Vibrio fischeri*; however, the measured effluent toxicity can mainly be attributed to persistent NAs, even though they contribute less than 5% of the total effluent COD in refinery A. These results further confirm that it is not possible to correlate Microtox® acute toxicity to NA concentration in NA-bearing,

complex wastewater streams and that depending on the composition of the wastewater stream, other components will contribute significantly to the measured toxicity.

In an effort to assess the contribution of NAs to the overall toxicity of complex refinery wastewater streams, attempts were made to remove NAs from a refinery desalter brine sample using organoclays, granular and powder activated carbon and anion exchange resins. Exposure of desalter brine samples to the adsorptive media resulted in a decrease in Microtox® acute toxicity (data not shown); however, it was not possible to verify if other toxic components of the desalter brine sample, removed by adsorption in addition to NAs, contributed to the measured decreased toxicity. As a result, a correlation of Microtox® toxicity to NA molecular descriptors (i.e., MW, *Z* and *n* number, and structure) was not achieved in the present study.

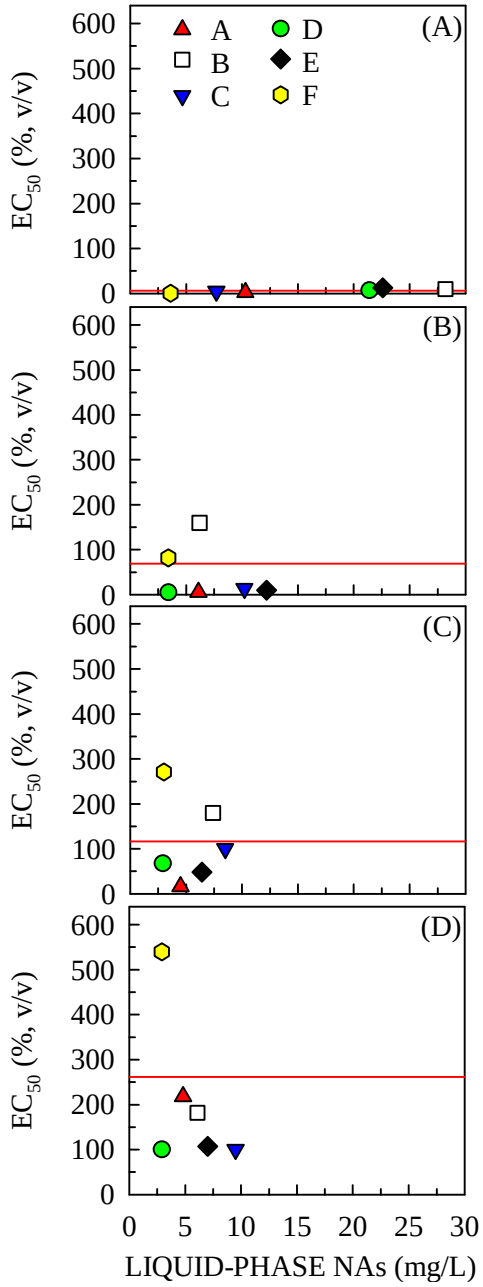


Figure 4.7. Measured 5-min Microtox® acute toxicity for desalter brine (A), influent (B), mixed liquor (C) and effluent (D) wastewater streams obtained from six refineries (A to F; horizontal red lines indicate mean EC_{50} values).

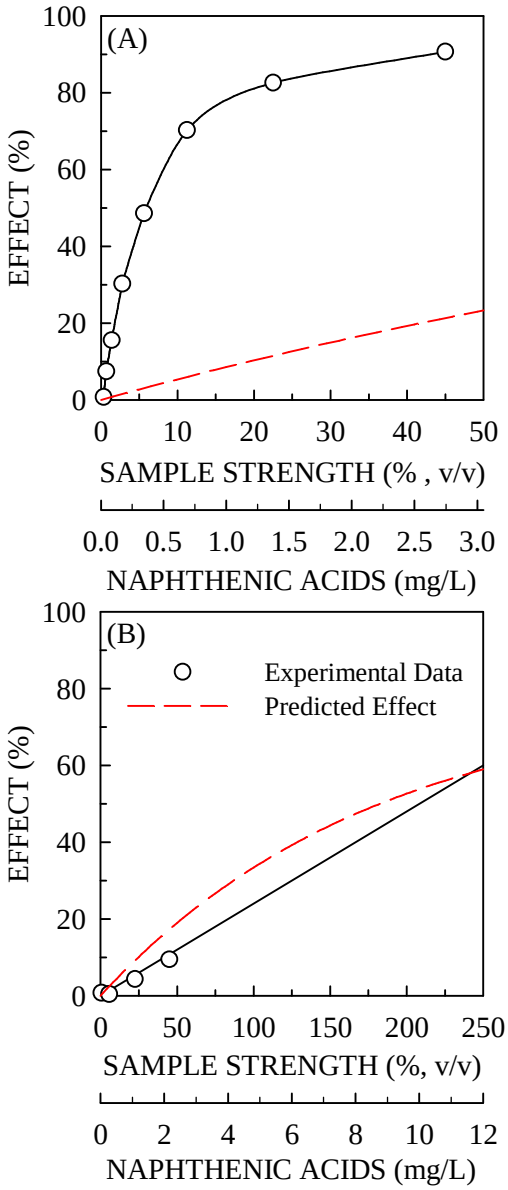


Figure 4.8. Measured and predicted 5-min Microtox® acute toxicity for influent (A) and effluent (B) wastewater streams obtained from refinery A (predicted effect based on measured NA concentration and TCI NA Microtox® acute toxicity effect).

4.3.3 NA Biotransformation and Inhibition of Refinery Microcosms

Aerobic batch tests were carried out to assess the inhibitory effect and biotransformation potential of NAs to the native refinery activated sludge microbial communities. Figure 4.9 shows the growth profiles of the six refinery microcosms with NAs as the only carbon and energy source. With the exception of the microbial community of refinery C (Figure 4.9C), which had a modest growth, all communities achieved significant growth on NAs, with the measured growth generally increasing with increasing initial NA concentration. Growth inhibition data at initial NA concentrations of 100 and 400 mg/L in each refinery microcosm, compared to respective control microcosms prepared without NAs, using the peptone/casein nutrient broth as carbon and energy source, are shown in Table 4.6. None of the activated sludge heterotrophic communities was completely inhibited up to 400 mg NA/L, though 16 to 22% and 10 to 59% growth inhibition was observed at 100 and 400 mg NA/L, respectively.

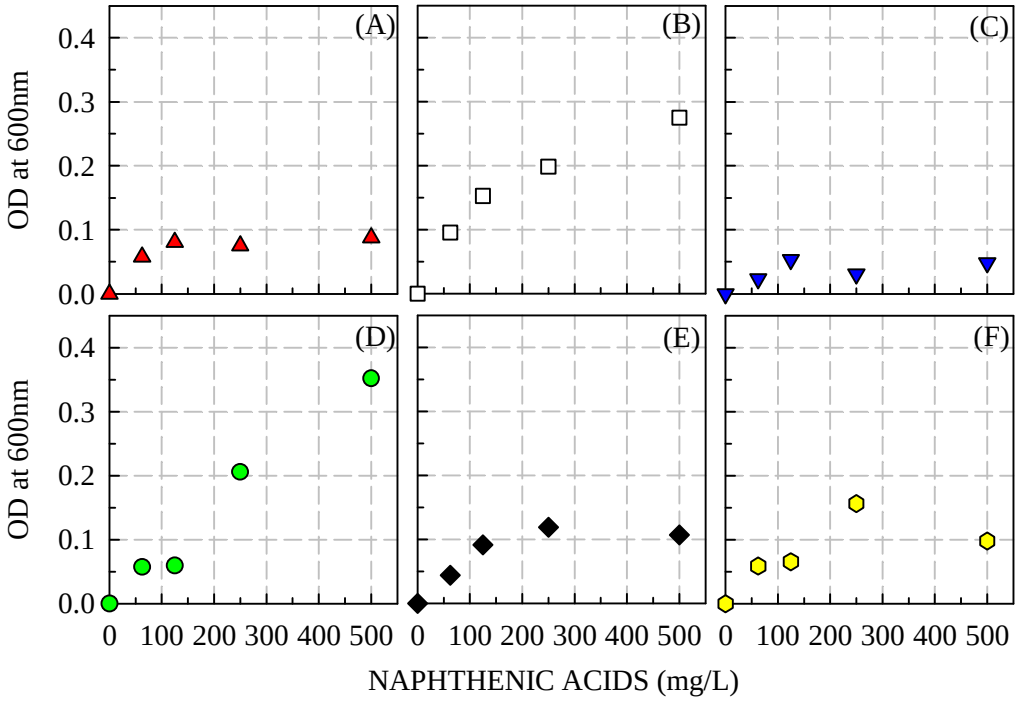


Figure 4.9. Growth profile of six aerobic refinery microcosms (A to F) at TCI NA concentrations from 0.5 to 500 mg/L with NAs serving as the sole carbon and energy source (corrected for growth of respective control microcosms containing nutrient broth and culture, but no NAs).

Table 4.6. Growth inhibition at two initial TCI NA concentrations in six refinery activated sludge microcosms.

Refinery Microcosm	Inhibition (%)[a] at an initial NA: 100 mg/L	400 mg/L
A	18.1	58.6
B	17.4	31.2
C	16.1	20.9
D	21.6	10.2
E	22.0	46.3
F	20.2	16.3

[a] Compared to respective control microcosms grown on peptone/casein nutrient broth

4.3.4 NA Desorption from Refinery Mixed Liquors

The initial total/liquid-phase NA concentrations in refinery A (with PAC) and refinery B mixed liquors were 83.4/2.4 mg NA/L and 82.6/3.6 mg NA/L, respectively. The results of the desorption assay are shown in Figure 4.10. Both mixed liquors exhibited poor desorption with greater than 80% of NAs remaining on the solid-phase at the end of the 10-day desorption period (five successive desorption steps). The desorption kinetics were very similar for both mixed liquor samples, indicating that desorption was equally limited in both mixed liquors, regardless of the presence or not of PAC.

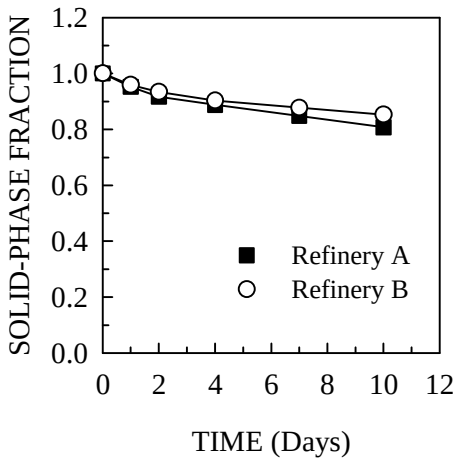


Figure 4.10. Fraction of solid-phase NAs remaining after each successive desorption step at pH 7 in two refinery activated sludge mixed liquor samples with chronically-sorbed NAs.

The initial NA congener distribution in the mixed liquors from refinery A and B is shown in Figure 4.11A and 4.12A, respectively. Acyclic and monocyclic NAs with carbon number 15 to 25 were predominant in refinery A mixed liquor, whereas dicyclic and tricyclic NAs with carbon number between 20 and 35 were predominant in refinery B mixed liquor. The results of the desorption assay indicate that, regardless of NA congener distribution, the majority of the NAs in activated sludge systems are associated with the solid-phase (greater than 80%). As a result, in refinery systems with high total NA concentrations, the liquid-phase NA concentrations remain very low, with minimum desorption even over an extended period under desorption-favorable conditions. The pKa of NAs is typically in the range of 5 to 6; therefore, increasing the pH above the tested pH 7 is unlikely to significantly increase the NA desorption rate and extent from activated sludge with chronically-sorbed NAs. A large fraction of the NAs that are not degraded by the biological treatment system will adsorb to the solids, increasing the overall NA removal and resulting in good effluent quality. Chronic exposure of biomass (and PAC) to NAs leads to accumulation and sequestration of NAs, thus contributing to their persistence as discussed below.

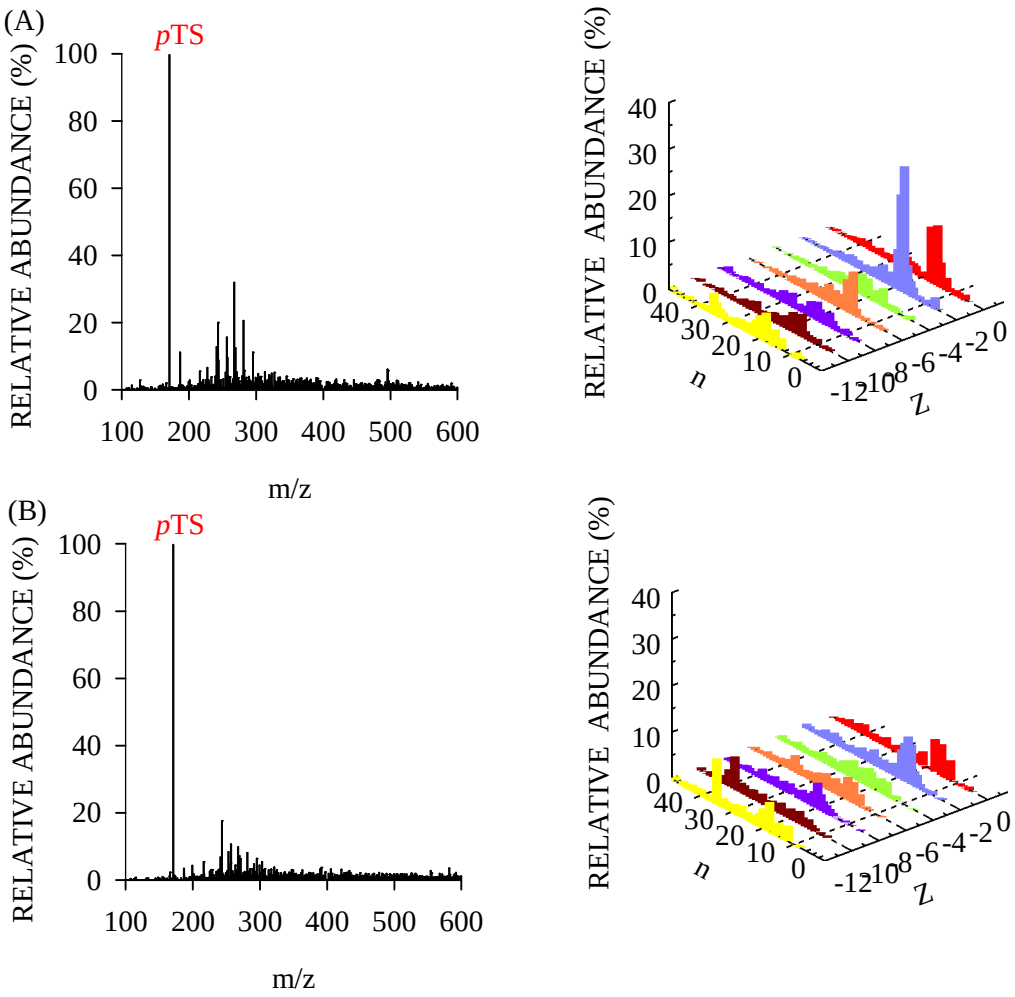


Figure 4.11. Total NA congener distribution in initial (A) and final (B) refinery A mixed liquor microcosm samples containing PAC (biotransformation assay with chronically-sorbed NAs).

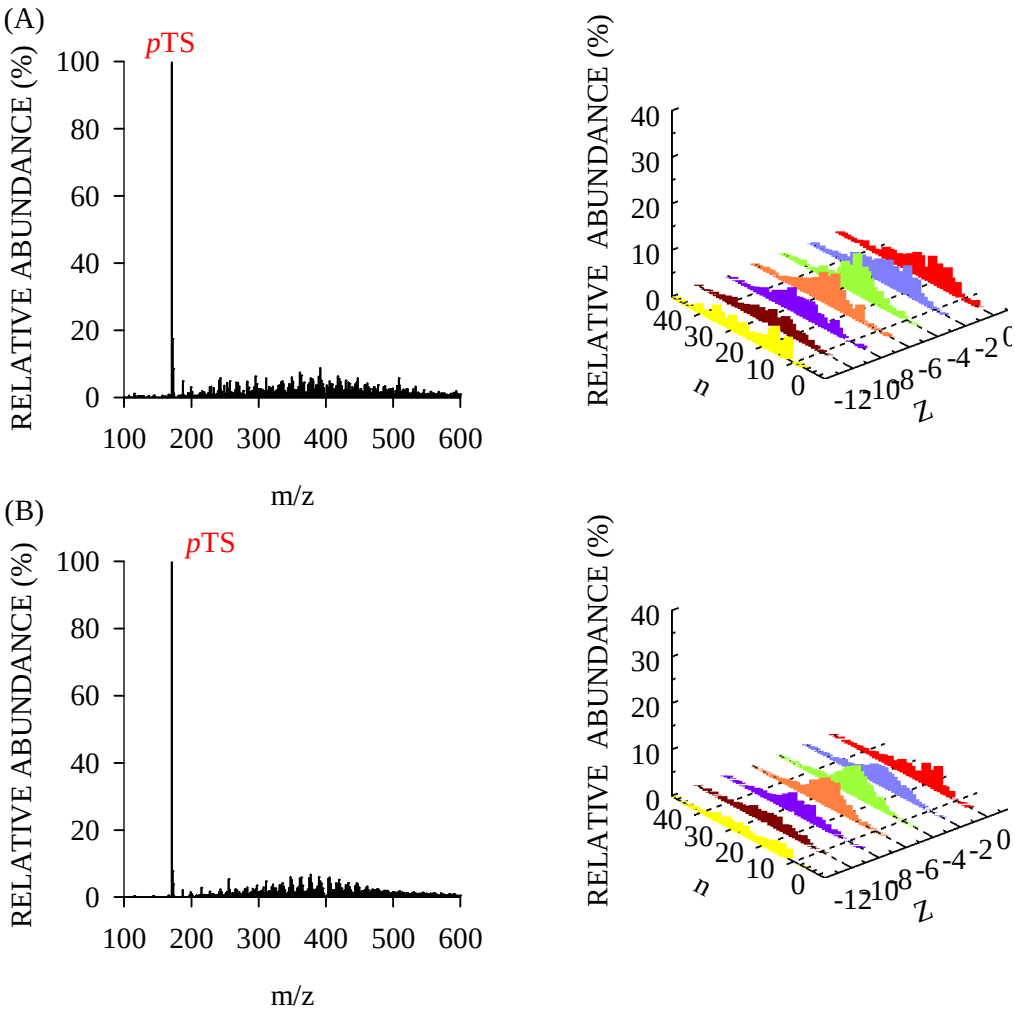


Figure 4.12. Total NA congener distribution in initial (A) and final (B) refinery B mixed liquor microcosm samples (biotransformation assay with chronically-sorbed NAs).

4.3.5 Biotransformation of Chronically-sorbed NAs

In order to determine the biotransformation potential of chronically-sorbed NAs, biotransformation assays were performed with activated sludge mixed liquors from refinery A (PAC-containing) and B (PAC-free). After the two mixed liquors were aerated and showed very low biological activity, indicating a very low level of available biodegradable substrates, the two mixed liquors were incubated at room temperature (22 to 24^{o}C) with continuous aeration, fed with a synthetic wastewater three times over the 90-day incubation period. Figure 4.13 shows the total and liquid-phase NAs as well as the soluble COD concentration over the incubation period. The NA liquid-phase concentrations were below 10 mg/L throughout the incubation period and their weighted average MW was lower by 40 to 160 Da compared to the MW of the solid-phase NAs (Table 4.7). The total NA concentration in the PAC-containing, refinery A mixed liquor decreased slowly, reaching an extent of NA degradation of 33% by the end of the 90-day incubation. In contrast, the total NA concentration in the PAC-free, refinery B mixed liquor decreased by 30% within the first 15 days of incubation and by 61% by the end of the 90-day incubation. The bi-phasic NA degradation pattern in the PAC-free mixed liquor is assumed to be due to NA fractions with a variable degree of bioavailability. On the other hand, the presence of PAC in the mixed liquor of refinery A, with its superior NA adsorption capacity compared to biomass, resulted in a much lower NA bioavailability. In view of the potential negative impact that degradable organic substrates may have on the NA degradation, long starvation periods between exogenous carbon amendments were maintained. However, an increase in the rate of NA degradation during the starvation periods was not observed (Figure 4.13). A comparison

of the NA congener distribution in both mixed liquors initially (Figure 4.11A and 4.12A) and at the end (Figure 4.11B and 4.12B) of the incubation shows an increase in the weighted average MW, *Z*, and carbon number, again indicating that the lower MW NA fraction was more degraded compared to the higher MW NAs (Table 4.7). These results agree with previously reported findings in which lower MW NAs were preferentially degraded over higher MW NAs with increased *Z* and carbon numbers (Biryukova et al., 2007; Clemente et al., 2004; Han et al., 2008; Holowenko et al., 2002; Scott et al., 2005; Watson et al., 2002).

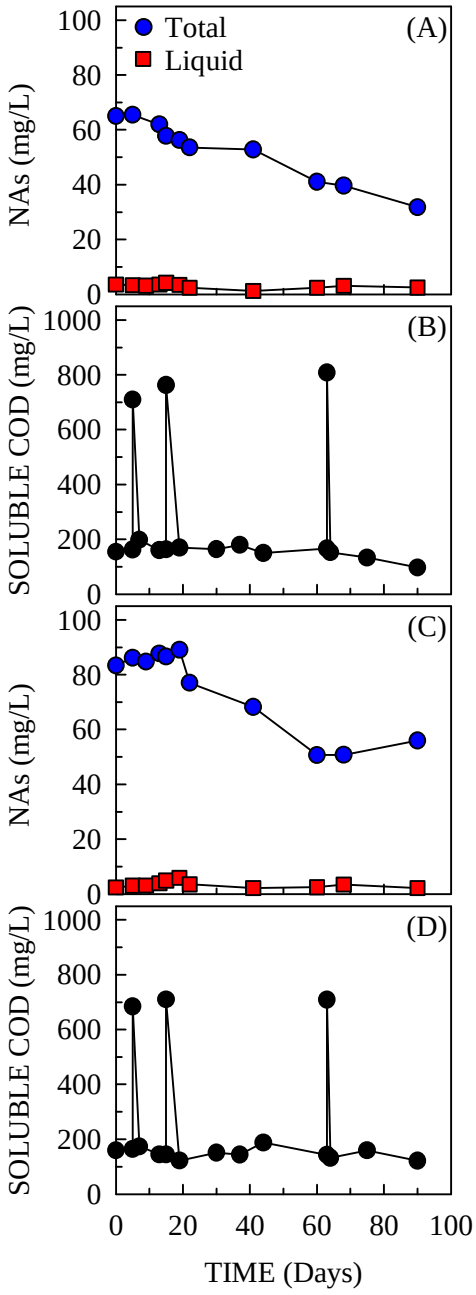


Figure 4.13. Total and liquid-phase NA concentrations and soluble COD in refinery A (A, B) PAC-containing and refinery B (C, D) PAC-free mixed liquor microcosms, respectively.

Table 4.7. Total and liquid-phase NA weighted average molecular parameters and formulae in mixed liquor microcosms from two refineries (initially and after 90 days of incubation).

Mixed Liquor	Time (Days)	Phase	*Z*	*n*	MW	Formula
Refinery A[a]	0	Total	-5.8	23.6	356.6	$C_{23.6}H_{41.4}O_2$
		Liquid	-5.4	20.4	312.6	$C_{20.4}H_{35.5}O_2$
	90	Total	-6.2	27.9	416.5	$C_{27.9}H_{49.6}O_2$
		Liquid	-6.1	24.1	362.9	$C_{24.1}H_{42.0}O_2$
Refinery B[b]	0	Total	-5.6	27.4	409.7	$C_{27.4}H_{49.1}O_2$
		Liquid	-5.8	19.7	302.6	$C_{19.7}H_{33.7}O_2$
	90	Total	-5.8	28.2	421.4	$C_{28.2}H_{50.6}O_2$
		Liquid	-4.1	16.7	262.0	$C_{16.7}H_{29.3}O_2$

[a] PAC-containing mixed liquor; [b] PAC-free mixed liquor

4.4 Summary

NAs are transferred from crude oil to the desalter brine, which is the major source of NAs in refinery wastewater. NAs were measured in all wastewater streams before, within and after the biological treatment systems (i.e., influent, mixed liquor and effluent). The activated sludge units of the six refineries included in this study were effective in lowering the effluent COD and NA concentrations as well as the effluent toxicity. Significant toxicity was measured in most wastewater streams tested, primarily in desalter brine and influent samples; however, it was not possible to correlate Microtox® acute toxicity values to NA concentrations in such complex samples. Other components contribute significantly to the measured toxicity and thus, the measured toxicity largely depends on the wastewater composition. Although NAs contributed less than 16% in the total COD of refinery effluents, the Microtox® acute toxicity effect of such effluents was primarily attributed to NAs.

Desorption and biodegradation of chronically-sorbed NAs in refinery mixed liquors were limited, resulting in sludge with elevated NA concentrations as compared to influent NA levels. The chronically-sorbed, lower MW of NAs were preferentially degraded in two mixed liquors tested in this study. The persistence of the higher MW residual NAs is likely due to a combination of molecular recalcitrance and decreased bioavailability when chronically-sorbed to the biomass and/or PAC. Therefore, refinery process water and wastewater, as well as solid wastes require proper treatment and disposal to minimize the environmental impact of NAs.

CHAPTER 5

PHASE PARTITIONING OF A NAPHTHENIC ACID MIXTURE TO BIOMASS AND ARTIFICIAL ADSORPTIVE MEDIA

5.1 Introduction

NAs are hydrophobic and partition to both natural and artificial solid media. Granular activated carbon (GAC) and powder activated carbon (PAC) have been used in treatment of refinery wastewater; however, their performance on NA removal has never been reported (IPIECA, 2010). Organoclays are also adsorptive media that could potentially be used as a tertiary treatment for hydrophobic, recalcitrant NAs; however, their ability to remove NAs is unknown.

The phase distribution in refinery samples (Chapter 4) indicated that approximately 50% of the NAs were associated with the solid phase in the desalter brine, influent and effluent wastewater streams. On the other hand, greater than 70% of the NAs were adsorbed to the biomass in the refinery mixed liquor samples and the NA partitioning was controlled by the concentration of solids in the samples. NAs accumulate on refinery mixed liquor biomass and desorption of chronically-sorbed NAs from refinery activated sludge mixed liquors was very limited. There is limited to non-existent information about the partitioning behavior of NAs to both natural (i.e., sediments, soil, sand) and artificial (i.e., organoclays, powder and granular activated carbon, resins) media.

The objective of this research was to investigate the phase partitioning behavior (i.e., kinetics and isotherms) of the TCI NA mixture to both biomass and artificial adsorptive media such as organoclays, GAC and PAC in order to determine the feasibility of using adsorption to artificial media as a tertiary treatment for recalcitrant, residual NAs.

5.2 Materials and Methods

5.2.1 Adsorptive Media and Culture Preparation

NA-enriched culture biomass and three adsorptive media including organoclays, GAC and PAC were used in adsorption kinetic, isotherm and residual NA adsorption assays. The organoclays and GAC were obtained from Ecologix Environmental Systems (Alpharetta, Georgia, USA). The organoclays (Product Number MCM-830P) were gray to tan granules of modified montmorillonite clay with a cationic quaternary amine salt surface with a specific gravity of 2.0 to 2.2 and a void volume of 35 to 45%. The GAC was coconut shell GAC 8x30 mesh (Product Number LCGAC8x30) with a surface area of 850 to 1350 m^2/g and an apparent density of 0.4 to 0.54 g/mL (Ecologix Environmental Systems). The PAC was provided by a US refinery and its properties were not reported. The NA-enriched biomass was washed three times with 10 mM phosphate buffer, as previously described in Chapter 6, Section 6.2.2, before use in the adsorption assays.

5.2.2 Adsorption Kinetic Assay

A 100 mg NA/L solution was prepared in 200 mg/L sodium azide-amended aerobic culture media at pH 7 as described in Chapter 6, Section 6.2.1. Portions of 100 mL of this solution were transferred to 250-mL Erlenmeyer flasks containing organoclays, GAC, PAC and biomass at concentrations of 1, 0.2, 0.05 and 0.3 g/L, respectively. The initial mass of adsorptive media was chosen based on preliminary assays (data not shown). Four individual sacrificial flasks having the same composition were prepared with each adsorptive media. A control flask was also prepared with 100 mg NA/L and no adsorptive media. The flasks were agitated on an orbital shaker at 190 rpm at room temperature (22 to 24°C) and sampled at predetermined times of 0, 1, 3, 6 and 24 hours. The liquid-phase NA concentration was measured and the solid-phase NAs were determined by difference between the total and liquid-phase NAs.

5.2.3 Adsorption Isotherm Assay

The adsorption of the TCI NA mixture to each of the four adsorptive media (organoclays, GAC, PAC and NA-enriched biomass) was tested after a 24 hour equilibration time, which exceeded the time to reach equilibrium determined by the above described kinetic assay. Triplicate samples were prepared in 250-mL Erlenmeyer flasks with 200 mg/L sodium azide-amended aerobic culture media at pH 7 (see Chapter 6, Section 6.2.1) and organoclays, GAC, PAC and biomass at concentrations of 1, 0.2, 0.05 and 0.3 g/L, respectively, were amended with the TCI NA mixture at concentrations of 10, 25, 50, 100, 200 and 400 mg/L. The flasks were covered and agitated on an orbital shaker at 190 rpm for 24 hours at room temperature (22 to 24°C). A control flask was also prepared at

each NA concentration, containing NAs and no adsorptive media. The phase distribution of NAs was determined at the end of the 24-hour batch adsorption assay by measuring the liquid-phase NA concentration and calculating the NA adsorbed to each media by the difference between total and liquid-phase NAs.

The Freundlich isotherm was used to describe the NA adsorption equilibrium data as follows: $q_e = K_F C_e^{n_F}$ where q_e is the NA concentration adsorbed to each adsorptive media/biomass at equilibrium (mg NA/g adsorptive media), C_e is the NA concentration in the liquid phase at equilibrium (mg NA/L), K_F is the adsorption capacity factor ((mg/g adsorptive media)(L/mg)^{nF}), and n_F is the Freundlich intensity parameter (exponent). The experimental data were fitted to the Freundlich isotherm equation and the adsorption parameter values (K_F and n_F) were estimated using non-linear regression (Sigma Plot, Version 8.02 software; Systat Software Inc., San Jose, CA, USA).

5.2.4 Adsorption of Residual NAs

A batch assay was performed to investigate the adsorption of residual NAs (discussed in Chapter 6) to the adsorptive media used in this study (organoclays, GAC and PAC). A portion of the NA-enriched culture was centrifuged at 5000 rpm and the supernatant (pH = 6.8) was filtered through a Whatman GF/C glass fiber filters (47 mm diameter and 1.2 μm nominal pore size; Whatman, Florham Park, NJ), amended with 200 mg/L sodium azide and added to 250-mL Erlenmeyer flasks containing different amounts of adsorptive media ranging from 0.05 to 1 g. A control series was also set up containing the sodium azide-amended culture supernatant and no adsorptive media. The flasks were loosely capped and agitated on an orbital shaker at 190 rpm for 24 hours at room temperature (22

to 24°C) before measuring the liquid-phase and control NA concentration. The solid-phase NA concentration was determined by difference between the total and liquid-phase NAs.

5.3 Results and Discussion

5.3.1 Adsorption Kinetics

The adsorption kinetics of the TCI NA mixture to the various adsorptive media and biomass were determined over 24 hours and the results are shown in Figure 5.1. The time required to reach equilibrium for all adsorptive media and biomass was less than 6 hours. Adsorption equilibrium was achieved with PAC, GAC and NA-enriched biomass within 1 hour. The rate at which equilibrium was reached between the NA mixture and organoclays was significantly lower, in between 1 and 6 hours.

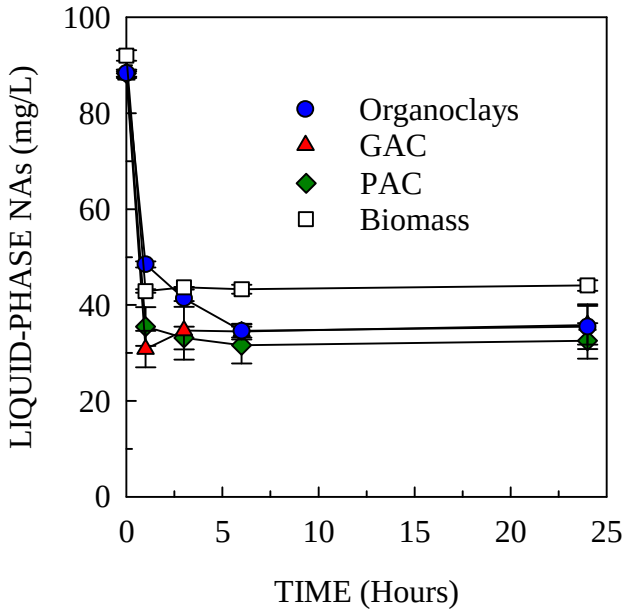


Figure 5.1. Adsorption kinetics for the non-biotreated TCI NA mixture to organoclays, GAC, PAC and NA-enriched biomass (Error bars represent mean values ± one standard deviation, n = 3).

5.3.2 Freundlich Isotherms

Adsorption kinetics showed that equilibrium to all adsorptive media and NA-enriched biomass was reached within 6 hours or less (see above). Therefore, adsorption of the TCI NA mixture to the four adsorptive media was measured at concentrations up to 400 mg/L after 24 hours equilibration. The Freundlich isotherm was used to describe the NA isotherms and was a good representation of the adsorption data as indicated by R^2 values greater than 0.954 (Figure 5.2). The estimated Freundlich adsorption capacity factor, K_F, and intensity parameter, n_F, values are summarized in Table 5.1. PAC has a higher adsorption capacity for the TCI NA mixture than GAC, organoclays and NA-enriched biomass as indicated by the high K_F value of 27.11. The observed difference in K_F values is likely due to an increased surface area of the PAC, which has a significantly smaller particle size than both organoclays and GAC, resulting in a larger adsorption surface for NAs (Snoeyink and Summers, 1999). All n_F values were less than 1, indicating that NAs are bound with weaker free energies as the solid-phase mass increases and thus, making it more difficult for NAs to adsorb (Schwarzenbach et al., 2003). NA adsorption to GAC was non-specific and the relative congener NA distribution of the liquid-phase NAs was very similar to the initial TCI NA distribution. The organoclays, PAC and biomass preferentially adsorbed the higher carbon number NAs; however, they were nonspecific in adsorption of different ring numbers, as indicated by the similar *Z* numbers compared to the congener NA distribution of the initial TCI NA mixture (Figure 5.3, Table 5.2).

There is very limited information available about NA partitioning to both natural and artificial solid phases. The phase distribution in the refinery samples (Chapter 4) indicated that chronic exposure of biomass (and PAC) to NAs leads to accumulation and

sequestration of NAs; however, NA desorption is very limited. Other studies have estimated the isotherms for oil sands NAs and model NA compounds to soils taken from an oil sands mining site in Alberta, Canada and found that the oil sands NAs rapidly adsorbed to the soil and the model compounds were weakly adsorbed and found that, in contrast to the Freundlich isotherms estimated in the present study, the oil sands NAs exhibited linear isotherms (Janfada et al., 2006 ; Peng et al., 2002). There is no information available about the partitioning behavior of NAs to PAC and GAC, despite the fact they are used in refinery wastewater treatment systems.

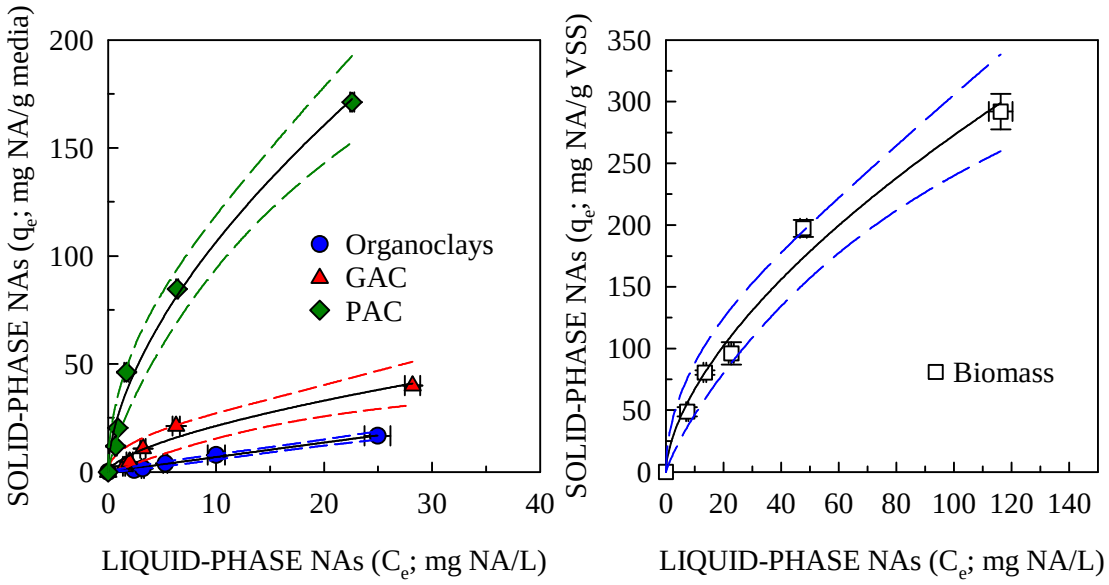


Figure 5.2. Freundlich isotherms for the adsorption of the non-biotreated TCI NA mixture to organoclays, GAC, PAC and NA-enriched biomass (dashed lines indicate 95% confidence intervals; error bars represent mean ± one standard deviation, *n* = 3).

Table 5.1. Freundlich capacity factor (K_F) and intensity parameter (n_F) values for TCI NAs to various adsorptive media.

Media	K_F	n_F	R^2
Organoclays	0.72±0.16[a]	0.98±0.16	0.989
GAC	4.95±1.43	0.63±0.10	0.954
PAC	27.11±3.52	0.59±0.05	0.989
Biomass	9.41±2.45	0.78±0.07	0.988

[a] Mean ± standard error ($n \geq 3$)

Table 5.2. NA congener distribution in the initial, non-biotreated TCI NA sample and the liquid-phase after adsorption equilibrium with each adsorptive media.

Sample	**Average *Z***	**Average *n***	**Average MW**	**Formula**
TCI (Initial)	-3.7	15.8	249.4	$C_{15.8}H_{27.9}O_2$
Organoclays	-3.8	13.3	214.8	$C_{13.3}H_{22.8}O_2$
GAC	-4.0	17.1	267.5	$C_{17.1}H_{30.2}O_2$
PAC	-3.7	13.4	216.4	$C_{13.4}H_{23.2}O_2$
Biomass	-3.6	14.4	229.8	$C_{14.4}H_{25.2}O_2$

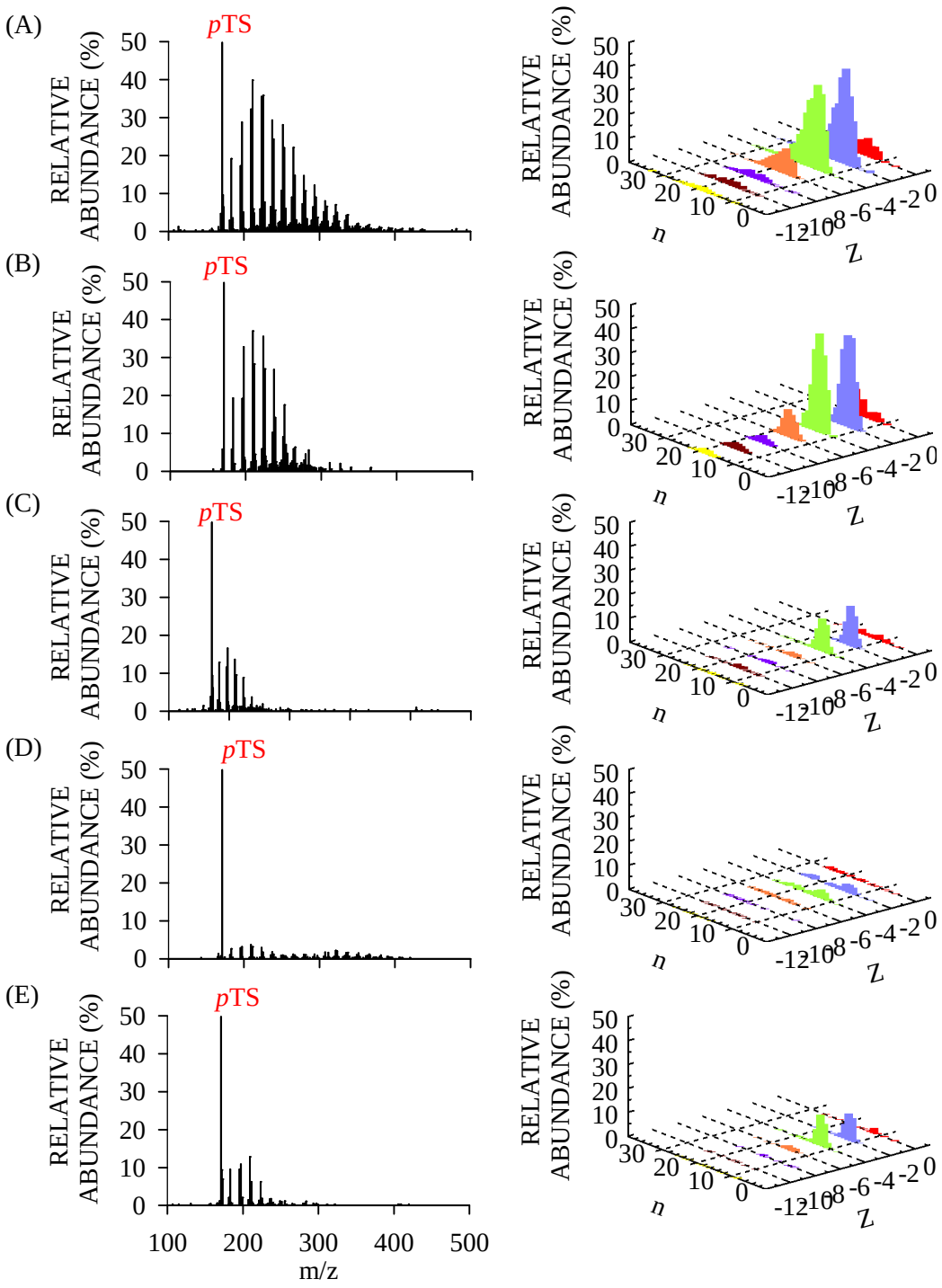


Figure 5.3. Liquid-phase NA congener distribution in 50 mg/L initial (A), biomass (B), organoclays (C), GAC (D) and PAC (E) adsorption samples at the end of the 24 hour equilibration period.

5.3.3 Residual NA Adsorption

Although the NA mixture was degraded under aerobic conditions, a residual fraction remained under all conditions studied, even after long incubation periods (Chapter 6). Thus, an alternative treatment method must be devised for complete removal of NAs. To investigate if the artificial adsorptive media can be used as a possible tertiary treatment for NA removal, batch adsorption assays were performed using the organoclays, GAC and PAC and the residual NA-bearing culture supernatant. Figure 5.4 shows the liquid-phase NA fraction (%) of residual NAs after adsorption to organoclays, GAC and PAC compared to the initial residual NA concentration (before adsorption). The results indicate that although organoclays were capable of adsorbing a fraction of the non-biotreated TCI NA mixture (see Section 5.3.2, above), the residual NAs are not adsorbed up to an organoclay concentration of 10 g/L. In contrast, the GAC and PAC are good adsorbents for removing the residual NAs as they removed 57 and 100% of the residual NAs at concentrations of 5 g adsorbent/L, respectively. PAC is a better adsorbent than GAC and removed more than 85% of the residual NAs with a PAC concentration of 2 g/L, while only 80% was removed at a GAC concentration of 10 g/L. Table 5.3 shows the liquid-phase NA congener distribution in the residual NA sample before and after adsorption to the artificial media. These results indicate that although the PAC preferentially adsorbed the higher carbon number NAs and they were not specific as to the *Z* number range when adsorbing the non-biotreated TCI NA mixture, adsorption of residual NAs was preferential to more cyclic NA structures (Table 5.3). These results indicate that activated carbon, especially PAC, is complementary to microbial biodegradation and a combination of physico-chemical and biological processes may be

necessary to achieve the complete removal of NAs from the petroleum refineries effluents.

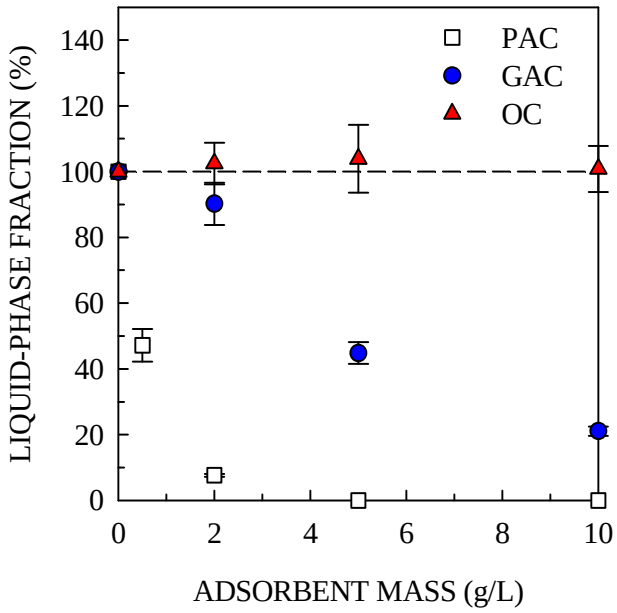


Figure 5.4. Liquid-phase NA fraction of post-biotreatment residual NAs after adsorption to organoclays, GAC and PAC (adsorbent mass indicates mass added to 100 mL liquid volume; error bars represent mean ± one standard deviation, n = 3).

Figure 5.3. NA congener distribution in the residual NAs before and after adsorption to organoclays, GAC and PAC.

Adsorbent	**Adsorbent Mass (g)**	**Average *Z***	**Average *n***	**Average MW**	**Formula**
Residual NAs	-	-5.5	17.3	268.1	$C_{17.3}H_{29.0}O_2$
Organoclays	0.2	-5.3	17.1	265.7	$C_{17.1}H_{28.8}O_2$
	0.5	-5.3	16.9	263.8	$C_{16.9}H_{28.6}O_2$
	1	-5.2	16.7	260.3	$C_{16.7}H_{28.2}O_2$
GAC	0.2	-5	17.1	266.6	$C_{17.1}H_{29.2}O_2$
	0.5	-5.3	17	264.4	$C_{17.0}H_{28.7}O_2$
	1	-4.7	16.6	259.9	$C_{16.6}H_{28.5}O_2$
PAC	0.05	-5.1	16.4	256.5	$C_{16.4}H_{27.7}O_2$
	0.2	-3.9	15.4	244.2	$C_{15.4}H_{26.9}O_2$

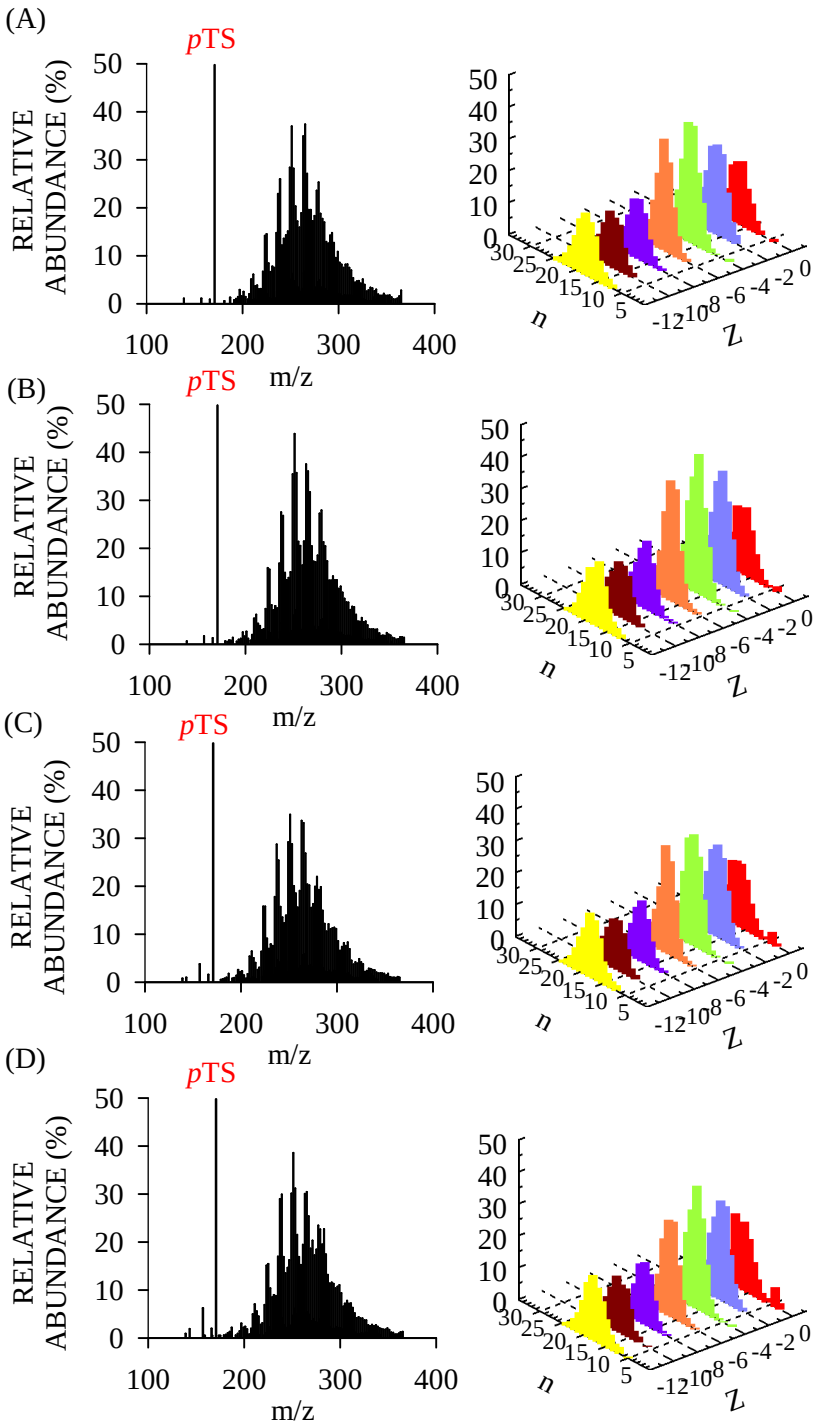


Figure 5.5. Liquid-phase NA congener distribution of initial residual NAs (A) and residual NAs after adsorption to 0.2 (B), 0.5 (C) and 1 g (D) of organoclays in 100 mL liquid volume.

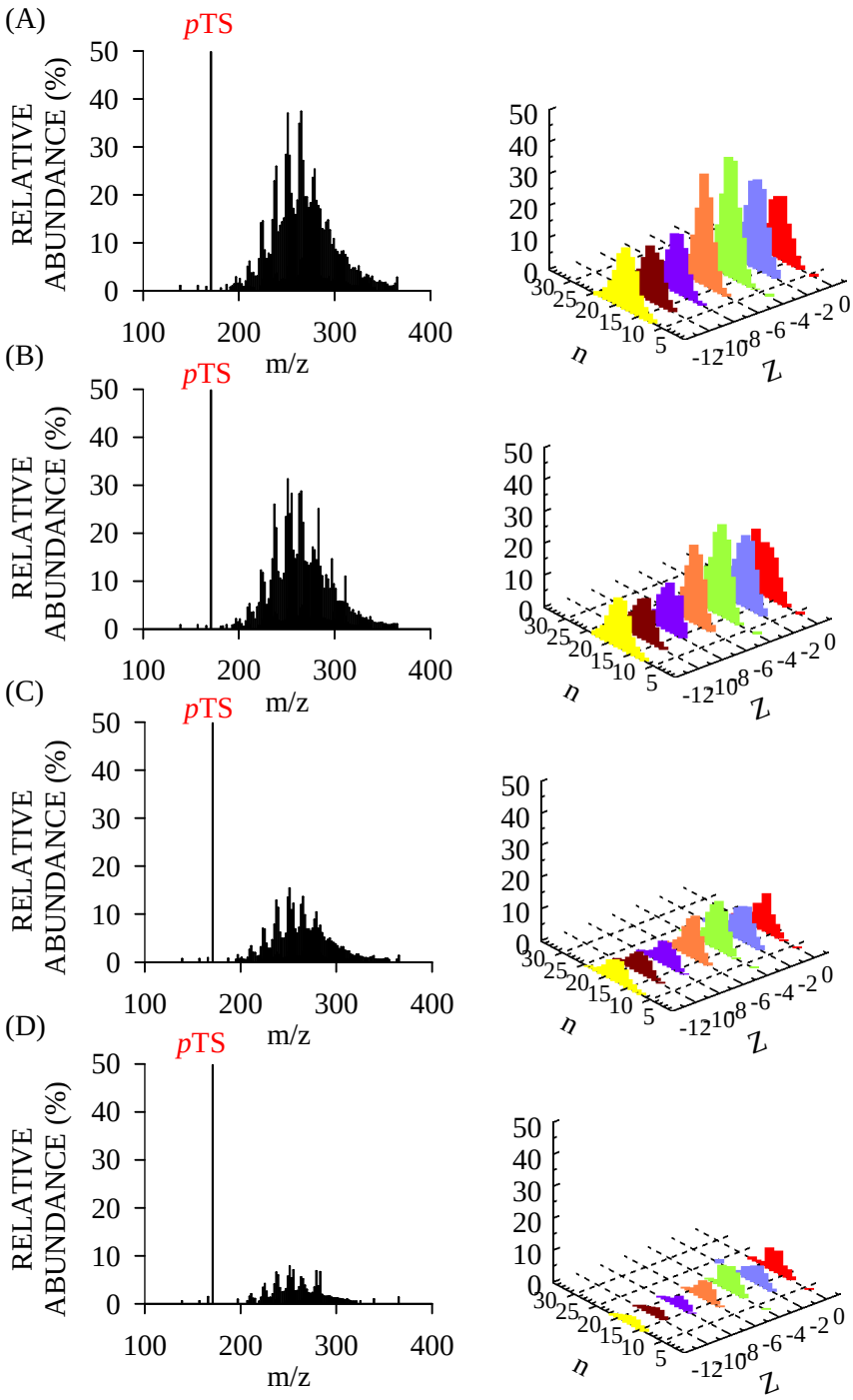


Figure 5.6. Liquid-phase NA congener distribution of initial residual NAs (A) and residual NAs after adsorption to 0.2 (B), 0.5 (C) and 1 g (D) of GAC in 100 mL liquid volume.

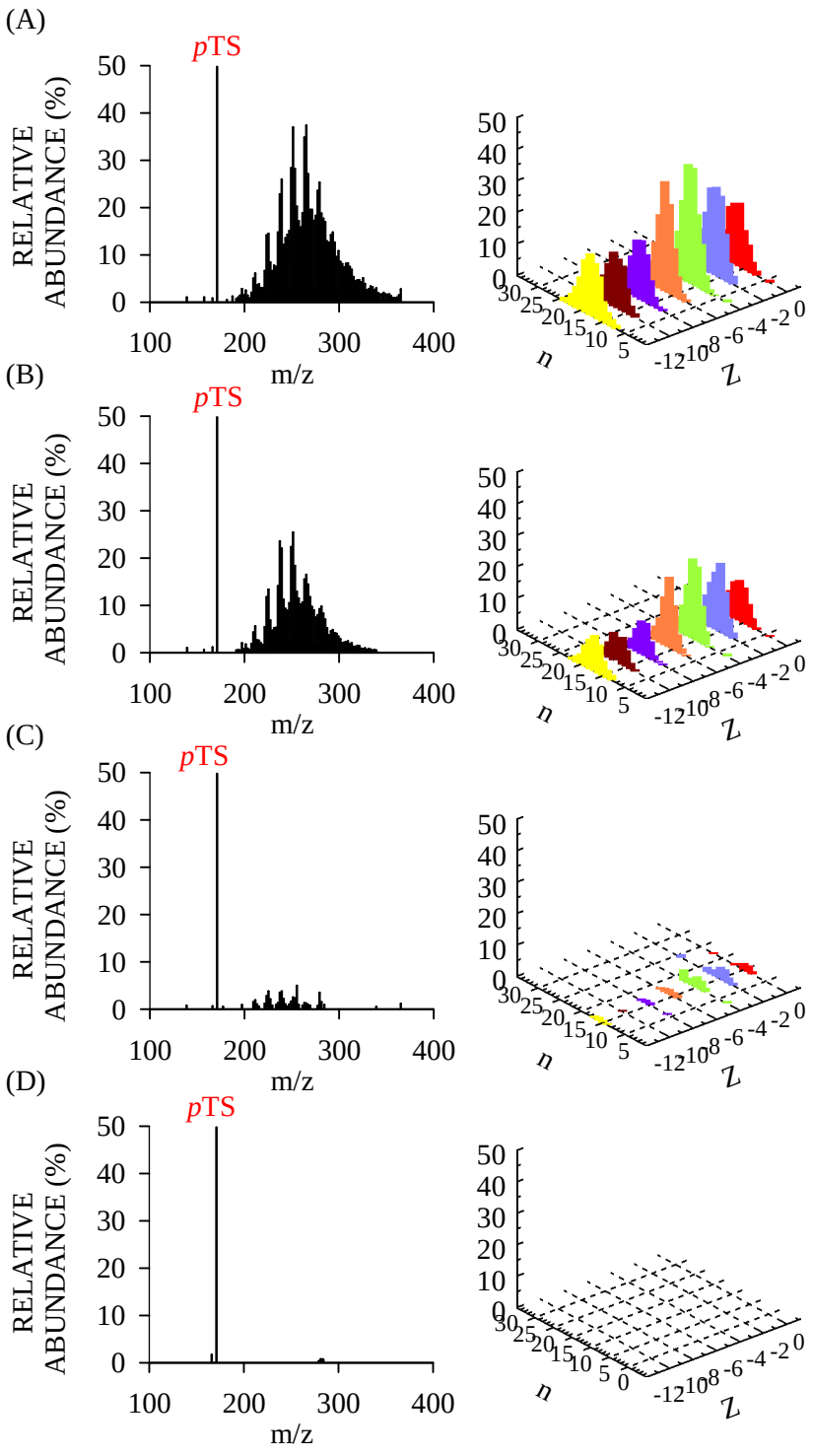


Figure 5.7. Liquid-phase NA congener distribution of initial residual NAs (A) and residual NAs after adsorption to 0.05 (B), 0.2 (C) and 0.5 g (D) of PAC in 100 mL liquid volume.

5.4 Summary

Despite the fact that activated carbon is commonly added to refinery activated sludge or tertiary treatment units, there are no reports on the partitioning behavior of NAs or the effectiveness of artificial media in removing NAs from refinery wastewater streams. The results of this study indicate that activated carbon, especially PAC, is the best adsorbent for the removal of NAs, especially those that are not easily biodegraded. The aerobic bioassays performed in this study (see Chapter 6) and previously published literature data showed that the higher molecular weight NAs are not biodegradable; thus, combination of both physical and biological may be required to treat NA-bearing wastewaters to completely remove NAs Biryukova et al., 2007; Clemente et al., 2004; Han et al., 2008; Holowenko et al., 2002; Scott et al., 2005; Watson et al., 2002). The results of the adsorption assays using both non-biotreated and residual NAs indicate that the higher molecular weight NA fraction is preferentially adsorbed to PAC. Thus, a post-biotreatment PAC filter is a promising tertiary treatment option for the removal of recalcitrant NAs.

CHAPTER 6

INHIBITION AND BIOTRANSFORMATION POTENTIAL OF NAPHTHENIC ACIDS UNDER DIFFERENT ELECTRON ACCEPTING CONDITIONS

6.1 Introduction

NAs can be degraded under aerobic conditions; however, many NAs are persistent and remain in the wastewater systems even after biotreatment (Clemente and Fedorak, 2005; Del Rio et al., 2006; Quagraine et al., 2005a; Scott et al., 2005). The toxicity of NAs is relatively well-studied; however, there is little to no information available about the inhibitory effect of NAs to biological processes, such as nitrification, denitrification and methanogenesis, and the biotransformation potential of NAs under these conditions. NAs are found in crude oil, especially in heavy crudes, and transferred to the desalter brine in the crude oil desalting process, which is the major source of NAs in refinery wastewater (see Chapter 4). Refinery wastewater treatment plants most commonly utilize aerobic activated sludge and attached growth processes to treat refinery process waters (IPIECA, 2010). Ammonia has been reported in oil sands and refinery process wastewater at concentrations up to 80 mg/L (Allen, 2008; Fang et al., 1993). However, it is unknown if NAs inhibit ammonia removal in the aerobic activated sludge units of refinery wastewater treatment plants.

In addition to refinery wastewater treatment plants, NAs are frequently found in oil sands tailings ponds and the environment. NAs are hydrophobic, adsorbing to organic

matter, which is then deposited to the bottom of oil sands tailings ponds and aquatic natural sediments, where in both cases anoxic and anaerobic conditions exist. Methane production in oil sands tailings ponds is significant; however the substrates utilized for methane production are not completely understood. Information on the fate and effect of NAs under anoxic and anaerobic conditions is thus very limited (Holowenko et al., 2000; Siddique et al., 2011; Whitby, 2010). NAs from oil sands process waters caused short-term inhibition of methanogenesis from H_2 or acetate and methane yields measured in microcosms amended with two surrogate NAs suggested complete mineralization under anaerobic conditions (Holowenko et al., 2001).

Although NAs are found in refinery process waters and impacted natural systems, there is limited information about the inhibitory effect and biotransformation potential of NAs in such systems and the environment. The objective of this study was to assess the inhibitory effect and biotransformation potential of a commercial NA mixture under nitrifying, denitrifying and fermentative/methanogenic conditions. Bioassays were performed using laboratory developed nitrifying, denitrifying and methanogenic cultures at a range of NA concentrations, with and without an organic co-substrate.

6.2 Theoretical Considerations

6.2.1 Free Energy Calculations

The standard Gibb's free energies of formation ($\Delta G_f^{0'}$) of NAs have not been reported in the literature and were calculated using the group contribution method developed by Mavrovouniotis (1990). The $\Delta G_f^{0'}$ of the TCI NA mixture (see Section 6.3.1, below) was estimated using the average formula for TCI NAs, $C_{17.4}H_{30.9}O_2$, and assuming various

possible NA structures with Z = 0, -2, -4, -6 and -8. The TCI NA $\Delta G_f^{0'}$ was used to estimate the free energies of reaction ($\Delta G_r^{0'}$) in order to determine if biodegradation of the TCI NA mixture is thermodynamically feasible under aerobic, denitrifying and methanogenic conditions

The standard Gibb's free energies of formation **($\Delta G_f^{0'}$)** of NAs were estimated using the group contribution method developed by Mavrovouniotis (1990) using the following equation:

$$\Delta G_f^{0'} = \sum_1^i a_i \Delta G_{f,i}^{0'} \qquad (6.1)$$

where, a is the number of occurrence of group *i* and $\Delta G_{f,i}^{0'}$ is the standard Gibb's free energy of formation of group *i*. The calculated group $\Delta G_{f,i}^{0'}$ values are given in Mavrovouniotis (1990). The standard Gibb's free energies of half-reactions ($\Delta G_r^{0'}$) under aerobic, nitrate-reducing and methanogenic conditions were obtained from Rittmann and McCarty (2001). The standard Gibb's free energies of NA reactions (without cell synthesis) were calculated by using the following equation (Rittmann and McCarty, 2001):

$$\Delta G_r^{0'} = \sum_1^j p_j \Delta G_{f,p_j}^{0'} - \sum_1^k r_k \Delta G_{f,r_k}^{0'} \qquad (6.2)$$

where, p is the molar fraction of product j; r is the molar fraction of reactant k; $\Delta G_{f,p}^{0'}$ and $\Delta G_{f,r}^{0'}$ are the standard Gibb's free energies of formation of products and reactants, respectively.

The calculated free energies of reaction for NAs ranged from -103.4 to -106.9, -96.9 to -100.4 and -1.2 to -4.6 kJ/electron equivalent (e^- eq) under aerobic, nitrate-

reducing and methanogenic conditions, respectively and are summarized in Table 6.1. The negative free energy values indicate that biodegradation of NAs is thermodynamically feasible under all three conditions (Figure 6.1).

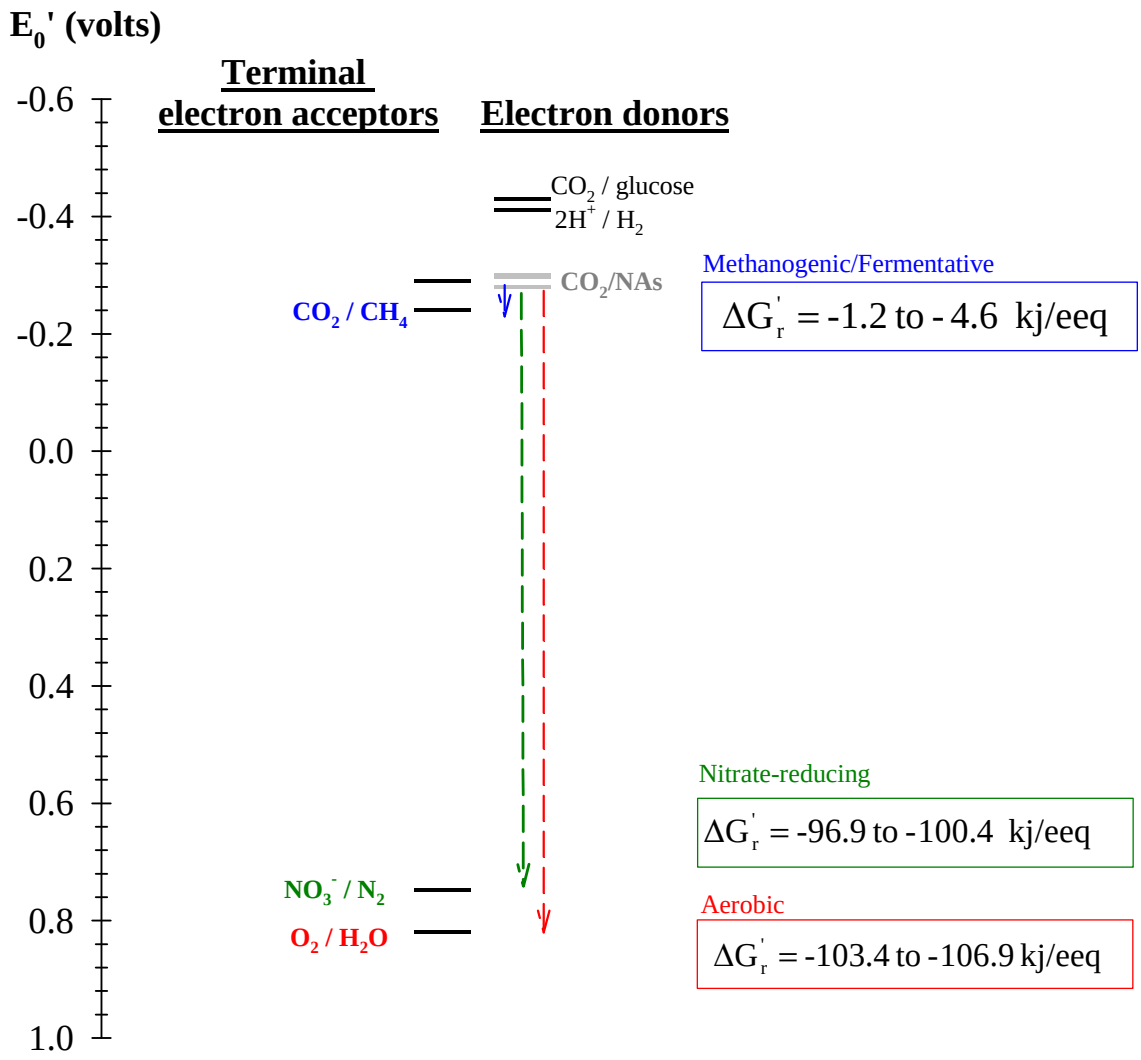


Figure 6.1. Schematic showing the positions of CO_2/NAs on the electron tower and the standard free energy upon complete oxidation of NAs to CO_2 under aerobic, nitrate-reducing and anaerobic/fermentative conditions.

Table 6.1. Standard Gibb's free energies of formation for TCI NA mixture and standard Gibb's free energies of reaction under aerobic, nitrate-reducing and fermentative/methanogenic conditions.

NAs	$\Delta G_f^{0'}$	$\Delta G_{f,r}^{0'}$ **(kJ/e⁻eq)**		
***Z* number**	**(kJ/mol)**	**Aerobic**	**Nitrate-reducing**	**Fermentative/Methanogenic**
0	-262.76	-106.87	-100.35	-4.61
-2	-319.25	-106.04	-99.52	-3.78
-4	-371.13	-105.22	-98.70	-2.96
-6	-424.27	-104.35	-97.83	-2.09
-8	-476.99	-103.45	-96.93	-1.19

6.2.2 Nitrification Simulation

The nitrification rates under the experimental conditions of this study were estimated by modeling nitrification as a two-step process, i.e., ammonia oxidation to nitrite followed by nitrite oxidation to nitrate, assuming ammonia to be the only rate-limiting substrate. The following four differential equations were considered for ammonia consumption, nitrite formation/consumption, nitrate formation, and biomass growth of nitrifying bacteria:

$$\frac{dS_{nh3}}{dt} = -\left(\frac{k_{nh3}S_{nh3}}{K_{snh3}+S_{nh3}}\right)X_a \tag{6.3}$$

$$\frac{dS_{no2}}{dt} = \left(\frac{k_{nh3}S_{nh3}}{K_{snh3}+S_{nh3}}\right)X_a - \left(\frac{k_{no2}S_{no2}}{K_{sno2}+S_{no2}}\right)X_a \tag{6.4}$$

$$\frac{dS_{no3}}{dt} = \left(\frac{k_{no2}S_{no2}}{K_{sno2}+S_{no2}}\right)X_a \tag{6.5}$$

$$\frac{dX_a}{dt} = \frac{Y_a k_{nh3} X_a S_{nh3}}{K_{snh3}+S_{nh3}} + \frac{Y_n k_{no2} X_a S_{no2}}{K_{no2}+S_{no2}} - bX_a \tag{6.6}$$

where, S_{nh3}, S_{no2} and S_{no3} are ammonia, nitrite and nitrate concentrations (mg N/L), respectively; t is time (days); k_{nh3} is the maximum specific ammonia utilization rate (MAUR; mg N/g VSS·day); k_{no2} is the maximum specific nitrite utilization rate (MNUR; mg N/g VSS·day); K_{snh3} is the half-saturation constant for ammonia (mg N/L); K_{sno2} is the half-saturation constant for nitrite (mg N/L); X_a is the active nitrifying biomass concentration (g VSS/L); Y_a and Y_n are the true yield coefficients for ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (mg VSS/mg N), respectively; and b is the biomass decay coefficient (d^{-1}). Simulations were used to determine the extent of inhibition in order to compare the effect of NAs on the different cultures under the experimental conditions of this study. Values for K_{snh3}, K_{sno2}, Y_a, Y_n, and b were chosen

based on stoichiometry, bioenergetics and literature and were kept constant throughout all simulations. The values chosen for K_{snh3}, K_{sno2}, Y_a, Y_n, and b were 1.25 mg N/L, 2 mg N/L, 0.27 mg VSS/mg N, 0.08 mg VSS/mg N and 0.1 day^{-1}, respectively (Rittmann and McCarty, 2001). Then, the MAUR and MNUR values were estimated.

The fraction of the active autotrophic biomass in the mixed nitrifying culture was determined by the oxygen uptake rate (OUR) values of the seed culture obtained when only dextrin or only ammonia was provided as substrate (Ginestet et al., 1998). Based on this procedure, the initial autotrophic biomass constituted 20% of the culture total biomass, corresponding to 0.14 g VSS/L. On the other hand, the heterotrophic biomass in the enriched nitrifying culture was found to be negligible (i.e., below 3%). The biomass concentrations of the nitrosofying (AOB) and nitrifying (NOB) bacteria were initially considered separately for simulations with the enriched nitrifying culture series.

For the enriched nitrifying culture, the ratio of AOB to NOB in the seed biomass was determined by the oxygen uptake rate (OUR) values of the seed culture obtained under the following conditions: 1) without any substrate (for endogenous OUR); 2) only ammonia as substrate; 3) only ammonia as substrate and nitrite oxidation inhibited by the addition of 24 mM sodium azide; 4) only nitrite as substrate; and 5) only glucose as substrate to quantify any heterotrophic population in the mixed enriched culture (Ginestet et al., 1998). The OUR values obtained under endogenous and glucose-amendment conditions were very similar. Therefore, the fraction of heterotrophs in the enriched nitrifying culture was considered to be negligible. Additionally, based on the OUR results, the seed culture consisted of 70% AOB and 30% of NOB. Initially, the biomass

concentrations of ammonia oxidizing (AOB) and nitrite oxidizing (NOB) bacteria were considered separately in the simulation, as follows:

$$\frac{dS_{nh3}}{dt} = -\left(\frac{k_{nh3}S_{nh3}}{K_{snh3}+S_{nh3}}\right)X_a \tag{6.8}$$

$$\frac{dS_{no2}}{dt} = \left(\frac{k_{nh3}S_{nh3}}{K_{snh3}+S_{nh3}}\right)X_a - \left(\frac{k_{no2}S_{no2}}{K_{sno2}+S_{no2}}\right)X_n \tag{6.9}$$

$$\frac{dS_{no3}}{dt} = \left(\frac{k_{no2}S_{no2}}{K_{sno2}+S_{no2}}\right)X_n \tag{6.10}$$

$$\frac{dX_a}{dt} = \frac{Y_a k_{nh3} X_a S_{nh3}}{K_{snh3}+S_{nh3}} - b_a X_a \tag{6.11}$$

$$\frac{dX_n}{dt} = \frac{Y_n k_{no2} X_n S_{no2}}{K_{no2}+S_{no2}} - b_n X_n \tag{6.12}$$

where, X_a is the AOB biomass concentration (g VSS/L) and X_n is the NOB biomass concentration. In the biomass growth equation, Y_a and Y_n are the true yield coefficients for AOB and NOB (mg VSS/mg N), respectively, and b_a and b_n are the decay coefficients (d^{-1}) for AOB and NOB, respectively. The values chosen for K_{snh3}, K_{sno2}, Y_a, Y_n, b_a and b_n were 1.25 mg N/L, 2 mg N/L, 0.27 mg VSS/mg N, 0.08 mg VSS/mg N, 0.1 day^{-1} and 0.1 day^{-1}, respectively (Rittmann and McCarty, 2001). For the two-populations simulation, X_a and X_n were 476 and 205 mg VSS/L.

Time course data of ammonia, nitrite and nitrate, obtained from the enriched culture nitrification assay were fitted to the model (Equations 6.8 – 6.12) using Berkeley-Madonna Version 8.3 BTBA software (Macey and Oster, 2006). The MAUR and MNUR values were estimated by minimizing the deviation between the model output and the experimental data. The 4th order Runge-Kutta algorithm with a step size of 0.01 day was used in the simulations. The root mean square deviation (RMSD) was used as a measure of the goodness of fit in each simulation. MAUR and MNUR values normalized to the

NA-free control series (i.e., NMAUR and NMNUR) were then calculated. Based on the two-biomass simulations, the extent of inhibition to MAUR and MNUR was not significantly different when considering a single nitrifying biomass or two populations (AOB and NOB)(Table 6.2). Therefore, the single biomass model was used throughout the simulations and the time course data of ammonia, nitrite and nitrate, obtained from the two nitrification assays were fitted to the model (Equations 6.3 – 6.6) using Berkeley-Madonna Version 8.3 BTBA software as described above.

Table 6.2. Nitrification simulation for enriched culture series assuming one and two (AOB and NOB) biomass populations.

Population	Parameter	Initial NA (mg/L)					
		0	**20**	**40**	**80**	**200**	**400**
Single Biomass	RMSD[a]	19.2	17.3	15.9	12.4	19.7	17.1
	MAUR[b]	0.359	0.337	0.334	0.354	0.321	0.199
	MNUR[c]	0.213	0.191	0.197	0.195	0.172	0.098
	NMAUR[d]	100	93.9	93.0	98.6	89.4	55.4
	NMNUR[e]	100	89.7	92.5	91.5	80.8	46.0
Two Biomass	RMSD	19.4	17.6	16.2	12.7	19.4	17.1
	MAUR	0.512	0.481	0.476	0.504	0.457	0.284
	MNUR	0.731	0.657	0.674	0.669	0.589	0.341
	NMAUR	100	93.9	93.0	98.4	89.3	55.5
	NMNUR	100	89.9	92.2	91.5	80.6	46.6

[a]RMSD, root mean square deviation

[b]MAUR (mg N/mg VSS-day)

[c]MNUR (mg N/mg VSS-day)

[d]NMAUR, normalized MAUR (%) compared to NA-free control series

[e]NMNUR, normalized MNUR (%) compared to NA-free control series

6.3 Materials and Methods

6.3.1 Naphthenic Acid Mixture

A commercial mixture of NA sodium salt was purchased from TCI Chemicals (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and used throughout this study. The mixture contains approximately 8.6% (w/w) sodium. The characteristics of the TCI NA mixture are summarized below (Section 6.4.1).

6.3.2 Culture Development and Maintenance

6.3.2.1 Nitrifying Cultures

Two aerobic nitrifying cultures, with no previous exposure to NAs, were used to investigate the effect of NAs on nitrification. In the first nitrification assay, a mixed aerobic nitrifying/heterotrophic culture was used as seed, which was developed from a mixed liquor sample collected at a poultry processing wastewater treatment plant. The mixed nitrifying culture was maintained by feeding untreated dissolved air floatation (DAF) underflow wastewater collected at the same poultry processing facility. The mixed, aerobic nitrifying culture was maintained in a continuous flow system with an hydraulic retention time (HRT) and solids retention time (SRT) of 2.5 and 15 days, respectively. The steady-state total suspended (TSS) and volatile suspended (VSS) solids were 1.6±0.3 and 1.3±0.2 g/L, respectively (Hajaya and Pavlostathis, 2012).

In the second nitrification assay, an enriched nitrifying culture was used, which was originally developed from a contaminated estuarine sediment sample by feeding only ammonia as substrate at a loading rate of 0.2 g N/L-d (Yang, 2007). The enriched nitrifying culture was maintained fed-batch with an HRT and SRT of approximately 20

days. Approximately 65% of the culture mixed liquor was wasted once per week and the wasted volume replaced with a salt medium described in detail in Chapter 3, Section 3.5.1. The pH in both cultures was controlled between 7.0 and 7.5 using a pH controller (Type HD PH-P, Barnant Company, Barrington, Illinois, USA) and a 42 g/L $NaHCO_3$ solution. Both nitrifying cultures were aerated with pre-humidified compressed air passed through a fine pore diffuser maintaining a dissolved oxygen concentration of 6 mg/L and higher.

6.3.2.2 Denitrifying Culture

A mixed, aerobic culture fed only with dextrin and peptone (D/P) and with no previous exposure to NAs was used in all denitrifying assays reported here. The culture was maintained fed-batch with an HRT and SRT of 14 days, fed twice per week with 2 g COD/L of D/P corresponding to an average loading rate of 0.57 g COD/L-day. Approximately 25% of the culture mixed liquor was wasted twice per week and replaced with salt media (Chapter 3, Section 3.5.1). The steady-state TSS and VSS concentrations were 2.7±0.1 and 1.7±0.1 g/L, respectively. The culture was aerated with pre-humidified compressed air passed through a fine pore diffuser and maintained with a pH of 7.0 to 7.5. A preliminary test confirmed that when the mixed culture was provided with nitrate and D/P as an electron donor and incubated under anoxic conditions, denitrification proceeded without any lag.

6.3.2.3 Methanogenic Culture

A mixed, methanogenic culture developed with inoculum obtained from a mesophilic, municipal anaerobic digester and maintained fed-batch with a HRT/SRT of 35 days at 35°C was used as seed in the fermentation/methanogenesis assay reported here. The culture was fed with 8 g/L dextrin and 4 g/L peptone (in the feed) and pre-reduced culture media, corresponding to an average loading rate of 0.34 g COD/L-day as previously reported by Tezel et al. (2006). The culture media is described in detail in Chapter 3, Section 3.5.2. The steady-state methane and carbon dioxide concentrations were 60.7±0.5% and 39.2±0.4% (mean±standard deviation), respectively. The steady-state total (TS) and volatile solids (VS) concentration of this culture were 6.9±0.3 and 2.2±0.1 g/L, respectively.

6.3.3 Inhibition and Biotransformation Assays

6.3.3.1 Nitrifying Conditions

The nitrification assay conducted with the mixed nitrifying culture was divided into two phases: with and without the addition of dextrin (600 mg COD/L) as an external carbon source. The seed culture, phosphate buffer (10 mM), trace metals, ammonium chloride, NAs and dextrin were mixed to make fourteen culture series prepared in duplicate. The NA-amended culture series were prepared with initial NA concentrations of 20, 40, 80, 200, and 400 mg NA/L. One culture series did not receive NAs and dextrin and served as a nitrification control. All dextrin-amended culture series had an initial dextrin concentration of 600 mg COD/L. One culture series received dextrin but no NAs and served as a control for the dextrin-amended culture series. Similarly to the above

described nitrification assay, another nitrification assay was set up using the enriched nitrifying culture as the seed to prepare six culture series with initial NA concentrations of 20, 40, 80, 200, and 400 mg/L. One culture series did not receive NAs and served as a nitrification control.

Both nitrification assays were performed using 300-mL Erlenmeyer flasks (150 mL liquid volume) and incubation was carried out at room temperature (22 - 24^{o}C). Before the assay, ammonia was not detected in either culture. The initial ammonia concentration in all culture series was 100 mg N/L and the initial pH was in the range of 7.1 – 7.3. The initial VSS concentration in all mixed and enriched culture series was 680 mg/L. Pre-humidified compressed air was passed through fine pore diffusers in order to maintain dissolved oxygen (DO) concentration at or above 6 mg/L in all culture series. The flasks were placed on an orbital shaker and continuously mixed at 190 rpm during the test period. The pH was monitored periodically and maintained above 7.0 by addition of sodium bicarbonate. Ammonia, nitrite, and nitrate were monitored throughout the incubation period. For the dextrin-amended cultures, soluble COD was also monitored over time. In addition, initial and final soluble COD, total NAs, TSS and VSS were measured.

6.3.3.2 Nitrate-reducing Conditions

A batch assay was conducted to investigate the biodegradation potential and inhibitory effect of NAs on nitrate reduction. The assay included twelve culture series prepared with NAs at initial concentrations of 0 (control), 20, 40, 80, 200, and 400 mg NA/L, with and without addition of dextrin/peptone (D/P) as an external carbon source. The assay was

conducted in 160 mL serum bottles (100 mL liquid volume) sealed with rubber stoppers and aluminum crimps and flushed with helium for 15 min before any liquid addition. An aliquot of 80 mL of culture seed taken at the end of a 7-day feeding cycle and 15 mL of culture media (Chapter 3, Section 3.5.1) were added to each serum bottle. Specific volumes of sodium nitrate, D/P, and NAs stock solutions were added to achieve target concentrations and the total liquid volume was adjusted to 100 mL with deionized water (DI). The initial nitrate and D/P concentrations were 100 mg N/L and 600 mg COD/L, respectively, in all culture series (COD:N = 6:1). Two additional series, seed blank (culture seed, denitrifying media and DI water) and D/P reference (culture seed, denitrifying media, DI water and D/P) were also prepared. The initial TSS and VSS concentrations of each culture series were 0.80 and 0.64 g/L, respectively.

All culture series were prepared in triplicate and incubated for 35 days at room temperature (22 to 24°C). Throughout the incubation period, total gas, headspace composition, nitrate and nitrite were monitored. At the end of the incubation, pH, nitrate, nitrite, ammonia, VSS, TSS and total and liquid-phase NAs were measured.

6.3.3.3 Methanogenic Conditions

A batch assay was performed to investigate the inhibitory effect and biotransformation potential of NAs in a mixed, mesophilic (35°C) methanogenic culture. The assay was conducted in 160-mL serum bottles (100 mL liquid volume) sealed with rubber stoppers and aluminum crimps and flushed with helium gas for 15 min before any liquid addition. An aliquot of 80 mL of the mixed methanogenic culture taken at the end of a 7-day feeding cycle was anaerobically transferred to each serum bottle along with 15 mL of

pre-reduced culture media (Chapter 3, Section 3.5.2). D/P, which served as carbon/energy source, and NAs at target concentrations were added and the total liquid volume was adjusted to 100 mL with deionized water (DI). The D/P COD concentration in the bottles was 1200 mg/L. The assay included twelve culture series with NAs at 0 (control), 20, 40, 80. 200 and 400 mg/L, with and without D/P added. Two additional culture series were prepared: seed blank and reference which consisted of only seed, culture media and DI water, and seed, culture media, DI water and D/P (1200 mg COD/L), respectively. Each culture series, including seed blank and reference, was prepared in triplicate. The initial pH in all culture series was 7.1±0.1. All culture series were incubated in the dark at 35°C and the bottles were agitated every few days by hand. Throughout the incubation period, the total gas volume produced and headspace composition were measured. At the end of the incubation period, pH, total and liquid-phase NAs, volatile fatty acids (VFAs), soluble and total COD concentrations were measured.

6.4 Results and Discussion

6.4.1 Commercial NA Mixture

The TCI NA salt mixture used in all biotransformation assays consists of mostly 0, 1 and 2 ring NA structures (i.e., Z = 0, -2, -4) with carbon numbers (n) ranging between 10 and 25 and has a weighted average MW of 271.4 Da. The congener distribution of a 100 mg/L solution of the TCI NA mixture is shown in Figure 6.2. The TCI NA mixture is representative of the types of NAs found in refinery wastewater streams, having a similar NA congener distribution to refinery desalter brine samples, which were identified as the main source of NAs in refinery wastewater (Chapter 4, Section 4.3.1). The general NA

congener distribution of refinery desalter brine samples (Chapter 4, Figure 4.3C and Table 4.5) was similar to the TCI NA mixture, shown in Figure 6.2, with carbon numbers ranging from 5 to 25 and the most abundant *Z* numbers of 0, -2, -4 and -6. The TCI NA characteristics are summarized in Table 6.3. Ammonia and anions, such as nitrite, nitrate, chloride, fluoride, bromide, sulfate and phosphate, were not detected in the 100 mg NA/L working solution. The theoretical oxygen demand (ThOD) and carbon content were calculated based on the weighted average formula of the NA mixture, $C_{17.4}H_{30.9}O_2$, and were 2.93 mg O_2/mg NA and 0.77 mg C/mg NA, respectively. The ThOD was lower than the measured COD value of 3.45 mg O_2/mg NA, while the calculated C content was higher than the measured DOC of 0.67 mg C/mg NA. The variation between the calculated and measured COD and DOC values is likely a result of the estimates being based on the weighted average molecular formula, while the mixture contains hundreds of NAs with varying structure and formula. Additionally, the toxicity of the TCI NA mixture was measured by the standard acute Microtox® toxicity assay and the effective concentration which causes 50% inhibition of *Vibrio fischeri* (EC_{50}) was between 8.1 and 10.2 mg NA/L (30 to 37 μM based on a mean MW of 271.4 Da).

Table 6.3. Characteristics of the TCI NA mixture.

Parameter	**Value**
pH	6.02
Sodium (%, w/w)	8.6
DOC (mg/mg NA)	0.67±0.04[a]
COD (mg/mg NA)	3.45±0.14
Microtox EC_{50} (mg NA/L)	8.9 (8.1 – 10.2)[b]
Average *Z*	-3.9
Average *n*	17.4
Average MW (Da)	271.7
Average Formula	$C_{17.4}H_{30.9}O_2$

[a]Mean ± standard deviation (n = 3)

[b]Mean (low – high)

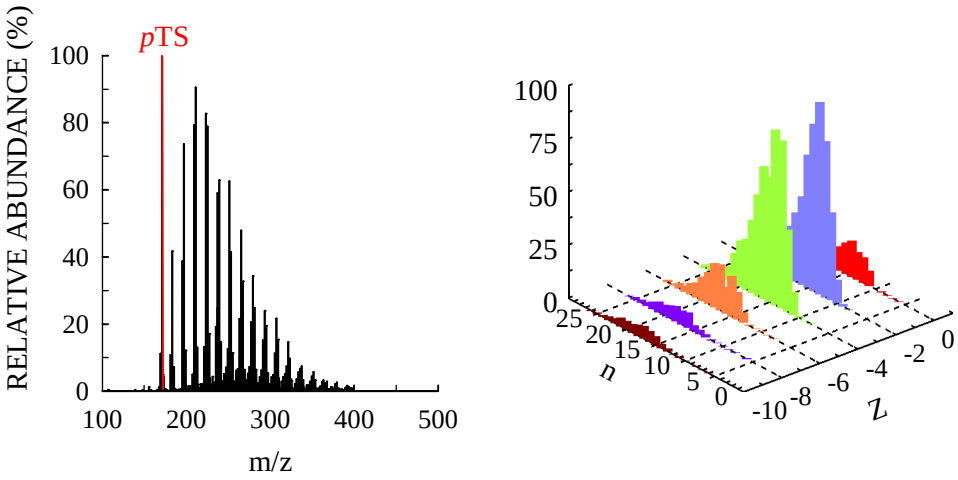


Figure 6.2. Congener distribution of the TCI NA mixture (100 mg/L solution in DI water).

6.4.2 Inhibition and NA Biotransformation Potential Under Nitrifying and Heterotrophic Conditions

6.4.2.1 Mixed Nitrifying and Heterotrophic Culture Series

To investigate the effect of NAs on nitrification, ammonia removal was monitored in the mixed nitrifying/heterotrophic culture with and without the addition of dextrin as an external carbon source. The batch assay performed with the mixed nitrifying culture amended with NAs and dextrin lasted 4.7 days. Figure 6.3A shows the ammonia consumption and Figure 6.3B shows the nitrate formation (nitrite was not detected). Table 6.4 summarizes the MAUR values estimated by fitting Equations 6.3 – 6.6 to the experimental data obtained from the mixed nitrifying culture series amended with NAs and dextrin. Significant inhibition (greater than 20%) was observed at an initial NA concentration of 40 mg/L and above. Compared to the nitrification rate of the NA-free, dextrin-amended control culture series, the MAUR was reduced by 18.4, 34.4, 52.7, 65.6 and 75% for the cultures with initial NA concentrations of 20, 40, 80, 200, and 400 mg NA/L, respectively. The estimated MAUR values ranged from 0.064 – 0.256 g N/g VSS-day (Table 6.4). Because nitrite was not detected in any of the dextrin-amended culture series, the extent of NA inhibition to MNUR could not be quantified. The estimated (i.e., simulated) MNUR values were relatively constant for all NA-amended culture series, ranging from 4.7 – 5.3 g N/g VSS-day, which were higher than the simulated MAUR values (Table 6.4). RMSD values ranged from 17 – 23, indicating a good model fit.

NAs were not degraded at or below 80 mg NA/L; however, approximately 50% of NAs were degraded in culture series amended with 200 and 400 mg NA/L (Table 6.5). The substrate COD consumption was also measured in dextrin-amended culture series

(Figure 6.4). All culture series with initial NA concentrations of 80 mg/L and lower had less than 300 mg soluble COD/L remaining at the end of the 4.7-days incubation. The culture series amended with dextrin at 200 and 400 mg/L NA had residual soluble COD of 470 ± 83 and 1,018 ± 126 mg/L, respectively. The elevated residual COD is attributed to the significantly higher residual NA concentrations of 112 and 200 mg NA/L, respectively, compared to the other culture series (Table 6.5), as well as to cell lysis at such relatively high NA concentrations.

The nitrification assay conducted with the mixed nitrifying culture, amended with NAs and no dextrin lasted 3.3 days and results are shown in Figure 6.3. Figure 6.3C shows the ammonia consumption and Figure 6.3D shows the nitrate formation over time in the dextrin-free culture series. Nitrite was not detected in any culture series, expect at the beginning of the incubation in the control and the 400 mg/L NA-amended culture series (transient nitrite at less than 2 mg N/L; data not shown). Similarly to the results obtained with the dextrin- and NA-amended nitrifying culture series (see above), nitrification was not significantly inhibited by NAs up to an initial NA concentration of 80 mg/L; however, at an initial NA concentration of 200 and 400 mg/L, the nitrification rate decreased significantly. Table 6.4 summarizes the MAUR values obtained in the dextrin-free mixed nitrifying culture series. As mentioned above, the MAUR values were obtained by fitting equations 6.3 – 6.6 to the experimental data (Section 6.2.2). Compared to the nitrification rate of the dextrin- and NA-free control culture, the decrease in MAUR was 8.8, 5.5, 10.6, 30.9 and 46.5% at an initial NA concentration of 20, 40, 80, 200 and 400 mg/L, respectively. The estimated MAUR values ranged from 0.116 – 0.217 g N/g VSS-day (Table 6.4). Similarly to the dextrin-amended culture series, because

nitrite was not detected in the dextrin-free, NA-amended culture series, the extent of NA inhibition to MNUR could not be quantified. The estimated (i.e., simulated) MNUR values were relatively constant for all NA-amended culture series, ranging from 4.7 – 5.0 g N/g VSS-day, which were higher than the simulated MAUR values (Table 6.4). RMSD values ranged from 16 – 31.

Although the mixed nitrifying culture had never been exposed to NAs, it was capable of some NA degradation, both with and without the addition of an external carbon source. It was observed that the residual NA concentrations in dextrin-free culture series were significantly lower compared to those in culture series fed both dextrin and NAs. This observation was most pronounced in culture series amended with 200 and 400 mg/L NA, in which the residual NA concentration was below 90 mg/L in the dextrin-free series. The removal of total NAs in dextrin-free culture series was in the range of 40 to 80%, excluding the 20 mg/L culture series in which NA removal was very low (Table 6.5). On the contrary, higher residual NA concentrations were observed in dextrin-amended cultures, in which NA degradation was not observed at initial NA concentrations of 20, 40, and 80 mg/L and approximately 50% degradation was observed in dextrin-amended cultures with initial NA concentration of 200 and 400 mg/L. Figure 6.5 shows an example of the NA mass spectra and congener distribution in culture series amended with an initial NA concentration of 400 mg/L. The NAs were significantly degraded in the dextrin-free culture series, whereas the NA degradation was less than 50% in the dextrin-amended culture series.

These observations suggest that in dextrin-free culture series, in which NAs were the only available, external carbon source, heterotrophic microorganisms degraded NAs

lowering the NA concentration. Consequently, the NA inhibition to nitrifiers was not very significant up to an initial NA concentration of 80 mg/L. On the contrary, in dextrin-amended culture series, heterotrophic microorganisms preferentially consumed dextrin over NAs, leaving NAs at relatively high concentrations for a longer duration. These results also indicate that the presence of a co-substrate decreases or completely inhibits NA degradation in cultures not previously exposed to NAs. The longer exposure of autotrophs to the relatively higher NA concentrations resulted in the higher degree of nitrification inhibition observed in dextrin-amended as compared to dextrin-free culture series.

6.4.2.2 Enriched Nitrifying Culture Series

The nitrification assay conducted with the enriched culture lasted for 1.7 days. Figure 6.6 shows the time course of ammonia, nitrite, and nitrate in each culture series amended with different initial NA concentrations. The nitrification activity by the enriched nitrifying culture was not significantly affected by NAs below 400 mg NA/L (Table 6.4). The measured and simulated ammonia, nitrite and nitrate concentration profiles in the enriched nitrifying culture series are shown in Figure 6.7.

Compared to the nitrification rate of the NA-free control culture, the MAUR reduction in the cultures amended with 20 to 200 mg NA/L was in the range of 2 – 11%. However, 45% reduction in MAUR was observed in the culture amended with 400 mg NA/L. In the enriched nitrifying culture series, nitrite oxidizers were more affected by NAs than ammonia oxidizers as the MNUR was reduced by 54% in the culture series amended with 400 mg NA/L compared to that of the NA-free control culture (Table 6.4).

The MAUR and MNUR values ranged from 0.199 – 0.359 and 0.098 – 0.213 g N/g VSS-day, respectively, and RMSD values ranged from 13 – 20.

The results of the initial and final NA analysis suggest that NAs were not degraded during the test period (Table 6.5). Thus, these cultures were exposed to the initial NA levels throughout the nitrification test. As stated above, the fraction of heterotrophic bacteria in the enriched culture was negligible and the nitrosofying (AOB) and nitrifying (NOB) autotrophs do not have the ability to degrade any organic matter. Regardless of the NA concentration and in contrast to the results obtained with the mixed nitrifying culture series, the nitrification activity was not significantly impacted in the cultures amended with 20 to 200 mg NA/L. One possible reason for the lower nitrification inhibition in the assay conducted with the enriched nitrifying culture is the higher population size of nitrosofying and nitrifying autotrophs. The ratio of NA to AOB and NOB biomass was significantly lower in this assay compared to the assay conducted with the mixed nitrifying culture, thus resulting in lower inhibition.

Table 6.4. Maximum specific ammonia utilization rate (MAUR) and maximum specific nitrite utilization rate (MNUR) at different initial NA concentrations in both the mixed and enriched nitrifying culture series.

Culture Series	**Initial NAs (mg/L)**					
	0	**20**	**40**	**80**	**200**	**400**
Dextrin-amended mixed culture						
MAUR[a]	0.256	0.209	0.168	0.121	0.088	0.064
NMAUR[b]	100.0	81.6	65.6	47.3	34.4	25.0
Dextrin-free mixed culture						
MAUR	0.217	0.198	0.205	0.194	0.150	0.116
NMAUR	100.0	91.2	94.5	89.4	69.1	53.5
Dextrin-free enriched culture						
MAUR	0.359	0.337	0.334	0.354	0.321	0.199
NMAUR	100.0	93.9	93.0	98.6	89.4	55.4
MNUR	0.213	0.191	0.197	0.195	0.172	0.098
NMNUR[c]	100.0	89.7	92.5	91.5	80.8	46.0

[a]MAUR and MNUR, g N/g VSS-day

[b]NMAUR, normalized MAUR (%) compared to NA-free control series

[c]NMNUR, normalized MNUR (%) compared to NA-free control series

Table 6.5. NA removal in mixed and enriched nitrifying culture series.

Initial NA (mg/L)	Dextrin-amended mixed culture series		Dextrin-free mixed culture series		Dextrin-free enriched culture series	
	Residual Total NA (mg/L)	NA Removal (%)	Residual Total NA (mg/L)	NA Removal (%)	Residual Total NA (mg/L)	NA Removal (%)
20	21.8 ± 3.6[a]	NR[b]	19.6 ± 0.9	2	24.6±1.5	NR
40	41.0 ± 4.5	NR	25.6 ± 0	36	43.3±1.2	NR
80	83.3 ± 9.4	NR	42.3 ± 6.3	47	82.4±2.7	NR
200	112.3 ± 26.2	44	51.9 ± 0.3	74	209.5±8.8	NR
400	199.7 ± 45.5	50	88.3 ± 28.2	78	409.9±3.2	NR

[a] Mean ± standard deviation (n = 2).

[b]NR, not removed

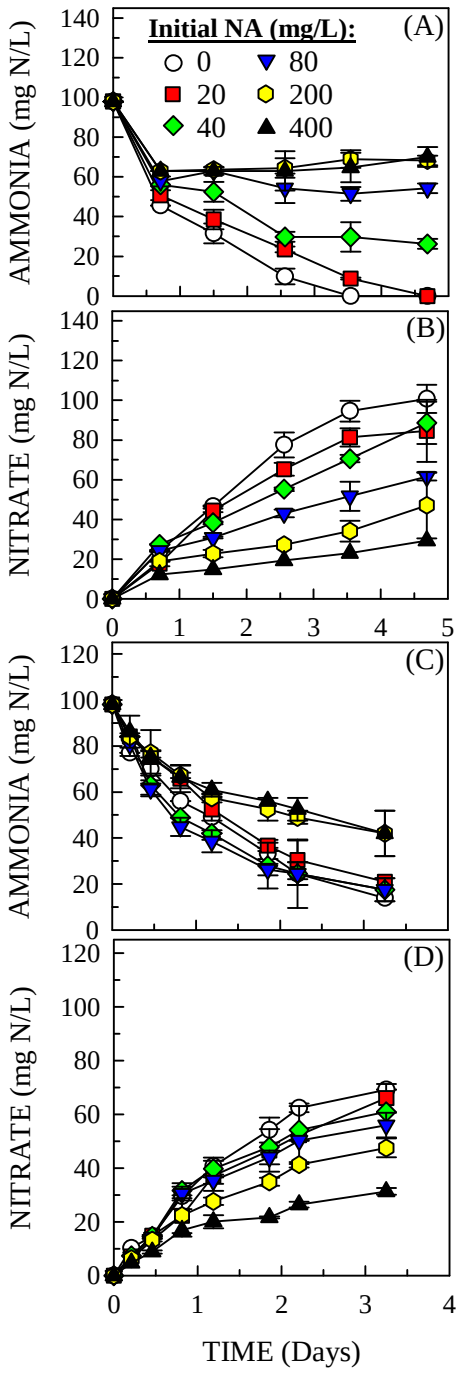


Figure 6.3. Ammonia consumption and nitrate formation in dextrin-amended (A, B) and dextrin-free (C, D) culture series at different initial NA concentrations. Error bars represent mean values ± one standard deviation, $n = 3$.

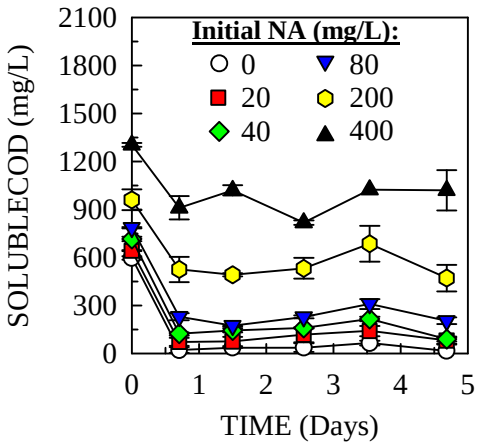


Figure 6.4. Soluble COD in dextrin-amended mixed nitrifying culture series.

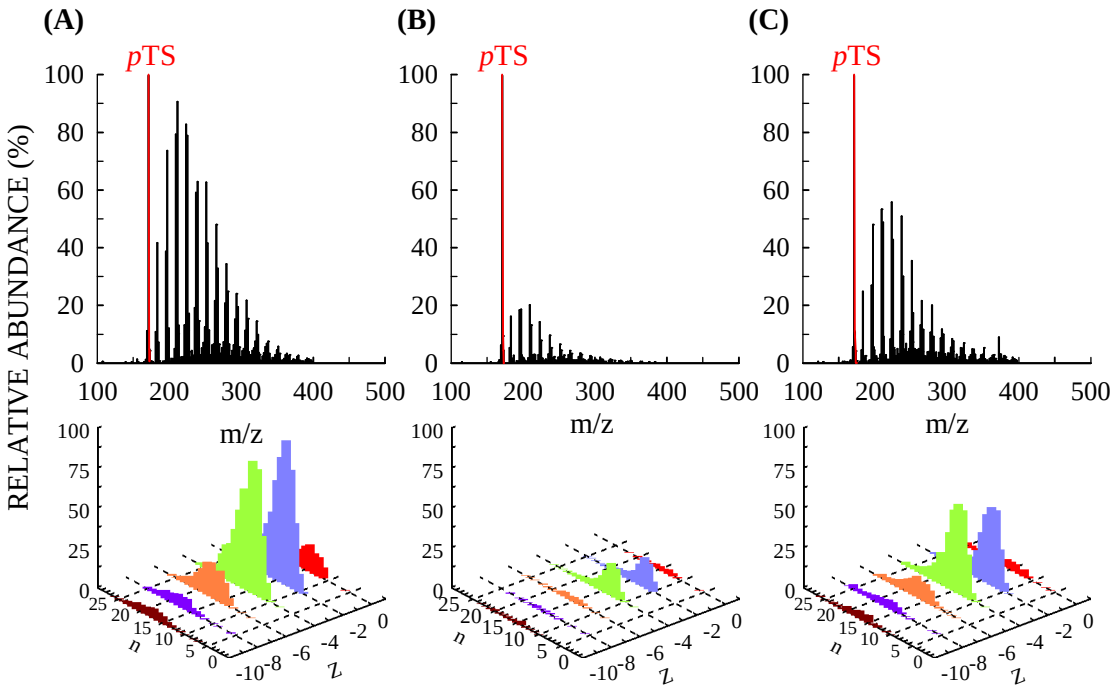


Figure 6.5. NA mass spectra and congener distribution in NA-amended cultures with an initial NA concentration of 400 mg/L. (A) Initial NAs, (B) residual NAs in dextrin-free culture series and (C) residual NAs in dextrin-amended culture series (*p*TS, *p*-toluene sulfonate used as a surrogate standard).

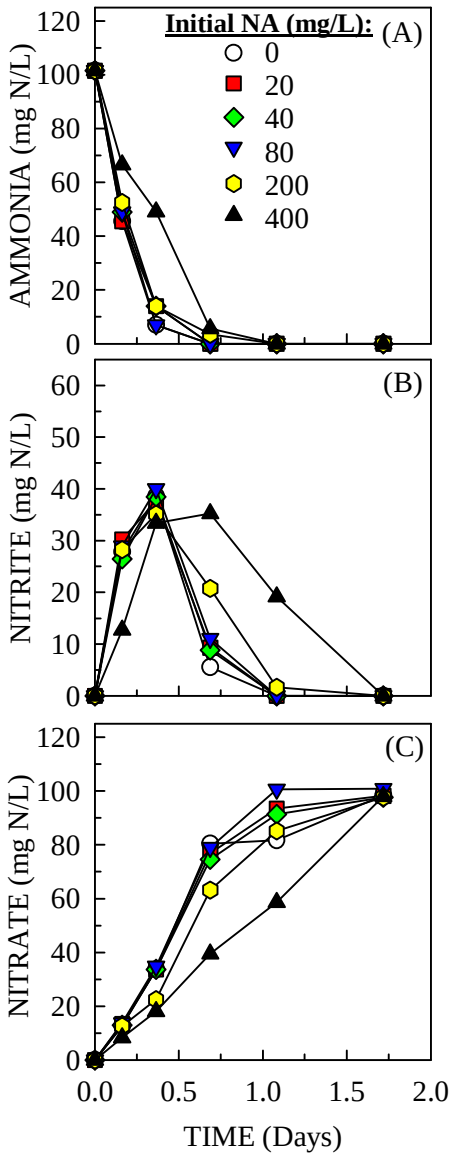


Figure 6.6. Ammonia consumption (A), nitrite formation/consumption (B), and nitrate formation (C) in enriched nitrifying culture series amended with different initial NA concentrations (0 to 400 mg/L).

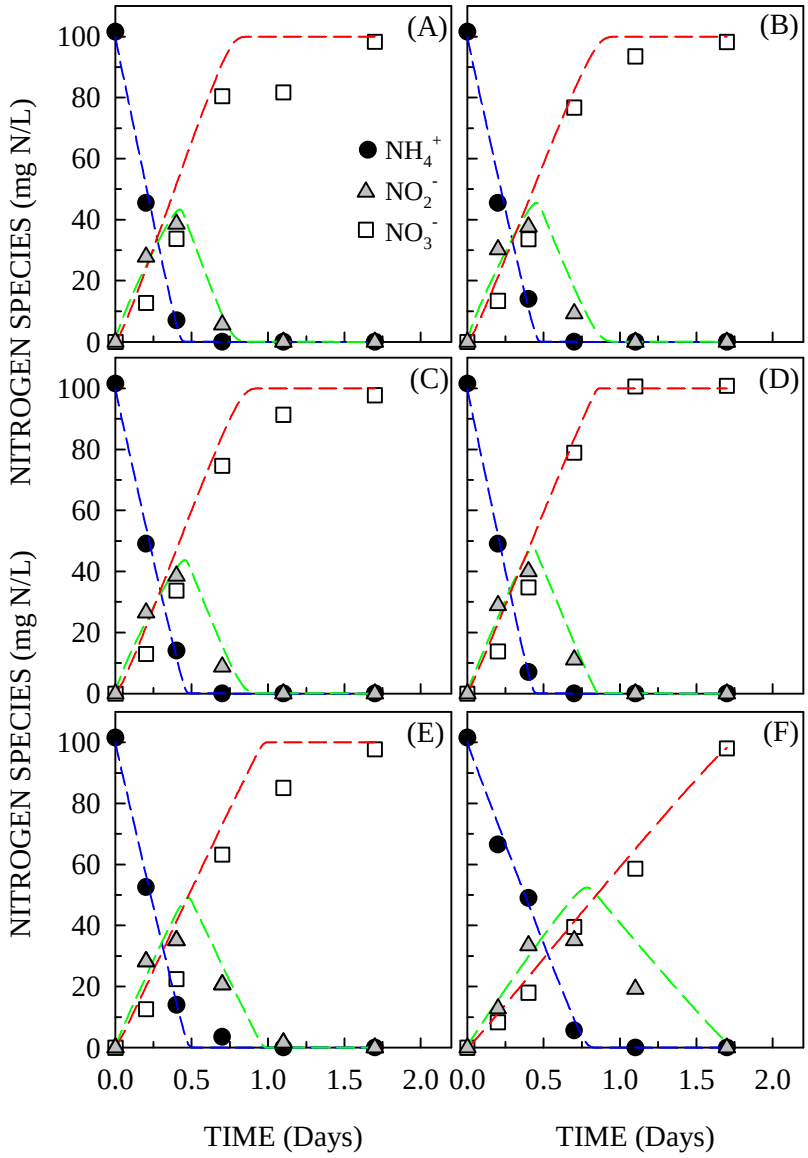


Figure 6.7. Measured (symbols) and simulated (dashed lines) ammonia, nitrite and nitrate profiles in the enriched nitrifying culture series at initial NA concentrations of 0 (A), 20 (B), 40 (C), 80 (D), 200 (E) and 400 mg NA/L (F).

6.4.3 Inhibition and NA Biotransformation Potential Under Nitrate-Reducing Conditions

Figure 6.8A and 6.8B show the nitrate removal and seed-corrected nitrogen gas production in the culture series amended with both NAs and D/P. The rate of nitrate reduction was the same in all culture series and even slightly increased at initial NA concentrations of 200 and 400 mg/L, indicating that NAs are not inhibitory to denitrifiers. Similarly, the nitrogen gas production was the same or slightly higher at increased NA concentrations (Figure 6.8B). All D/P-amended culture series contained sufficient degradable carbon to drive denitrification to completion (COD:N $\geq$ 6), which is shown by the complete removal of nitrate by day 4 (Figure 6.8A). The added COD (D/P and NAs) and initial COD:N ratio for each culture series are shown in Table 6.6.

Figure 6.9A shows the nitrogen balance for the D/P- and NA-amended culture series. The nitrogen balance shows that 100% of the added nitrate-N was recovered in the form of nitrogen gas in all D/P-amended culture series. The culture media used for the denitrification assays contain ammonia. In addition, ammonia production as a result of peptone fermentation is expected. However, reference-corrected ammonia concentrations increased with increasing NA concentrations (Figure 6.9A). Similar ammonia concentrations were found in all culture series amended with NAs and D/P, excluding the 400 mg NA/L culture series, in which a higher ammonia concentration was observed. The higher ammonia concentration is a result of cell lysis at higher NA concentrations which is also corroborated by the much lower final VSS concentrations in the 200 and 400 mg NA/L culture series. The final VSS concentrations decreased with increasing NA

concentrations, further indicating that cell lysis occurred at the higher NA concentrations (Table 6.6).

The total and liquid-phase NA concentrations at the end of the incubation were measured in all culture series and the results are summarized in Table 6.6. The ratio of the liquid to total NA concentrations was relatively constant in all D/P-amended culture series, ranging from 0.46 to 0.58. Figure 6.10A shows that the initial and final total NA concentrations were the same in all culture series, indicating that NAs were not degraded under nitrate reducing conditions during the 28-day incubation period. The NA congener distribution for each culture series is shown in Figure 6.11. Although NA degradation is energetically feasible under nitrate-reducing conditions (Section 6.2.1), the overall NA distribution did not change in all culture series, further indicating that NAs were not degraded or even partially transformed during the 28-day incubation period.

To investigate the biotransformation potential of NAs under anoxic, denitrifying conditions, similar culture series were set up with NAs as the only external carbon source. Figure 6.8C and 6.8D shows the nitrate removal and the seed-corrected nitrogen gas production in culture series amended with only NAs. Ignoring microbial growth, the theoretical electron donor requirement for complete nitrate reduction (i.e., denitrification) is 2.85 mg COD/mg nitrate-N. Assuming an electron equivalent fraction for energy (f_e) equal to the electron equivalent fraction for cell synthesis (f_s)(i.e., $f_e = f_s = 0.5$) (Rittmann and McCarty, 2001), the theoretical electron donor requirement for both complete denitrification and cell synthesis is approximately 5.7 mg COD/mg nitrate-N. Based on this electron donor requirement, the NA-free control series and some of the culture series amended with only NAs up to 80 mg NA/L were electron donor limited assuming full

NA degradation under nitrate-reducing conditions. However, as discussed below, NAs were not degraded, which makes all D/P-free culture series electron donor limited relative to the 100 mg/L of nitrate-N initially added. Table 6.6 summarizes the COD added and the COD:N ratio in each culture series. Nitrate removal and nitrogen gas production increased as the initial NA concentration increased, indicating that at higher NA concentrations, more biodegradable carbon was available for denitrification as explained below.

A nitrogen balance was performed for all D/P-free, NA-amended culture series (Figure 6.9B). In all culture series amended with NAs only, greater than 89% of the added nitrogen was recovered, with 3 – 28 and 39 – 61% recovered as nitrate and nitrogen gas, respectively. Similar to the D/P-amended culture series, a higher seed-corrected ammonia concentration was measured in culture series with higher NA concentrations. In addition, the final VSS concentrations decreased with increasing NA concentrations (Table 6.6), again indicating that cell lysis was more pronounced at higher initial NA concentrations. Thus, cell lysis products were used as electron donor(s), which explains the observed partial denitrification in culture series with a COD:N ratio lower than the above discussed theoretical value needed for both nitrate reduction and cell synthesis.

The total and liquid-phase NAs concentrations at the end of the incubation in the culture series amended with only NAs are listed in Table 6.6. The ratio of liquid to total NAs ranged from 0.45 to 0.64. Similar to the D/P- and NA-amended culture series, the initial and final NA concentrations were practically the same, indicating that NA degradation did not occur, even when no other external carbon source was present. The

initial and final NA concentrations are shown in Figure 6.10B and the NA congener distribution for each culture series is shown in Figure 6.12. The overall NA distribution did not change in all culture series, indicating that NAs were not degraded or even partially transformed, regardless of the addition or not of an external carbon source.

Table 6.6. Initial and measured final values for denitrifying culture series prepared with and without supplemental external carbon source (dextrin/peptone, D/P).

Culture Series	Initial NA (mg/L)	Initial COD[a] (mg /L)	COD:N Ratio[b]	Final VSS[c] (g/L)	Final Total NA (mg/L)	Final Liquid-phase NA (mg/L)	Liquid to Total NA Ratio
D/P-amended	0	1200	12.0	0.78±0.24[d]	-	-	-
	20	1270	12.7	0.73±0.01	28.4±2.6	13.0±0.8	0.46
	40	1340	13.4	0.63±0.02	35.5±8.9	18.4±0.2	0.52
	80	1480	14.8	0.66±0.04	84.5±9.0	42.3±5.9	0.50
	200	1900	19.0	0.42±0.06	231.0±25.5	132.7±1.5	0.57
	400	2600	26.0	0.31±0.05	414.0±21.1	240.6±3.7	0.58
D/P-free	0	0	0	0.74±0.02	-	-	-
	20	70	0.7	0.69±0.06	18.2±1.4	11.4±1.6	0.64
	40	140	1.4	0.62±0.17	39.2±1.3	21.1±0.9	0.54
	80	280	2.8	0.57±0.02	73.7±4.1	33.0±3.0	0.45
	200	700	7.0	0.48±0.07	193.9±17.1	105.4±8.5	0.54
	400	1400	14.0	0.35±0.07	417.8±18.9	239.2±15.9	0.57

[a]Includes both D/P (1200 mg COD/L) and NA COD calculated based on the measured COD of TCI NA mixture (3.5 mg COD/mg NA) and initially added NAs

[b]Assumes NAs are fully degradable

[c]The final VSS concentration of the seed blank (unfed) culture series was 0.6 g/L; all VSS values were corrected for the residual NA concentrations

[d]Mean ± standard deviation; n = 3

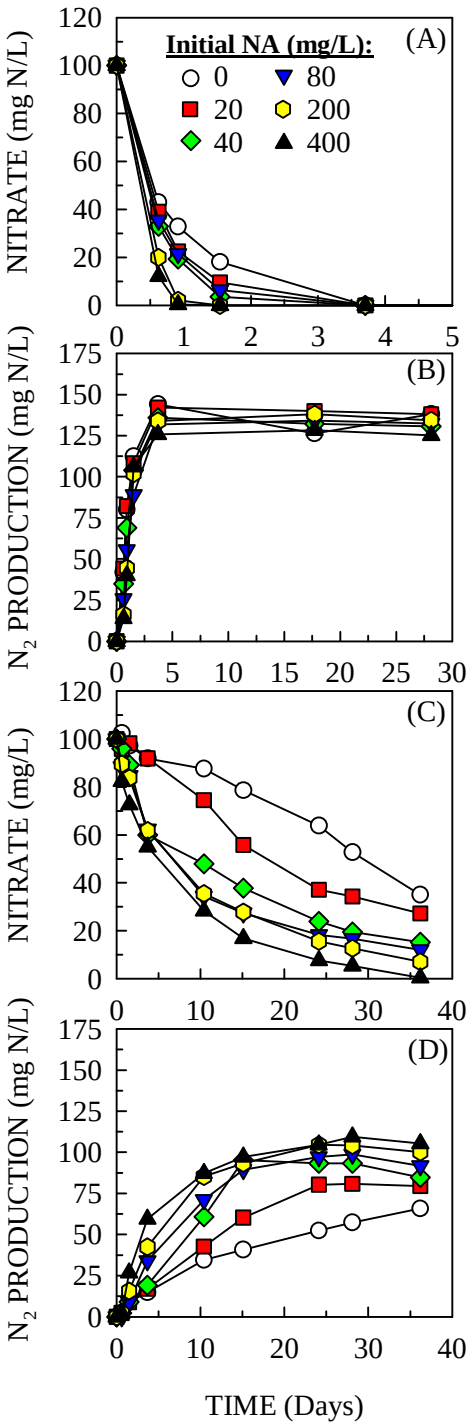


Figure 6.8. Nitrate removal and nitrogen gas production in culture series amended with both NAs and D/P (A, B) and NAs only (C, D).

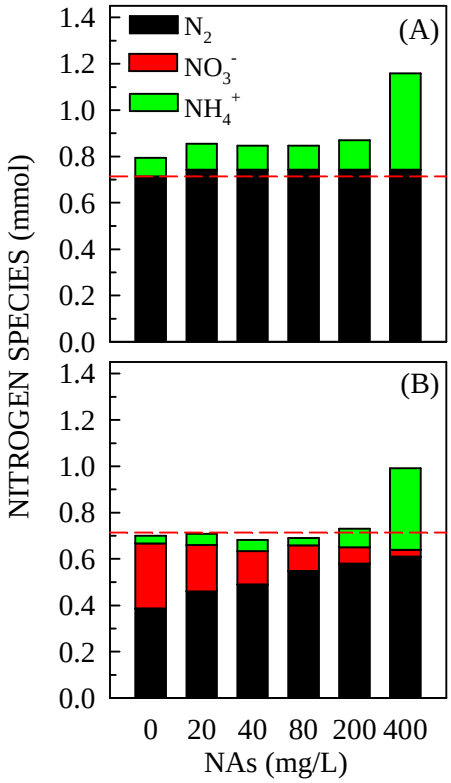


Figure 6.9. Nitrogen balance for denitrifying culture series containing NAs and DP (A) and NAs only (B) (dashed horizontal line represents total nitrogen added).

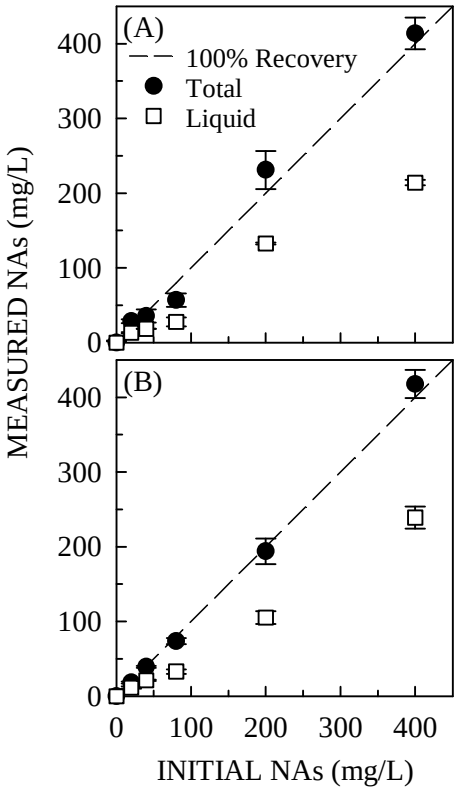


Figure 6.10. Total and liquid-phase NA concentrations in denitrifying culture series containing NAs and D/P (A) and NAs only (B) at the end of the 28-day incubation period. Error bars represent mean values ± one standard deviation, $n = 3$.

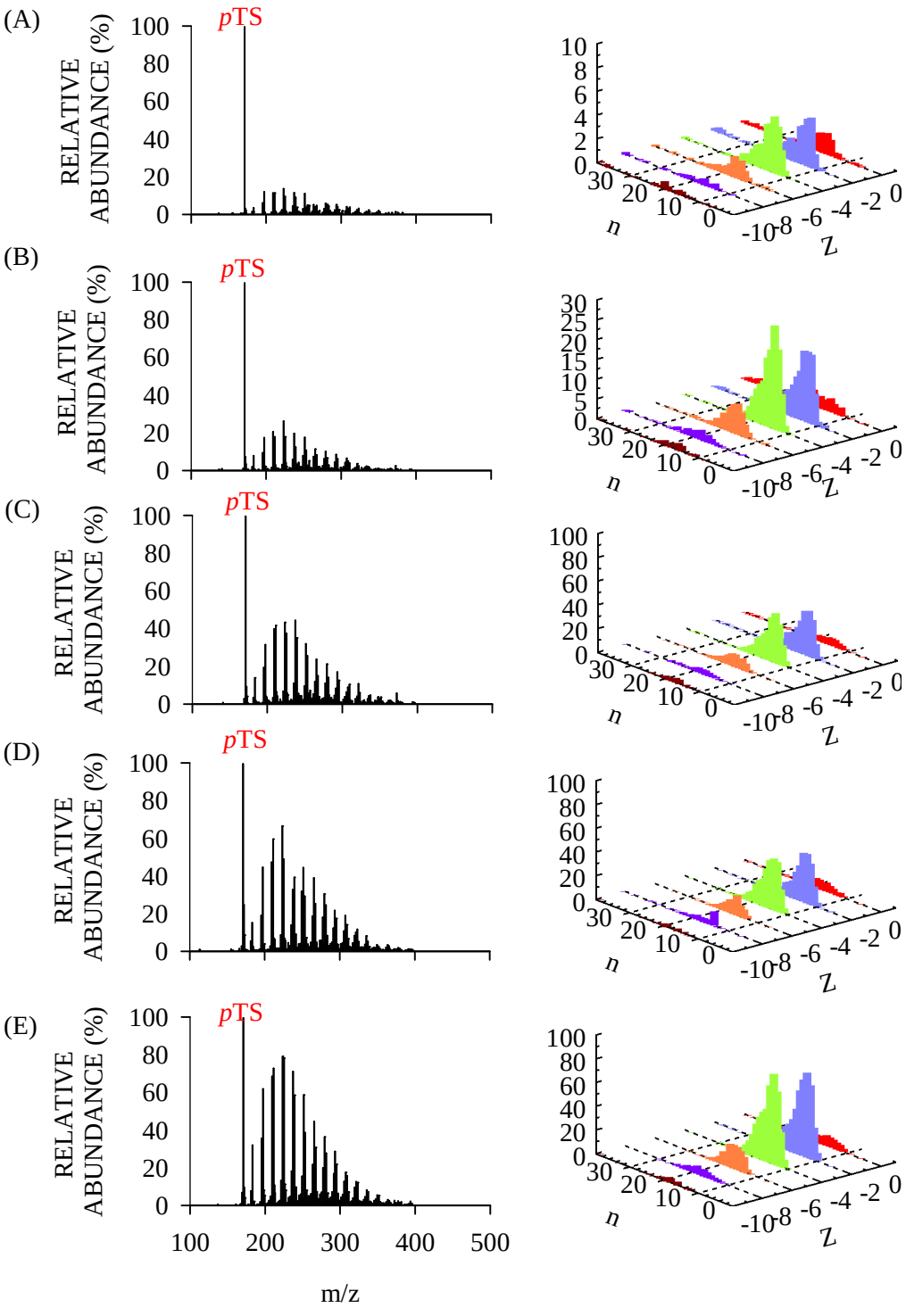


Figure 6.11. Congener NA distribution in D/P- and NA-amended denitrifying culture series at initial NA concentrations of 20 (A), 40 (B), 80 (C), 200 (D) and 400 mg/L (E).

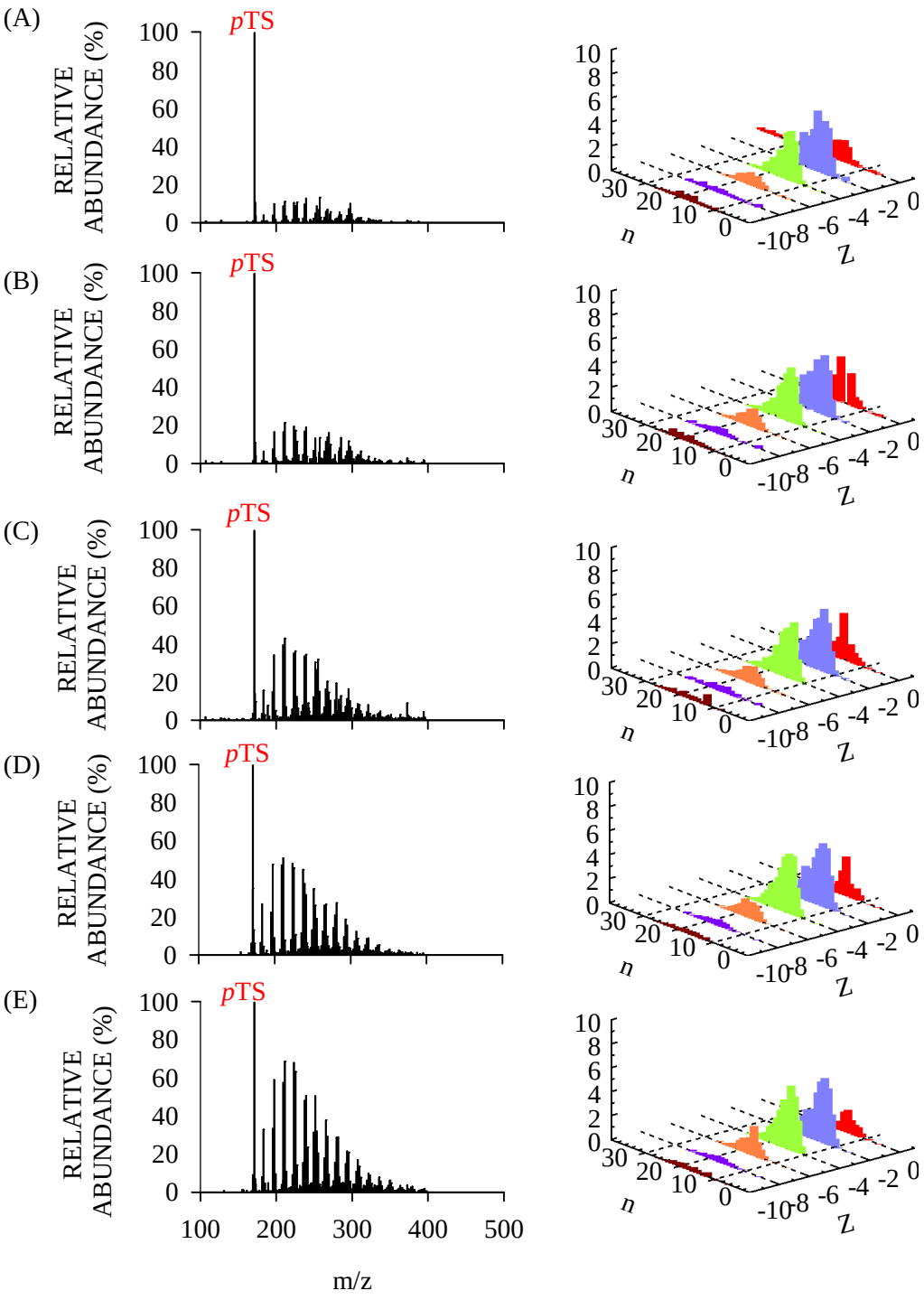


Figure 6.12. Congener NA distribution in D/P-free, NA-amended denitrifying culture series at initial NA concentrations of 20 (A), 40 (B), 80 (C), 200 (D) and 400 mg/L (E).

6.4.4 Inhibition and NA Biotransformation Potential Under Fermentative/Methanogenic Conditions

Total gas, methane and carbon dioxide production in culture series amended with D/P and NAs are shown in Figure 6.13. The TCI NA mixture was not inhibitory to methanogens up to 40 mg NA/L; however, inhibition was observed at 80 mg NA/L and above. While methane production was completely inhibited in culture series amended with NAs at 200 and 400 mg/L, culture series amended with NAs at 80 mg/L were only transiently inhibited. In the culture series with initial NAs at 80 mg/L, methane production was inhibited until day 136, upon which methane gas production recovered. These results agree with a previous study conducted by Holowenko et al. (2001), in which NAs from oil sands process waters had a short-term inhibition of methanogenesis from H_2 or acetate, but with prolonged incubation, methane production resumed. In contrast to methanogenesis, fermentation was not inhibited at all NA concentrations tested in our study indicated by the comparable carbon dioxide production by the reference and NA-amended culture series (Figure 6.13C). VFAs were detected at the end of the incubation in all culture series amended with 80 mg NA/L and higher (Table 6.7). The predominant VFAs measured in the NA-amended culture series were acetate, propionate, iso-butyrate and iso-valerate. The accumulation of VFAs also confirms that fermentation and acidogenesis were active even at relatively high NA concentrations. Therefore, methanogenesis was more susceptible to NA inhibition than fermentation/acidogenesis. Final measured total NA concentrations show that NA degradation did not occur (Figure 6.14). Although NA degradation is energetically feasible under methanogenic conditions (Section 6.2.1), the final NA congener

distributions were the same as in the NA- and D/P-amended denitrification culture series (Figure 6.11), indicating that NAs were not degraded or even partially transformed.

Total gas, methane and carbon dioxide production in culture series amended with NAs and no D/P are shown in Figure 6.15. Similar to the D/P-amended culture series, methane production was completely inhibited in culture series with initial NAs at 200 and 400 mg/L. Transient inhibition was also observed in culture series prepared with 80 mg NA/L; however, methane production recovered on day 41, approximately 100 days earlier than in the respective D/P-amended series. VFAs were also measured at inhibitory NA concentrations, excluding culture series containing 80 mg NA/L (Table 6.7). Similarly to the D/P-amended culture series, the predominant VFAs were acetate, propionate, iso-butyrate and iso-valerate. VFAs were likely not detected in the D/P-free culture series amended with 80 mg NA/L because methanogenesis recovered almost 100 days earlier than in the D/P-amended culture series, allowing time for VFA fermentation and conversion to methane.

The NA-only culture series amended with 20, 40 and 80 mg NA/L produced more methane and carbon dioxide than the NA-free seed blank. Thus, there was more readily available carbon for fermentation/acidogenesis and methanogenesis as the NA concentration increased; however, the final measured NA concentrations and NA congener distributions in each culture series indicate that no NA degradation or biotransformation occurred throughout the 181-day incubation period (Figure 6.14). The excess methane is attributed to degradable carbon resulting from cell lysis at the higher NA concentrations, similar to the results obtained under nitrate-reducing conditions (Section 6.4.3 above). In contrast to these results, when Holowenko et al. (2001) used

two NA surrogates (3-cyclohexylpropanoic and 4-cyclohexylbutanoic acid) in methanogenic microcosms containing fine tailings from an oil sand extraction process, the observed methane yield suggested complete mineralization of these compounds (i.e., mineralization of both the aliphatic chain and the ring).

The final measured VFAs, NAs and seed-corrected methane in each culture series are summarized in Table 6.7. The liquid to total NA ratio in all culture series ranged from 0.38 to 0.69, which is similar to the ratios measured in the denitrifying culture series. The COD balance, calculated by comparing the initial measured total COD to the sum of the produced methane-COD and the final measured total COD (including NAs, VFAs, and soluble COD) in all culture series is summarized in Table 6.7. On average, 103 and 91% of the COD was recovered in NA- and D/P-amended culture series and D/P-free, NA-amended culture series, respectively.

Table 6.7. Initial and measured final values for methanogenic culture series prepared with and without supplemental external carbon source (dextrin/peptone, D/P).

Culture Series	Initial NAs (mg/L)	Final Total NAs (mg/L)	Final Liquid-phase NAs (mg/L)	Liquid to Total NA Ratio	CH_4 (mg COD/L)	VFAs (mg COD/L)	COD Balance (%)
D/P-amended	0	-	-	-	753.3±42.8	ND	114±9
	20	22.8±4.8[a]	15.8±0.85	0.69	866.1±22.1	ND	95±7
	40	39.8±6.3	25.6±4.4	0.64	1123.7±59.9	ND	108±10
	80	82.9±5.0	51.4±5.4	0.61	209.3±191.7	426.6±27.5	88±12
	200	201.5±8.0	101.5±13.2	0.50	ND[b]	1023.4±23.9	106±11
	400	404.6±38.8	183.4±13.3	0.45	ND	1225.8±38.1	104±5
D/P-free	20	20.5±3.7	11.3±1.2	0.55	48.0±28.4	ND	91±6
	40	39.1±3.6	21.7±1.9	0.56	188.1±14.4	ND	86±9
	80	81.1±7.4	51.3±3.6	0.63	218.6±92.9	ND	83±13
	200	207.1±10.9	79.5±15.5	0.38	ND	362.3±3.6	94±10
	400	410±8.9	186.4±16.7	0.45	ND	476.6±17.8	102±7

[a]Mean ± standard deviation; n = 3

[b]ND, not detected

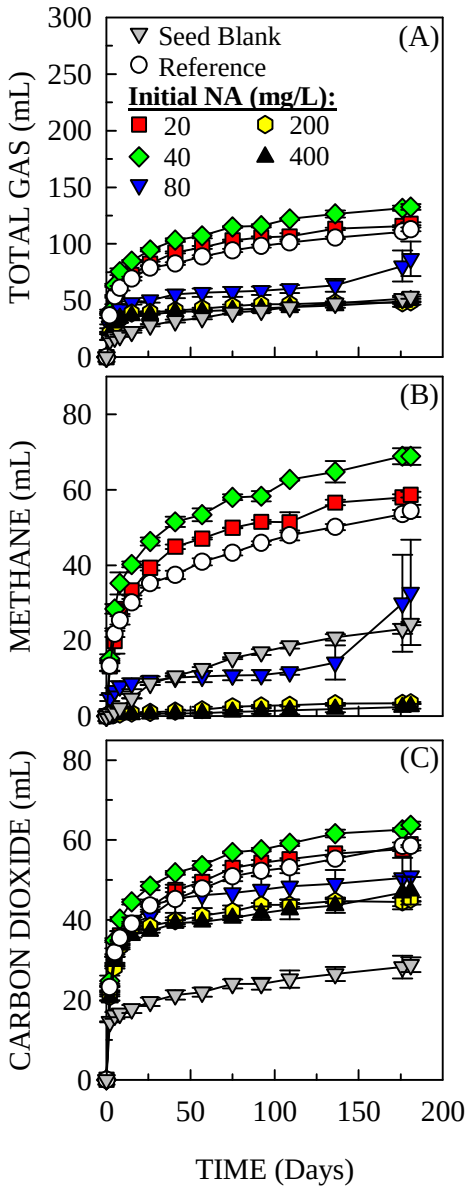


Figure 6.13. Total gas (A), methane (B) and carbon dioxide (C) profiles in methanogenic culture series amended with D/P and NAs at different initial NA concentrations (0-400 mg/L; gas data at 35°C and 1 atm). Error bars represent mean values ± one standard deviation, *n* = 3.

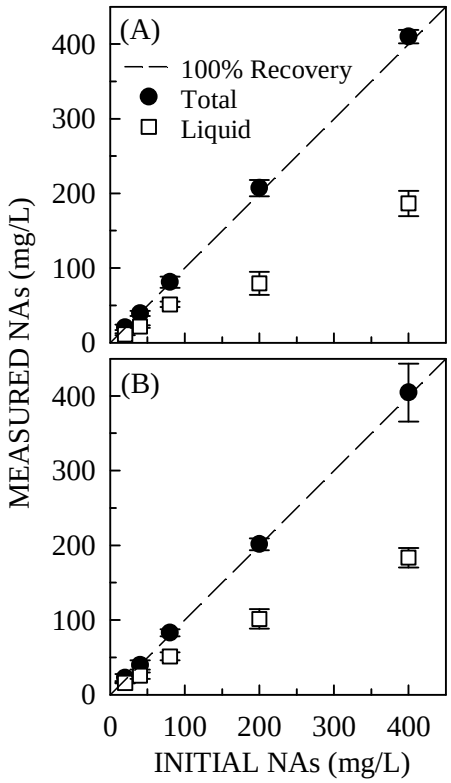


Figure 6.14. Total and liquid-phase NA concentrations in methanogenic culture series containing NAs and D/P (A) and NAs only (B) at the end of the 181-day incubation period. Error bars represent mean values ± one standard deviation, $n = 3$.

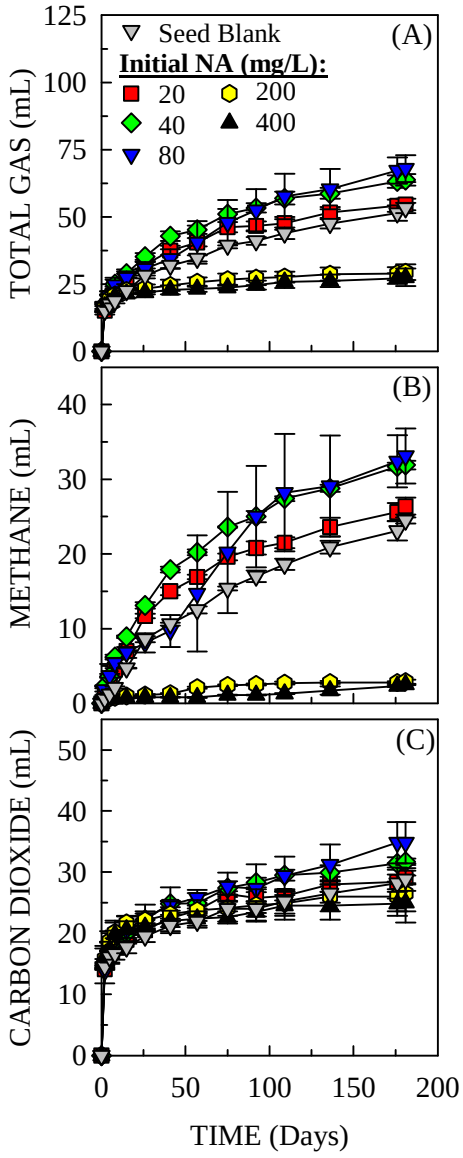


Figure 6.15. Total gas (A), methane (B) and carbon dioxide (C) profiles in methanogenic culture series amended with NAs only at different initial NA concentrations (0-400 mg/L; gas data at 35°C and 1 atm). Error bars represent mean values ± one standard deviation, *n* = 3.

6.5 Summary

NAs were found to be inhibitory to nitrifiers in both mixed and enriched nitrifying cultures. When dextrin was provided as an additional carbon source, the extent of NA degradation by the heterotrophs was lower than in dextrin-free mixed nitrifying culture series, resulting in longer exposure of nitrifiers to higher NA concentrations, which in turn resulted in a higher degree of nitrification inhibition. The nitrification activity by the enriched nitrifying culture was not significantly affected by NAs up to an initial concentration of 400 mg NA/L, which is attributed to the higher population size of AOB and NOB compared to the mixed culture series.

Inhibition and biotransformation assays showed that the TCI NA mixture was not inhibitory to denitrifiers at concentrations up to 400 mg/L. In contrast, NAs were found to be inhibitory to methanogens at and above 80 mg NA/L; however, carbon dioxide production indicated that fermentation and acidogenesis were not affected by NAs. NAs were not degraded under denitrifying or methanogenic conditions; however, significant cell lysis took place at high NA concentrations. The released lysis products were in turn used as electron donor(s) for nitrate reduction or converted to methane.

Refinery treatment systems typically use aerobic biotreatment units; however, understanding the fate and effect of NAs under anoxic and anaerobic conditions is relevant for the treatment of NA-bearing sludge and the fate of NAs in impacted natural sediments. NAs are introduced into many anoxic/anaerobic environments within oil sands tailings ponds, landfills and sediments. Thus, it is important to understand the potential toxic and inhibitory effect that NAs have under all conditions and other factors that may affect NA degradation in order to prevent disruption of refinery wastewater treatment

systems and/or negative effects on natural ecosystems in which NAs could potentially be introduced. The present study provides valuable information relative to the fate and effect of NAs in engineered and natural systems.

CHAPTER 7

AEROBIC BIOTRANSFORMATION POTENTIAL OF A COMMERCIAL MIXTURE OF NAPHTHENIC ACIDS

7.1 Introduction

Model NAs, commercial NA mixtures and NA-bearing refinery process water samples have been degraded aerobically using inocula obtained from refinery wastewaters and NA-exposed wetland waters; however, commercial NAs were found to be more readily degraded than the NAs found in refinery wastewaters (Clemente and Fedorak, 2005; Del Rio et al., 2006; Scott et al., 2005). Although NAs are prevalent in refinery wastewaters and oil sands tailings ponds, there is limited information about their biotransformation potential and toxicity. NAs can be degraded under aerobic conditions; however, many NAs are persistent and remain in wastewater effluents even after biotreatment (Clemente and Fedorak, 2005; Del Rio et al., 2006; Quagraine et al., 2005a; Scott et al., 2005). Partial biodegradation of NAs has been shown to result in more oxidized biotransformation intermediates, containing more than one carboxylic group or 3, 4 or 5 oxygen atoms (Grewer et al., 2010; Han et al., 2008; Johnson et al., 2011). Activated sludge wastewater systems are commonly used to treat refinery process water and wastewater. However, no reports have been made to the degree that activated sludge units are capable of reducing the NA concentration and the factors affecting NA biotransformation are not completely understood.

The objective of this research was to investigate the biodegradation potential of a commercial mixture of NAs under aerobic conditions using different cultures with

varying degrees of NA exposure history and at different time points in the culture development. Bioassays were performed to investigate the effect of co-substrate, prior NA exposure and NA concentration on the extent of NA biodegradation and mineralization.

7.2 Materials and Methods

7.2.1 Culture Development and Maintenance

Three mixed, aerobic cultures (un-amended, NA-amended and NA-enriched) were developed with the same inocula obtained from a refinery activated sludge system and then used as seed in the biotransformation assays reported here. The NA-amended culture was developed from the un-amended culture biomass after 3 months of development on an NA-free feed. Both the un-amended and NA-amended cultures were maintained fed-batch with a 40-day solids retention time (SRT) and 12-day hydraulic retention time (HRT) at room temperature (22-24°C) (Table 7.1). Each feeding cycle consisted of three phases: two 3-day oxic phases, followed by one 1-day anoxic phase. In the first oxic phase, the cultures were fed with a synthetic wastewater (1,200 mg COD/L), ammonia (100 mg N/L), and NAs (50 mg/L; NA-amended culture only). The composition of the synthetic wastewater feed was chosen in order to simulate to some degree the influent of refinery activated sludge units. The synthetic wastewater composition was as follows (in g COD/L): sodium acetate, 0.179; sodium propionate, 0.179; sodium butyrate, 0.089; sodium benzoate, 0.358; methanol, 0.269; naphthalene , 0.089; and phenol, 0.036. The synthetic wastewater and ammonia were consumed during the first oxic phase after which the second oxic phase began. In order to enrich and maintain a nitrifying autotrophic

population, at the beginning of the second oxic phase the cultures were fed with only ammonia at an initial concentration of 100 mg N/L. Complete nitrification was achieved in the second oxic phase, while nitrate accumulated. Then, aeration was stopped and the reactors were switched to the anoxic phase with the addition of the same synthetic wastewater (1,200 mg COD/L) which served as carbon source and electron donor for denitrification. Complete removal of nitrate via denitrification in the third, anoxic phase was achieved within one day. To maintain a relatively high SRT, wasting was alternated with and without biomass settling at the end of the anoxic phase. After wasting, the cultures were replenished with the same volume of a mixture of tap water amended with phosphate buffer (10 mM), synthetic wastewater, ammonia and NAs (NA-amended culture only). The un-amended and NA-amended feeding cycles corresponded to an average organic loading rate of 0.34 and 0.37 g COD/L-day, respectively and the NA-COD to wastewater COD (WW-COD) ratio in the NA-amended culture was approximately 0.074 mg NA-COD/mg WW-COD. The cultures were aerated with pre-humidified compressed air passed through a fine pore diffuser and maintained at a pH between 6.8 and 7.2 throughout all feeding phases.

A third culture, an aerobic NA-enriched culture, was developed using the same inoculum as the above-described un-amended, and the commercial TCI NA mixture was the sole carbon source. The culture was fed and wasted twice per week corresponding to an average organic loading rate of 0.2 g COD/L-day. The feed included 200 mg NA/L (TCI NA mixture), 25 mg NH_4-N/L and a salt medium containing the following (in g/L): K_2HPO_4, 1.07; KH_2PO_4, 0.524; $CaCl_2 \cdot 2H_2O$, 0.068; $MgCl_2 \cdot 2H_2O$, 0.135; $MgSO_4 \cdot 7H_2O$, 0.268; $FeCl_2 \cdot 4H_2O$, 0.068; and 0.67 mL/L trace metal stock solution. The culture

was maintained with an HRT and SRT of 14 days, aerated with pre-humidified compressed air passed through a fine pore diffuser and maintained at a pH between 6.5 and 7.5.

In order to assess the effect of non NA co-substrate and absence of NA in the culture feed on NA biodegradation, two additional cultures were developed from the NA-enriched culture biomass and fed either both NAs and synthetic wastewater or synthetic wastewater only. Synthetic wastewater was fed to both cultures to achieve an initial concentration of 700 mg COD/L. NAs were fed to the NAs and wastewater-fed culture in three 20-day phases at initial concentrations of 200, 50 and 15 mg NA/L, corresponding to NA-COD to WW-COD ratios of 1, 0.25 and 0.075 mg NA-COD/mg WW-COD (Table 7.1). The cultures were maintained for approximately 60 days with an HRT and SRT of 14 days, aerated with pre-humidified compressed air passed through a fine pore diffuser and maintained at a pH between 6.5 and 7.5.

Table 7.1. Characteristics of mixed aerobic cultures used in NA biotransformation assays.

Culture	Loading Rate (g COD/L-day)		Feed NA/WW Ratio[c]	Retention Time (Days)	
	NA mixture	Synthetic Wastewater		HRT	SRT
Un-amended	-	0.34	-	12	40
NA-amended	0.025	0.34	0.074	12	40
NA-enriched	0.2	-	-	14	14
Wastewater only[a]	-	0.2	0	14	14
NAs and Wastewater[b]	0.2/0.05/0.015	0.2	1/0.25/0.075	14	14

[a]NA-enriched side culture amended with synthetic wastewater only

[b]NA-enriched side culture amended with NAs and synthetic wastewater

[c]On a COD-basis

7.2.2 Biotransformation Assays

Six batch assays were performed to investigate the biotransformation potential of NAs by mixed aerobic cultures developed under various levels and duration of NA exposure. In the first biotransformation assay, which assessed the effect of culture enrichment and co-substrate, four culture series were set up as follows. Two cultures were prepared with the un-amended culture, one with and one without synthetic wastewater amendment. Another two cultures were prepared with the NA-enriched culture similarly to the two un-amended cultures (i.e., with and one without synthetic wastewater amendment). Prior to the bioassay, culture biomass was settled and washed twice with 10 mM phosphate buffer before being re-suspended in aerobic salt media (composition detailed in Section 7.2.1, above). All cultures were amended with 200 mg NA/L (equal to 700 mg COD/L). Nitrogen was added to each culture series without and with wastewater at concentrations of 35 and 70 mg NH_4^+-N/L, respectively (COD:N = 100:5). The synthetic wastewater was the same used to feed the un-amended and NA-amended cultures (see Section 7.2.1, above). At the time the first biotransformation assay was performed, the un-amended and the NA-enriched cultures had been developed and maintained for 3 and 15 months, respectively. The two cultures initially amended with the synthetic wastewater at an initial concentration of 700 mg COD/L were again amended on day 10 with the same wastewater and at the same initial concentration.

In the second biotransformation assay, three culture series were set up using the three, previously developed cultures (un-amended, NA-amended and NA-enriched culture) in order to assess the effect of culture acclimation and age on the biotransformation of the TCI NA mixture. At the time the second biotransformation assay

was performed, the un-amended, NA-amended, and the NA-enriched cultures had been maintained for 15, 12, and 27 months, respectively. The three culture series were prepared in the same manner as in the first bioassay and amended with 200 mg NA/L and 35 mg NH_4-N/L, without addition of the synthetic wastewater. In the above described, two biotransformation assays, the cultures were mixed using magnetic stirrers and aerated with pre-humidified, compressed air. Total and soluble NAs, pH, and soluble COD were measured regularly. Nitrogen species, including ammonia, nitrite, and nitrate, were measured periodically. The pH in all culture series was maintained around 7.

In the third biotransformation assay, the NA-enriched culture was used to assess the effect of initial NA concentration on the extent of NA biodegradation. Before use in this bioassay, NA-enriched culture biomass was washed three times with 10 mM phosphate buffer to remove biomass-sorbed, residual NAs. After the last washing, the biomass was suspended in aerobic salt media with the same composition as described in the NA-enriched culture development (see Section 7.2.1 above). The total NA concentration in the pre-washed and re-suspended culture was less than 3 mg/L and NAs were not detected in the liquid-phase. Four culture series were set up in 250-mL Erlenmeyer flasks with the pre-washed, NA-enriched culture, 35 mg NH_4^+-N/L and target, initial NAs concentrations of 50, 100, 150 and 250 mg/L. The initial VSS in all culture series was approximately 500 mg/L. During the incubation period, the flasks were aerated with pre-humidified air at room temperature (22 to 24°C) and agitated on an orbital shaker at 190 rpm. Total and soluble NAs, pH, and soluble COD were measured regularly throughout the incubation period. The pH in all culture series was maintained around 7.

To further investigate the biotransformation potential of the persistent, residual NA concentration remaining at the end of each feeding cycle of the NA-enriched culture, a fourth biotransformation assay was performed as follows. A portion of the NA-enriched culture waste was maintained with approximately the same NA loading rate (0.2 g COD/L-day) as the NA-enriched culture through five feeding cycles without culture wasting, fed approximately 200 mg NA/L and 35 mg NH_4^+-N/L, biweekly for three weeks. The total and liquid-phase NA concentrations were monitored before and after each feeding and the pH was maintained around 7.

A fifth biotransformation assay was conducted using the NA-enriched culture under closed bottle, aerobic conditions to determine to what extent the NA mixture was mineralized to CO_2 following a modified, previously published method (Clemente et al., 2004). The NA-enriched culture biomass was washed as described in the third biotransformation assay, re-suspended in aerobic salt media and amended with 35 NH_4-N/L. Two sacrificial culture series were set up in 70-mL serum bottles amended with 40-mL NA-enriched culture without and with 200 mg NA/L, representing the seed control and NA-amended culture series, respectively. Both the NA-amended and seed culture series were amended with 35 mg NH_4^+-N/L, sealed and 15 mL pure O_2 was injected into the headspace containing air to maintain a positive pressure and to ensure oxygen was in excess. The culture series were agitated on an orbital shaker at 190 rpm and sacrificially sampled for CO_2, O_2, total and soluble NAs, total and soluble COD and pH on day 0, 1, 2, 3 and 6. On each sampling day, one NA-amended and one NA-free seed culture bottle were acidified with 3 mL of 6 N H_2SO_4, agitated for 4 hours, and the headspace CO_2 concentration was measured in triplicate. Prior to opening for liquid analysis, the

headspace O_2 concentration in the un-acidified serum bottles was quantified. The pH was maintained in between 6.5 and 7 in all culture series. The extent of NA mineralization was determined based on carbon mass balance calculations and the weighted average molecular weight (MW) of the NA mixture. The biomass incorporated carbon in the NA-amended culture series was determined assuming $C_5H_7O_2N$ as the biomass molecular formula.

Lastly, a sixth biotransformation assay was conducted using the side culture developed from the NA-enriched culture biomass fed only synthetic wastewater. A portion of the culture biomass was used in a batch biotransformation assay and amended with NAs at 200 mg NA/L, 35 mg NH_4^+-N/L and aerobic media (see Section 7.2.1, above). Total and liquid-phase NAs were measured periodically throughout the incubation period and the pH was maintained in between 6.5 and 7. The flask was aerated with pre-humidified air at room temperature (22 to 24°C) and agitated on an orbital shaker at 190 rpm.

7.2.3 Microbial Community Analysis

Microbial community analysis of the NA-enriched culture was performed when this culture was maintained for 27 months. The biomass was washed several times with a saline phosphate buffer before extracting genomic DNA using soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA). The 16S rRNA genes were amplified by PCR using a pair of universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCARCCGCA-3′). The PCR amplification was carried out with the following conditions: single cycle denaturation at 94 °C for 5 min, 30 cycles of

30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C, and final extension for 7 min at 72 °C. Cloning was performed using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. The clones were then grouped into different operational taxonomic units (OTUs) after digesting with restriction enzymes Msp1/Taq1 and based on the restriction patterns. Representative clones of each OTU were selected for sequencing of nucleotides after PCR amplification and purification. The 16S rRNA gene sequences were then queried against the National Center for Biotechnology Information (NCBI) *GenBank* sequence database using MEGABLAST algorithm. Sequence alignment was performed with the program CLUSTALW. The sequence-based phylogenetic tree of the dominant bacteria was constructed by applying the neighbor-joining algorithm using the program MEGA4.1. The tree topology was evaluated by bootstrap resampling analysis of 1000 resampling data sets. The sequences have been submitted to the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/) for deposition.

7.3 Results and Discussion

7.3.1 Culture Development

7.3.1.1 Un-Amended and NA-Amended Cultures

The operational cycles of the un-amended and NA-amended cultures are described in Section 7.2.1. Figure 7.1 shows the nitrogen species and soluble COD concentrations throughout the three operational phases of one complete culture feeding cycle of the un-amended culture. All ammonia was consumed within 1 and 1.5 days during the first and second oxic phases, respectively. At the end of the second oxic phase, the nitrate

concentration was approximately 105 mg N/L. Nitrite initially accumulated during both oxic cycles and was then quickly consumed. The subsequent anoxic phase started with the addition of 1,000 mg COD/L and complete denitrification was achieved within a day in this reactor. The pH was maintained between 6.8 and 7.2 throughout all operational cycles. The steady-state biomass concentration in this reactor maintained as described above was 1.6 g VSS/L.

In the NA-amended culture, the oxic phase-1 lasted less than 2 days, during which ammonia, nitrite and COD were consumed (Figure 7.2). Ammonia was consumed at a slightly slower rate by the NA-amended culture than by the un-amended culture. Nitrite initially accumulated to approximately 10 mg N/L during this period and was then quickly consumed. During the second oxic phase, complete nitrification was observed within 2 days and nitrite accumulated to approximately 20 mg N/L before being rapidly consumed. The nitrate concentration at the end of the second oxic phase was approximately 100 mg N/L. During the anoxic phase, complete denitrification was observed within one day. The pH was maintained between 6.8 and 7.2 throughout all operational cycles.

Although NAs were fed to the NA-amended culture at the beginning of each feeding cycle, NA degradation did not occur and a steady-state total NA concentration between 100 and 120 mg/L was maintained throughout the life of the NA-amended culture. Based on NA addition and wasting through biomass and culture supernatant and assuming no NA degradation, the steady-state total NA concentration was estimated to be between 100 and 115 mg/L, which agrees with the above-mentioned, measured total NA concentration (Figure 7.3). The steady-state biomass concentration was (mean ± standard

deviation) 1.1 ± 0.2 g VSS/L for the NA-amended culture. The measured VSS concentration was corrected for the biomass-adsorbed NA concentration. The lower biomass concentration in the NA-amended culture compared to the un-amended culture indicates NA-induced cell lysis.

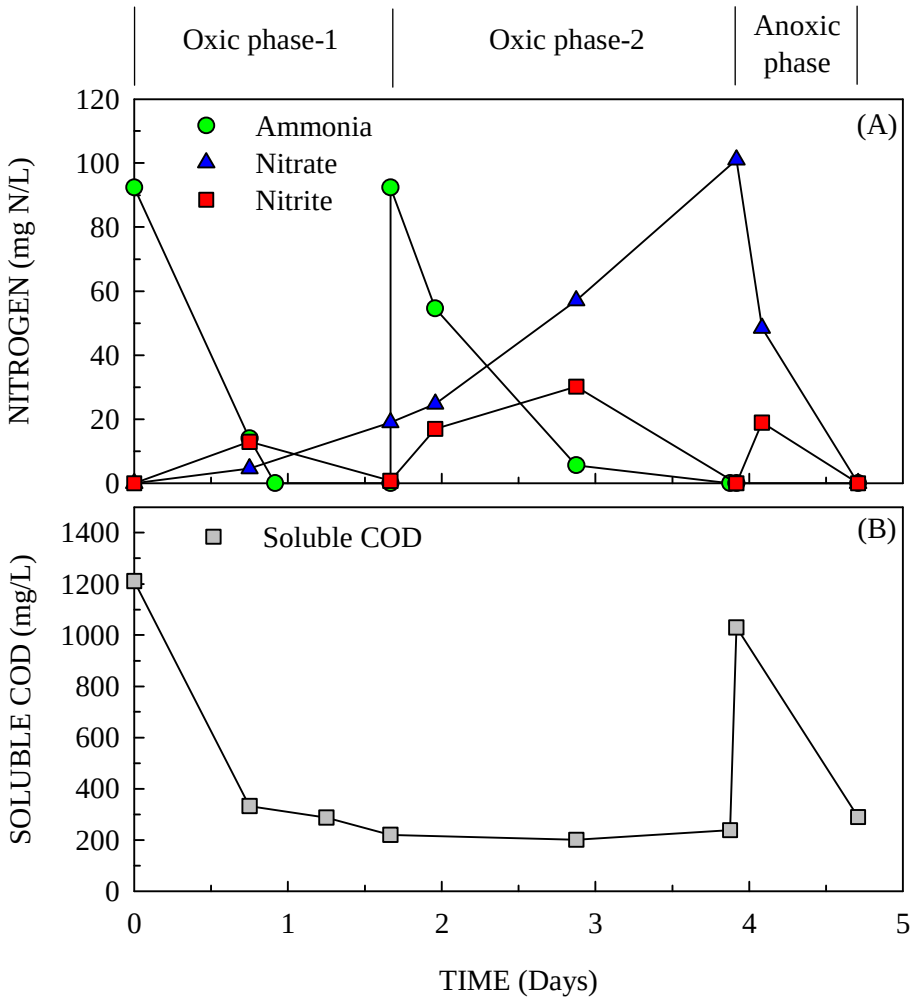


Figure 7.1. Nitrogen species (A) and soluble COD (B) in the un-amended culture throughout three operational cycles.

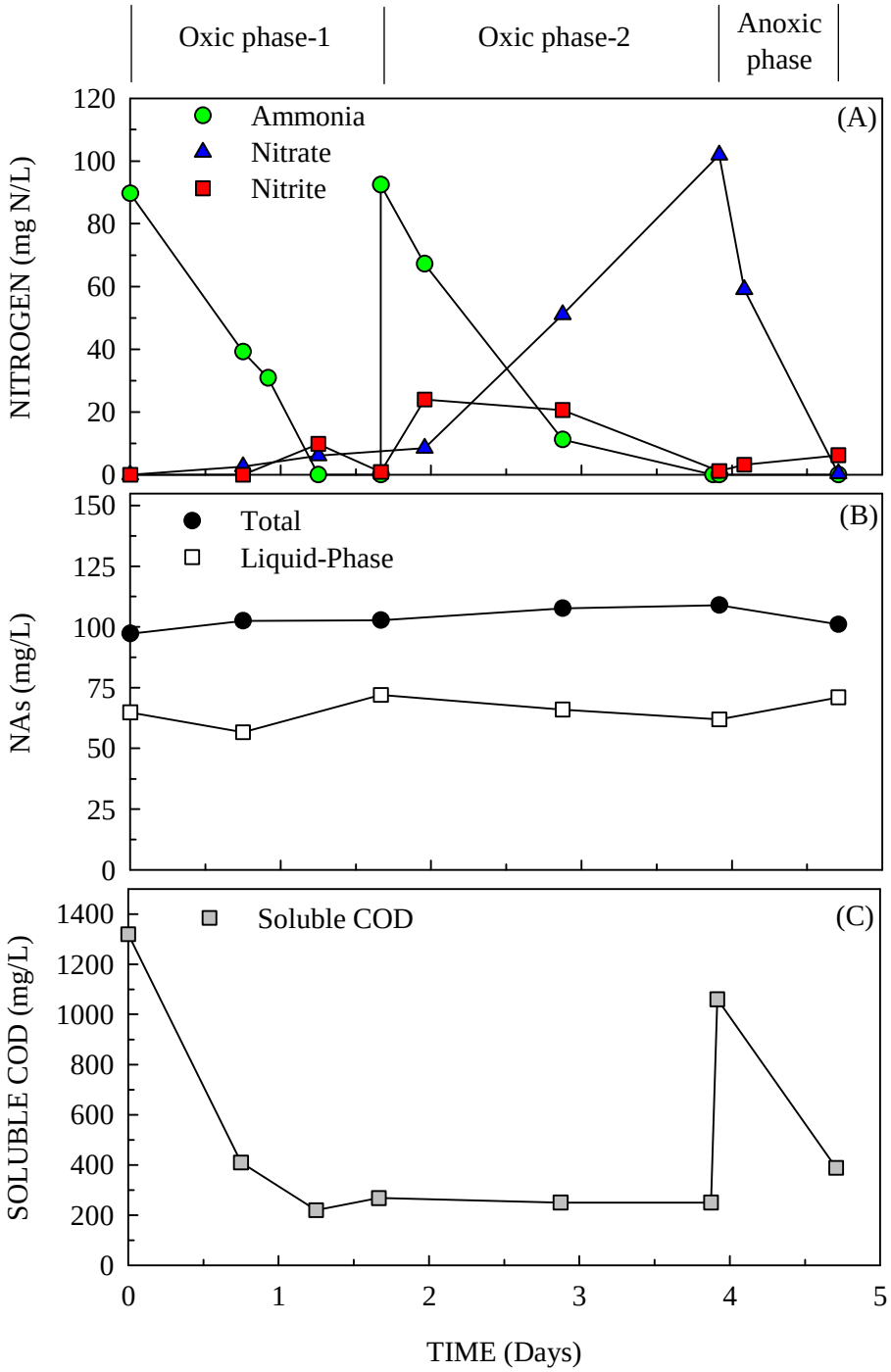


Figure 7.2. Nitrogen species (A), total and liquid-phase NAs (B) and soluble COD (C) in the NA-amended culture throughout three operational cycles.

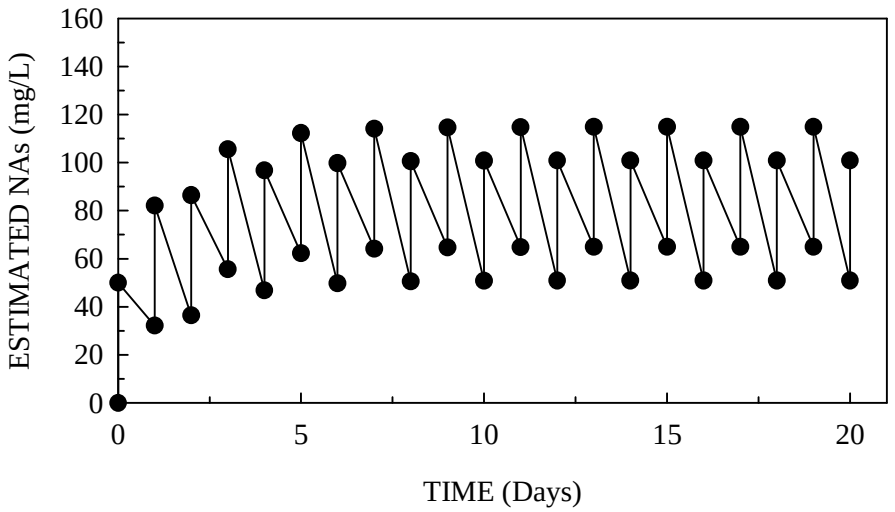


Figure 7.3. Estimated steady-state NA concentration in NA-amended culture based on fed NA concentration (50 mg/L), culture wasting method and assuming no NA degradation.

7.3.1.2 NA-Enriched Culture

Figure 7.4 shows the initial feeding cycles (immediately after inoculation with refinery mixed liquor biomass) of the NA-enriched culture, indicating that the biomass in the refinery mixed liquor inoculum was capable of degrading NAs. Initially, degradation of the TCI NA salt was slow, reaching a residual concentration of approximately 50 mg/L within 10 to 15 days. After the first few feeding cycles, the culture quickly degraded NAs to the same residual concentration within the 3 to 4 day feeding cycles. The culture was maintained with a mean total NA feed concentration of (mean ± standard deviation) 192.6±28.5 for approximately 40 months and degraded approximately 75% of the fed NAs, resulting in a mean total residual NA concentration of 55.5±13.3 mg NA/L. Approximately 50% of the total NAs was adsorbed to the biomass, with the remainder in the liquid-phase, regardless of the composition of NAs, fed (un-degraded) or residual. Figure 7.5 shows the nitrogen species and total and liquid-phase NA concentrations throughout one complete NA-enriched culture feeding cycle after 20 months of development. Before wasting and feeding, the ammonia and nitrate concentrations were 0 and 18 mg N/L, respectively. In addition to NA degradation, nitrification was occurring in this culture, indicated by the increase in nitrate throughout the feeding cycle (Figure 7.5A). Nitrite was not detected. The steady-state biomass concentration was (mean ± standard deviation) 366 ± 22 mg VSS/L.

The NA distributions at the beginning and end of the feeding cycle are shown in Figure 7.6. In the initial sample, monocyclic (Z = -2) and dicyclic (Z = -4) NAs were the most dominant NAs and at the end of the incubation period, the distribution of all acyclic and cyclic NAs was approximately the same.

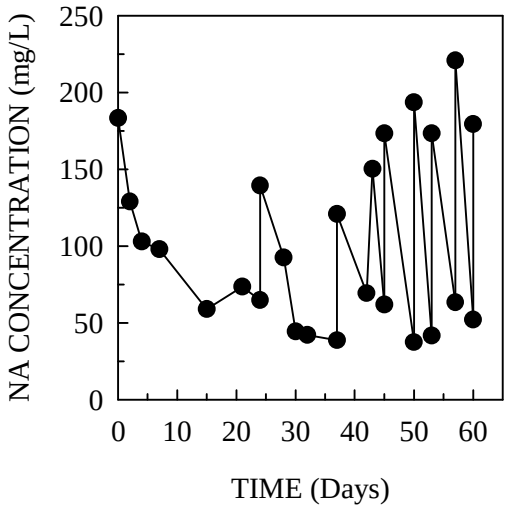


Figure 7.4. Initial feeding cycles of the NA-enriched culture immediately after inoculation with refinery activated sludge mixed liquor.

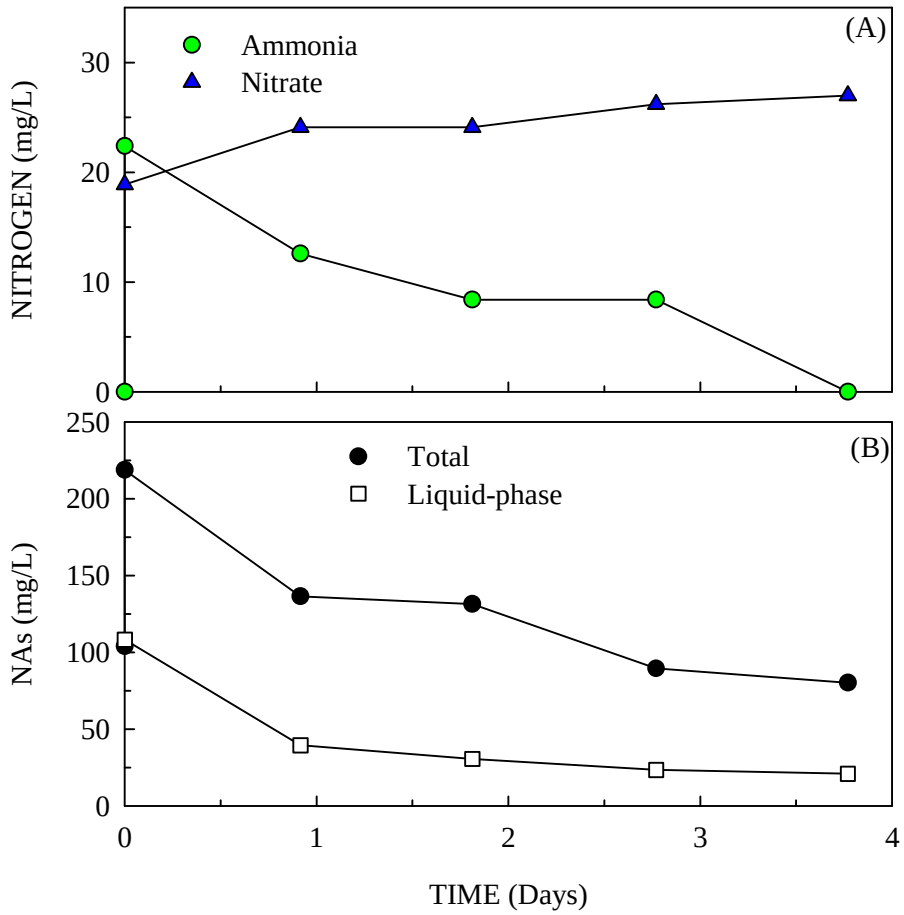


Figure 7.5. Nitrogen species (A) and total and liquid-phase NAs (B) in NA-enriched culture throughout one feeding cycle.

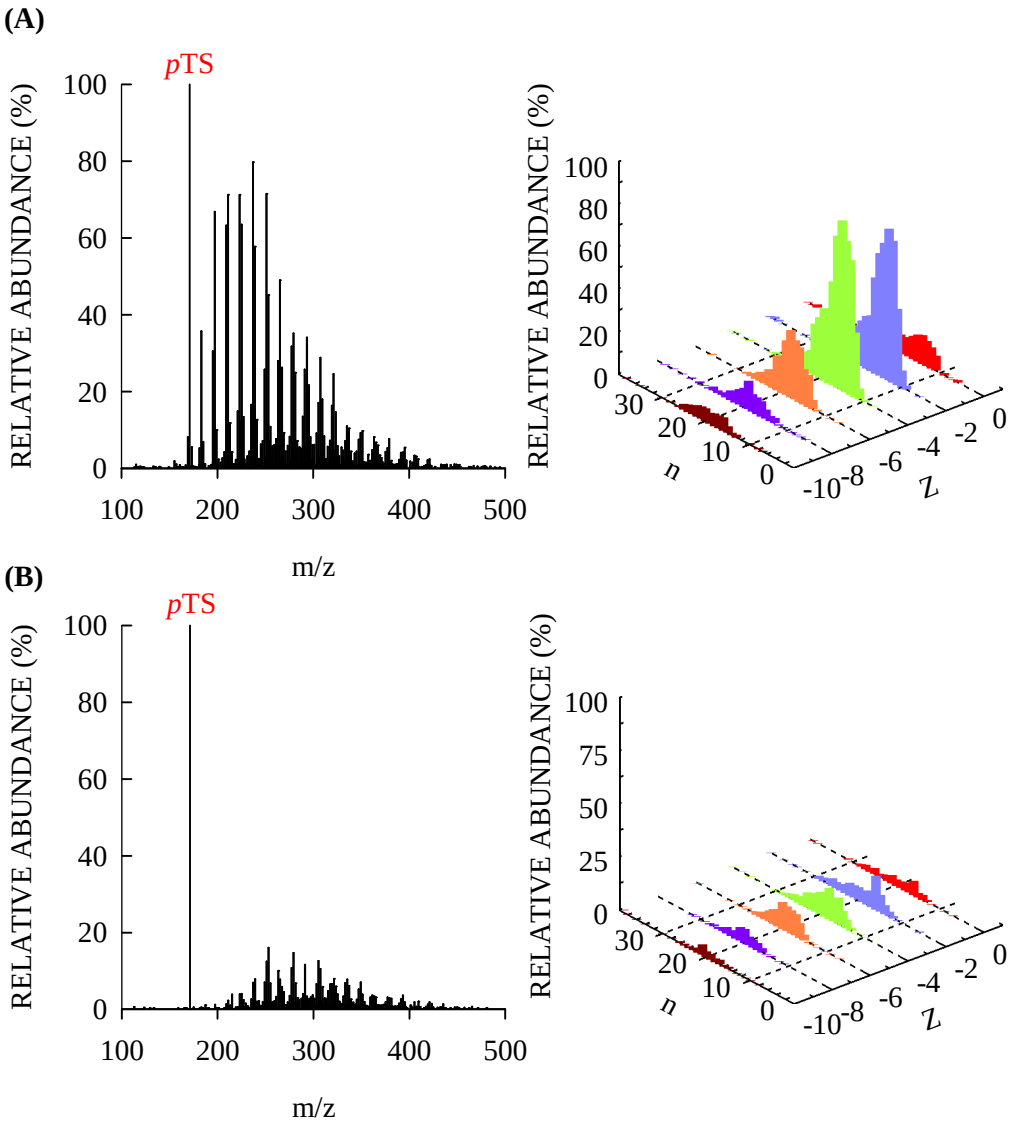


Figure 7.6. Congener NA distributions in the initial (A) and final samples (B) of a NA-enriched culture feeding cycle.

7.3.1.3 NA-Enriched Side Cultures

Two side cultures were developed from the NA-enriched culture waste and maintained for approximately 60 days. The NA-enriched culture biomass was left unfed for 7 days before the beginning of the first feeding cycle. The total and liquid-phase NAs and soluble COD, before and after feeding, in the cultures fed with synthetic wastewater-only and synthetic wastewater and NAs are shown in Figure 7.7 and 7.8, respectively. The NAs in the culture fed only synthetic wastewater were washed out within 14 days of incubation. The mean total NA concentrations at the beginning and end (residual) of the feeding cycles in the culture fed synthetic wastewater and NAs during three feeding phases of 200, 50 and 20 mg NA/L were 225.8±9.7 and 54.4±2.0, 75.5±13.8 and 31.4±6.1, and 53.2±4.4 and 25.9±2.6 mg NA/L, respectively. The steady-state biomass concentrations in the cultures fed synthetic wastewater only and NAs and synthetic wastewater were (mean ± standard deviation) 590.0±50.9 and 864.7±23.2 mg VSS/L, respectively.

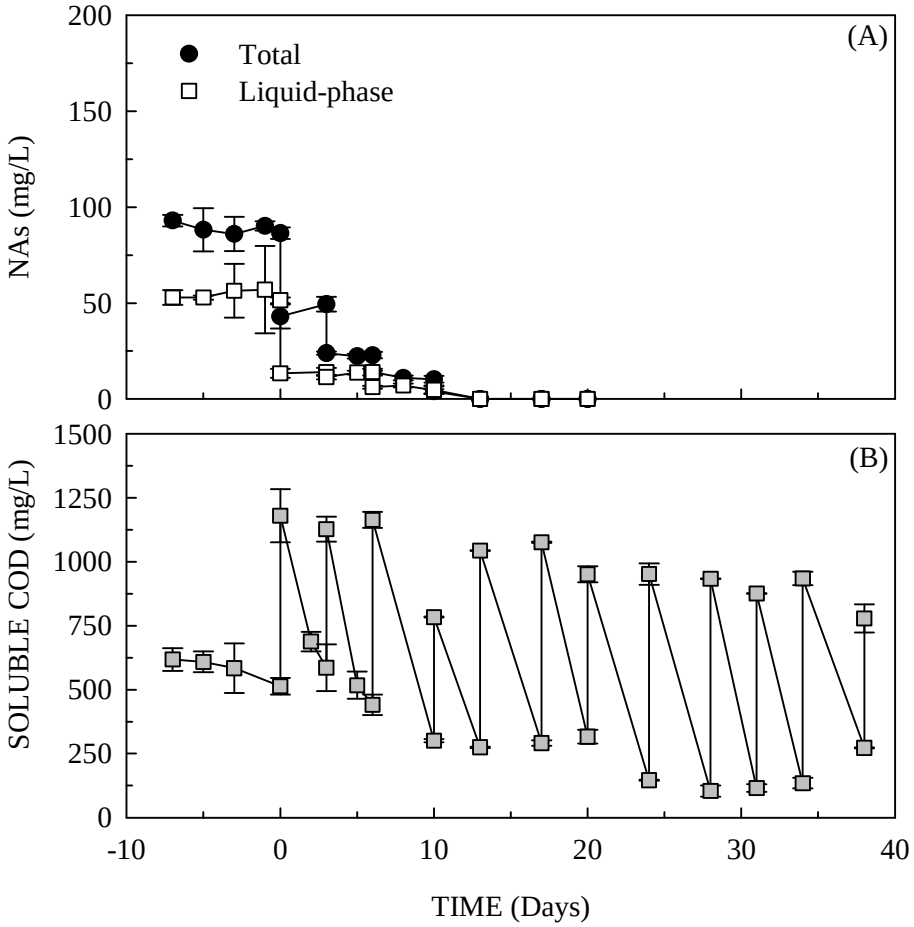


Figure 7.7. Total and liquid-phase (A) NAs and soluble COD (B) in the NA-enriched side culture fed only synthetic wastewater (Error bars represent mean values ± one standard deviation, n = 3).

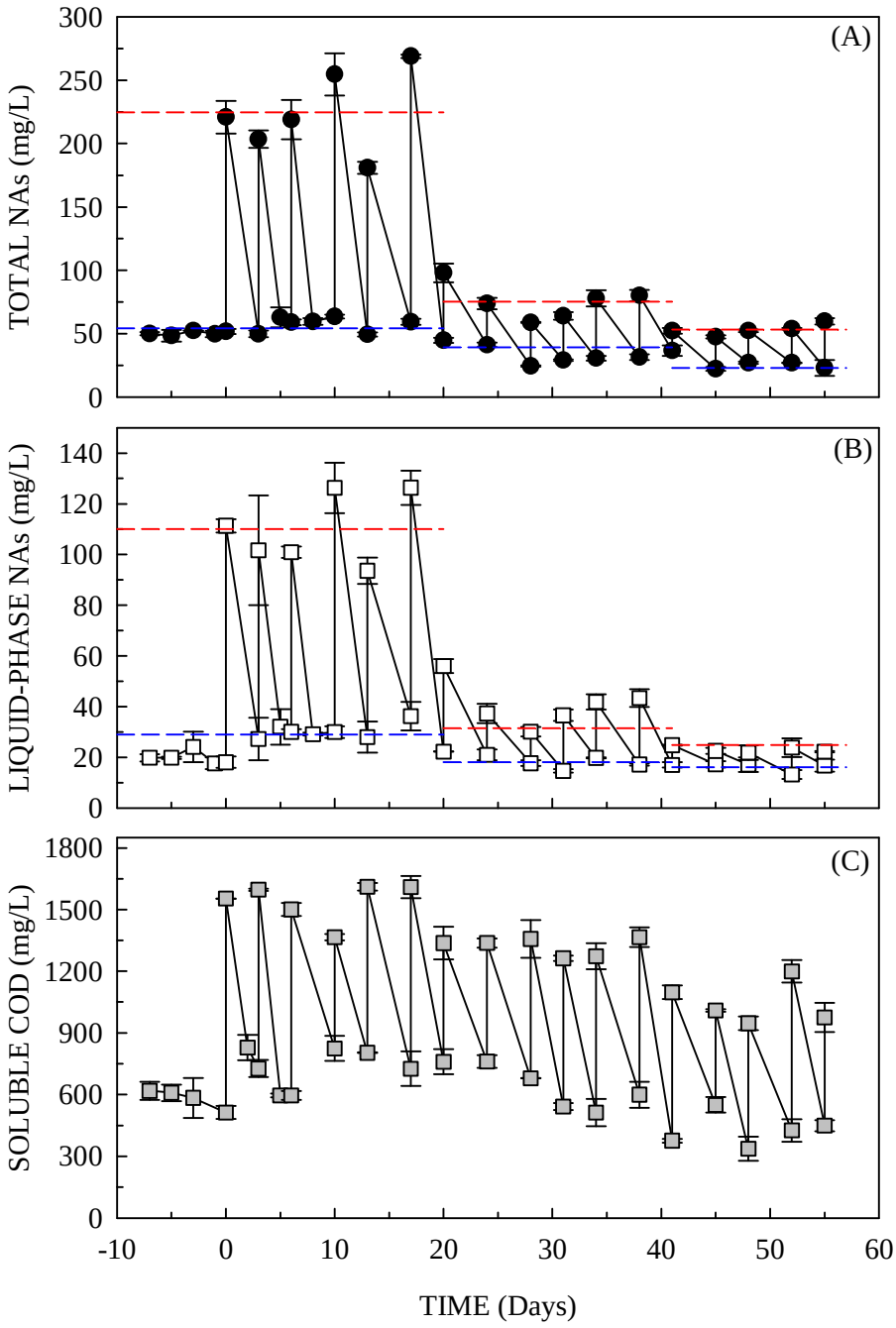


Figure 7.8. Total (A) and liquid-phase (B) NAs and soluble COD (C) in the NA-enriched side culture fed both synthetic wastewater and NAs at concentrations of 200, 50 and 15 mg/L (Red and blue lines represent the mean NA concentration before and after feeding at three different initial NA concentrations; error bars represent mean values ± one standard deviation, n = 3).

7.3.2 Biotransformation Assay I

The first batch NA biotransformation assay, which assessed the effect of culture enrichment and co-substrate, was conducted with the NA-enriched and NA un-amended cultures with and without the addition of a degradable synthetic wastewater as discussed in Section 7.2.2, above. The total and liquid-phase NA concentrations and soluble COD in the NA-enriched and un-amended culture series are shown in Figure 7.9. In both NA-enriched culture series (Figure 7.9A and C), the total NA concentration decreased from 200 to 50 mg/L within 3 days and remained stable for the rest of the incubation period. A short lag in total and soluble NA degradation was observed in the NA-enriched culture fed with the synthetic wastewater, but then NAs were degraded at a similar rate as in the culture without addition of synthetic wastewater. NA degradation was not observed in the un-amended culture fed with the synthetic wastewater (Figure 7.9G). In contrast, after 7 days without NA degradation in the un-amended culture not amended with the synthetic wastewater, NA degradation took place after 7 days and within another 3 days the total NA concentration decreased from approximately 200 to 50 mg/L (Figure 7.9E). Thus, when a readily degradable carbon source was available, the NA un-amended microbial population did not degrade NAs. Soluble COD data indicated that the synthetic wastewater was rapidly consumed in both culture series and on day 10, the same two cultures were re-amended with the synthetic wastewater (Figure 7.9D and H).

Figures 7.10 and 7.11 show the time course of *Z* number, average carbon number, *n*, and MW during the incubation period in the NA-enriched and un-amended culture series which were not amended with the synthetic wastewater. Initially, monocyclic (Z = -2) and dicyclic (Z = -4) NAs were the most dominant. Both cultures degraded similar

NA compounds and at the end of the incubation period, the concentration of the individual groups of acyclic (Z = 0) and cyclic NAs (Z = -2, -4, -6, -8 and -10) were the same at around 5 to 10 mg/L (Figure 7.10). The observed residual NAs may not be inherently recalcitrant, but rather below a concentration threshold, which was investigated as discussed below (see Section 7.3.5 and 7.3.6). The initial weighted average MW and carbon number in both cultures were around 275 and 18, respectively. As some of the NA groups were degraded, the weighted average MW and carbon number increased in both cultures, indicating that lower MW NAs were preferentially degraded, an observation which agrees with previous reports (Biryukova et al., 2007; Clemente et al., 2004; Han et al., 2008; Holowenko et al., 2002; Scott et al., 2005; Watson et al., 2002). Scott et al. (2005) found that the NAs with carbon numbers less than or equal to 17 were more readily biodegraded than the higher MW NAs. On the other hand, Han et al. (2004) found that carbon number had less of an effect on NA biodegradability than Z number, whereas lower biodegradation rates were observed for NAs with higher Z numbers. Similarly in this study, the more cyclic, higher MW NAs were not degraded and remained at the end of the incubation period in both cultures, regardless of prior NA enrichment or not. Figure 7.11 shows that the average carbon number, average Z number, and thus average MW, increased by approximately 5 carbons by the end of the 13-day incubation period. The congener NA distributions in all culture series in the initial and final samples are summarized in Table 7.2.

The Microtox® acute toxicity was measured for the initial and day 16 samples for each culture series and results are summarized in Table 7.3. The EC_{50} values in terms of % (v/v) and NA concentration (mg/L) increased from the beginning to the end of the

incubation period, indicating toxicity decrease throughout the incubation period. Previous NA biodegradation studies with commercial and oil sands NA-bearing wastewaters reported toxicity decrease after biotreatment, even though higher MW NAs were persistent (Clemente et al., 2004; Leung et al., 2003). NA toxicity reduction was observed in all cultures; however, toxicity was reduced to a lesser extent in the un-amended culture fed with the synthetic wastewater, in which NA degradation was not observed. In terms of NAs, the EC_{50} values were within 5 – 7 mg NA/L at the start of the bioassay and increased to 16 – 20 mg NA/L in all cultures, indicating that the degraded NAs are more toxic than the residual NAs remaining after biotreatment.

Table 7.2. Congener NA distributions in initial and final samples from four culture series amended with NAs and synthetic wastewater (WW) in batch biotransformation assay I.

Culture Series	Sample	*Z*	*n*	MW	Formula
NA-Enriched	Initial	-4.2	18.4	286.0	$C_{18.4}H_{32.7}O_2$
	Final	-5.3	23.3	353.1	$C_{23.3}H_{41.3}O_2$
NA-Enriched + WW	Initial	-4.0	17.4	272.3	$C_{17.4}H_{30.9}O_2$
	Final	-5.4	21.7	330.7	$C_{21.7}H_{38.1}O_2$
Un-amended	Initial	-4.3	17.8	276.9	$C_{17.8}H_{31.3}O_2$
	Final	-5.4	23.3	352.4	$C_{23.3}H_{41.2}O_2$
Un-amended + WW	Initial	-4.1	17.6	274.3	$C_{17.6}H_{31.1}O_2$
	Final	-3.9	16.7	261.9	$C_{16.7}H_{29.5}O_2$

Table 7.3. Microtox® acute toxicity in NA-enriched and un-amended cultures during the batch NA biotransformation assay I.

Culture Series	Day 0			Day 16		
	NA (mg/L)	**EC_{50} (%,v/v)**	**EC_{50} (mg NA/L)**	**NA (mg/L)**	**EC_{50} (%,v/v)**	**EC_{50} (mg NA/L)**
NA-enriched	105.6	4.8	5.1	20.5	80.0	16.4
NA-enriched + Wastewater	104.5	4.8	5.0	25.2	72.2	18.1
Un-amended	110.8	5.6	6.2	27.0	67.6	18.3
Un-amended + Wastewater	112.3	5.7	6.4	109.0	18.7	20.4

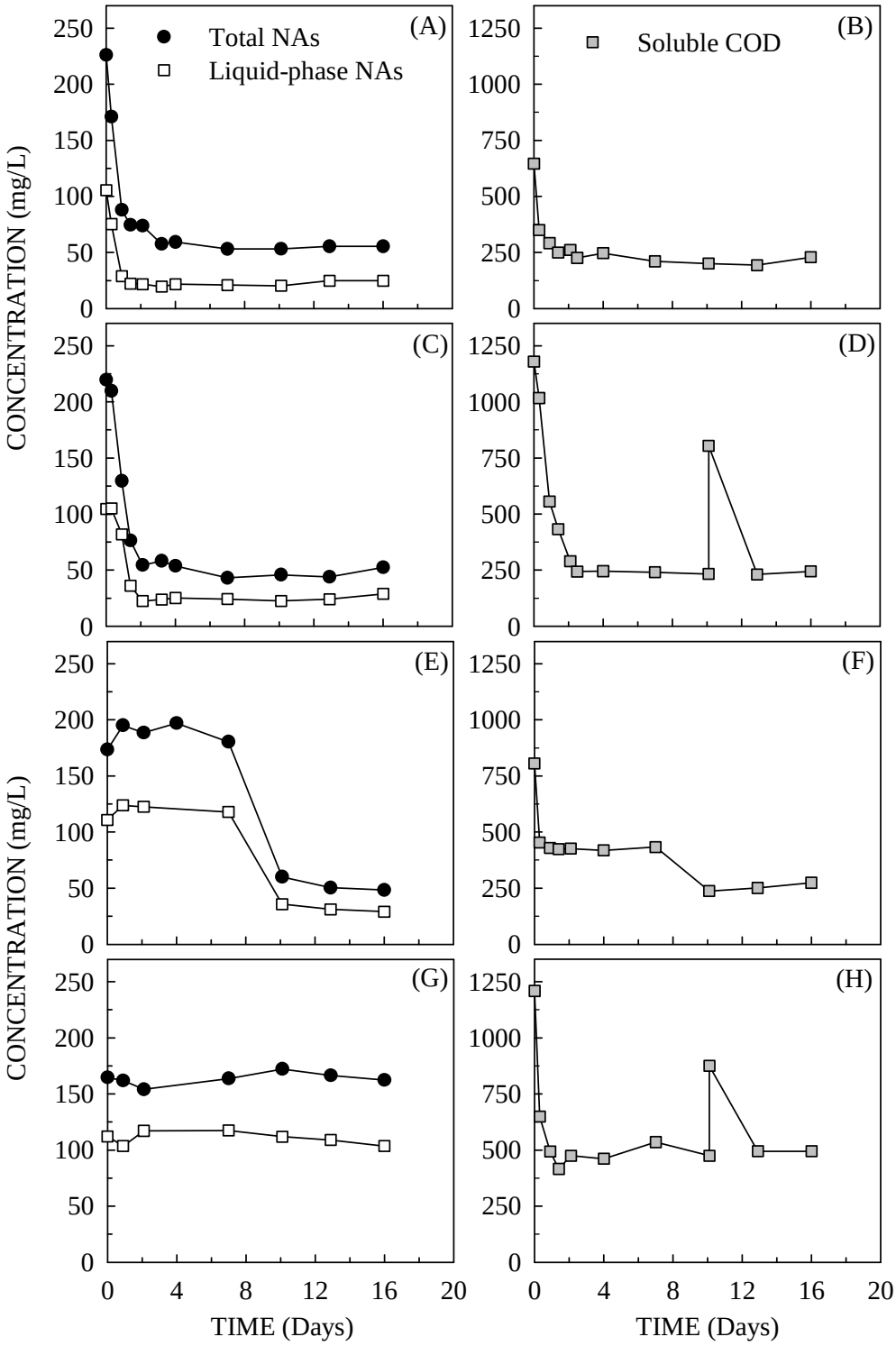


Figure 7.9. Total and liquid-phase NA concentrations and soluble COD in the NA-enriched and un-amended culture series as follows: NA-enriched culture without wastewater (A, B), NA-enriched culture with wastewater (C, D), un-amended culture without wastewater (E, F) and un-amended culture with wastewater (G, H) (biotransformation assay I).

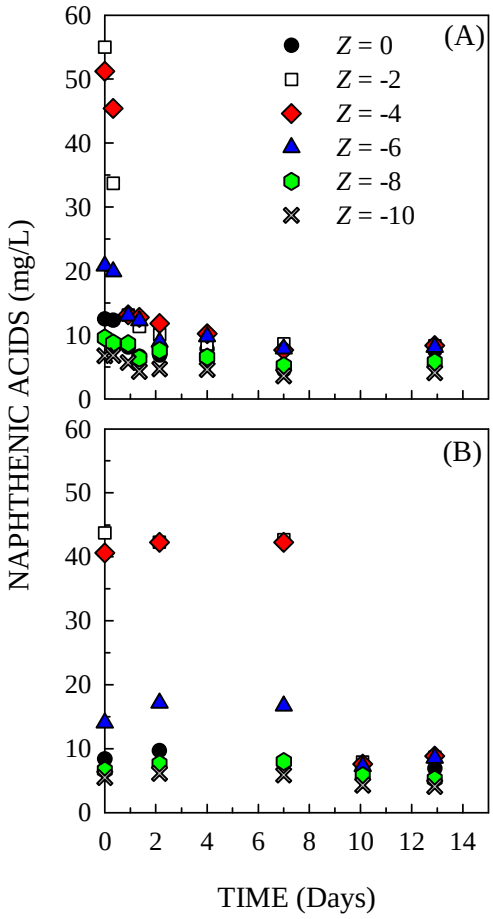


Figure 7.10. Time course of NA *Z* number during the incubation period of the NA-enriched (A) and un-amended (B) cultures (Cultures were not amended with synthetic wastewater; biotransformation assay I).

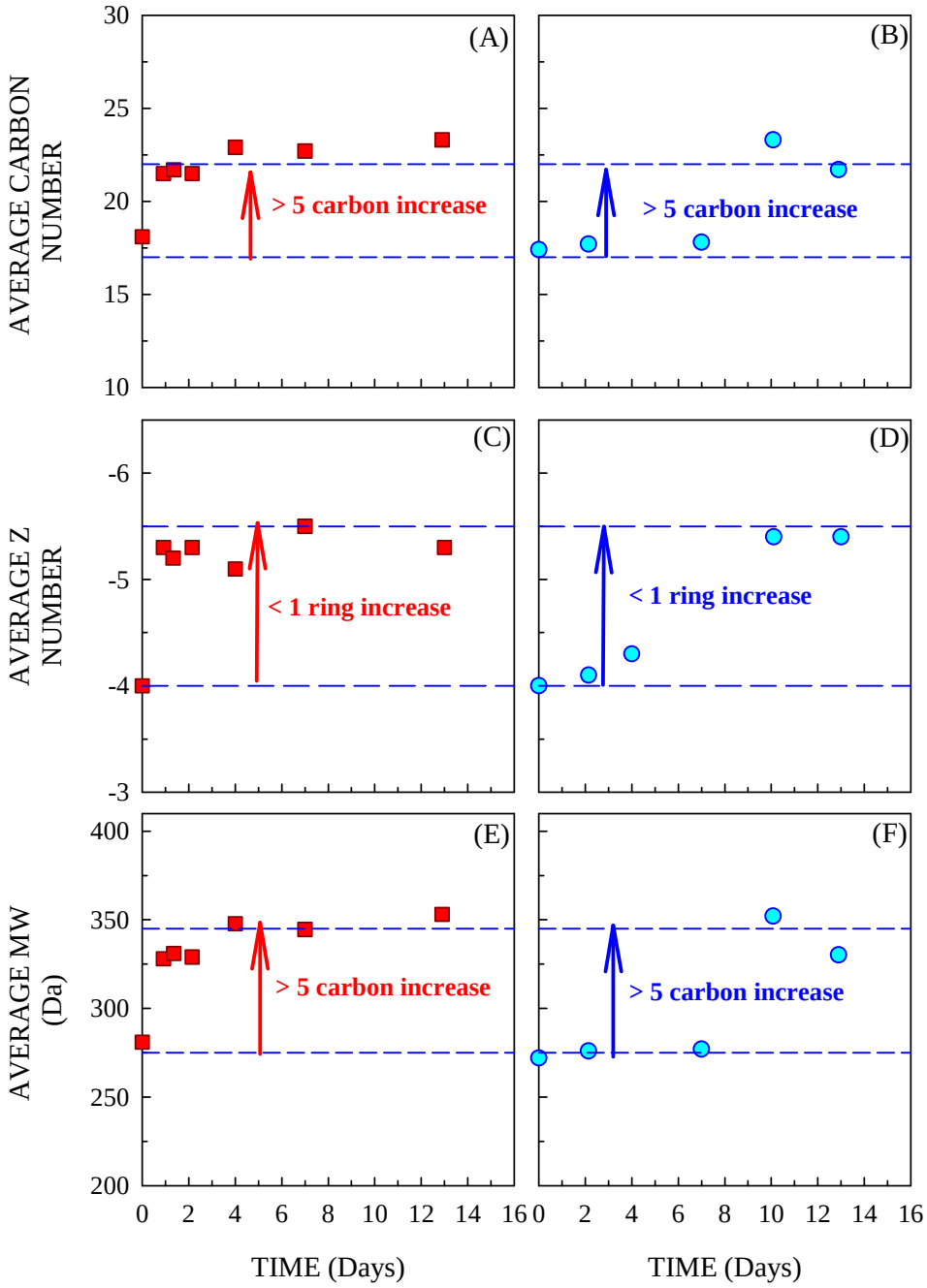


Figure 7.11. Time course of NA average carbon number, *Z* number, and molecular weight in the NA-enriched (A, C, E) and un-amended (B, D, F) cultures throughout the incubation period (Cultures were not amended with synthetic wastewater; biotransformation assay I).

7.3.3 Biotransformation Assay II

The second batch NA biotransformation assay, which assessed the effect of culture acclimation and age on NA degradation, was conducted with three cultures (NA un-amended, amended, and enriched) as discussed in Section 7.2.1, above. The total and liquid-phase NA concentrations were monitored throughout the 20-day incubation period and results are shown in Figure 7.12. Although the un-amended culture was able to degrade NAs in the first bioassay (Figure 7.9C), at some point within the 12 months without any NA exposure, this culture lost its ability to degrade NAs after 15 months of maintenance without NA in the feed (Figure 7.12B). Similarly, the NA-amended culture, which was developed from the un-amended biomass and had been exposed to NAs for 12 months without exhibiting any NA degradation (see Section 7.3.1.1, above), it was again confirmed that was not able to degrade NAs during the 20-day incubation cycle (Figure 7.12C). Regardless of prior NA exposure, both the un-amended and NA-amended cultures lost the ability to degrade NAs after 12 months of maintenance, fed with only the NA-free, synthetic wastewater. The first biotransformation assay confirmed that for a non-enriched culture, when a degradable carbon source is available, NAs are not degraded. Thus, in spite the fact that both the un-amended and NA-amended cultures were developed with oil refinery activated sludge samples which were capable of NA-degradation (see Section 7.3.1.2, above), maintenance with a NA-free, degradable synthetic wastewater resulted in loss of NA biodegradation. In contrast, the NA-enriched culture, which was maintained with a NA-only feed, degraded NAs at the same rate and extent as observed in the biotransformation assay I, which was conducted 12 months prior to the biotransformation assay II (Figure 7.12A). The NA congener distribution in

the NA-enriched culture at the end of the incubation was very similar to the one achieved by the same culture 12 months earlier (see Section 7.3.1.2, above).

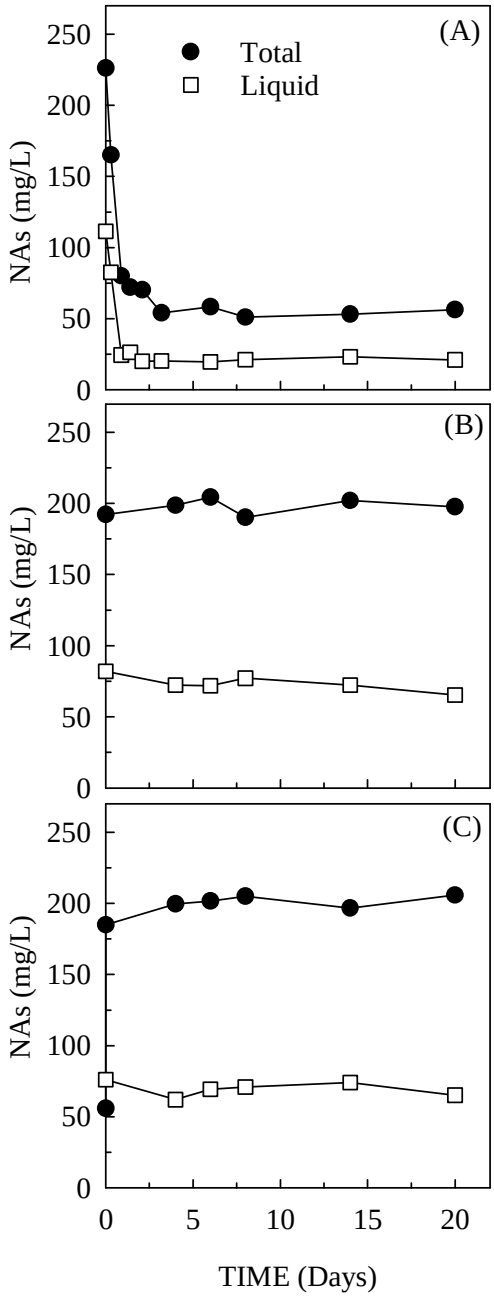


Figure 7.12. Total and liquid-phase NA concentrations in the NA-enriched (A), un-amended (B) and NA-amended (C) culture series (biotransformation assay II).

7.3.4 Biotransformation Assay III

To investigate the effect of initial NA concentration on the residual NA concentration and congener distribution, the washed NA-enriched culture (residual NA concentration less than 3 mg/L prior to feeding) was used in four culture series set up with different initial NA concentrations. Figure 7.13 shows the total and liquid-phase NA concentration in the NA-enriched culture series amended with NA at an initial concentration range from approximately 60 to 250 mg/L. Regardless of the initial NA concentration, 74 to 85% of NAs were degraded within 3 days in all culture series, resulting in total residual NA concentrations as low as 20 mg/L in the 60 mg/L culture series. The residual NA concentrations increased with increasing NA feed concentration and the residual fraction of the total NAs fed to the culture series amended with 60, 100, 135 and 270 mg NA/L was 25.8, 25.8, 23.0 and 16.7%, respectively. The weighted average MW, carbon number, *Z* number and molecular formula in the initial NA mixture and after biotransformation in each culture series are summarized in Table 7.4. Similarly to previous bioassays, the lower MW NAs were preferentially degraded in all culture series, evidenced by the increase in the NA weighted average MW by the end of the incubation.

Figure 7.14 shows the extent of biodegradation (%) of the NA mixture by *Z* numbers determined by averaging the fraction of NAs degraded in the four culture series amended with different initial NA concentrations. Regardless of the initial NA concentration, the extent of biodegradation was consistent for each NA group of the same *Z* number, indicating that a certain fraction of the NA mixture is biodegradable, while the other fraction is either recalcitrant or not bioavailable. NAs with *Z* numbers equal to -2 and -4 were degraded to the furthest extent, followed by NAs with *Z* numbers equal to 0

and -6. The biodegradation extent of each NA group of the same *Z* number correlates to their abundance in the TCI mixture (Figure 7.14), indicating that the residual NA fraction is likely the result of the low individual NA concentrations (i.e., threshold values; see below).

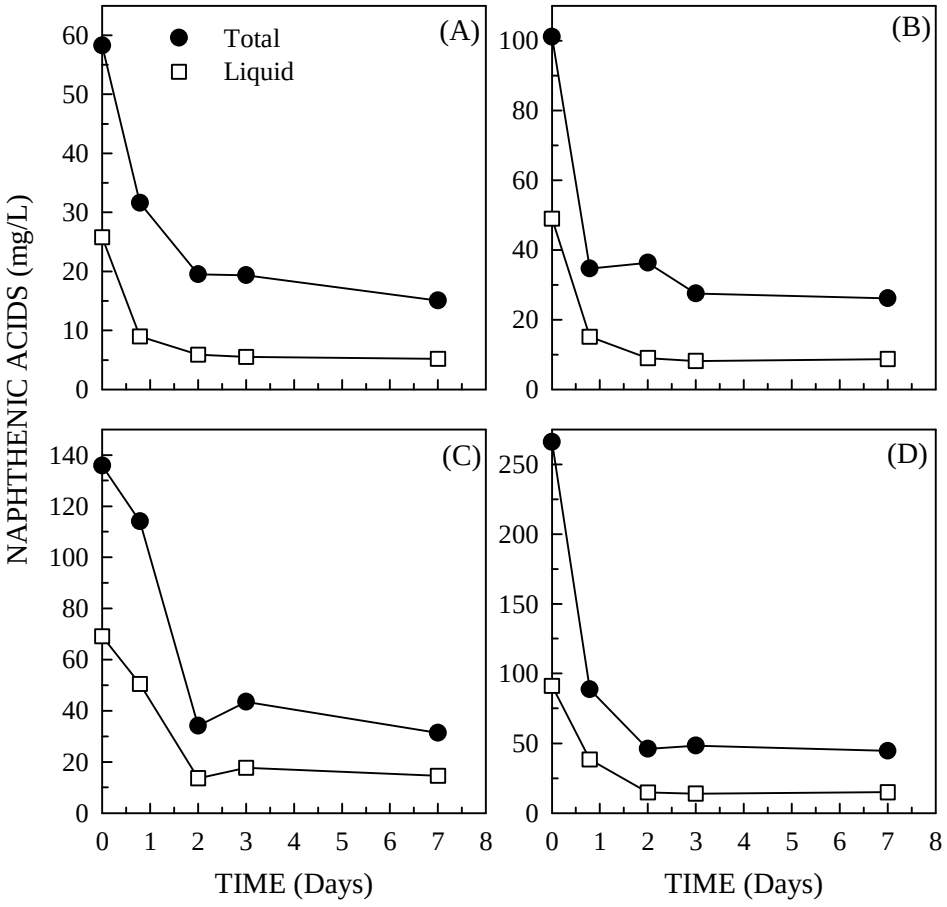


Figure 7.13. Total and liquid-phase NA concentration in NA-enriched culture series set up with different initial NA concentrations (biotransformation assay III).

Table 7.4. Weighted average *Z* number, carbon number (*n*), MW and molecular formula for the initial and residual NAs in the four culture series amended with different initial NA concentrations at the end of the 7-day incubation period.

NAs (mg/L)	*Z*	*n*	MW	Formula
Initial	-3.9	17.4	271.7	$C_{17.4}H_{30.9}O_2$
60	-5.3	19.5	299.0	$C_{19.5}H_{33.7}O_2$
100	-5.5	18.6	284.7	$C_{18.6}H_{31.7}O_2$
150	-5.5	18.9	291.1	$C_{18.9}H_{32.2}O_2$
250	-5.7	18.5	285.5	$C_{18.5}H_{31.3}O_2$

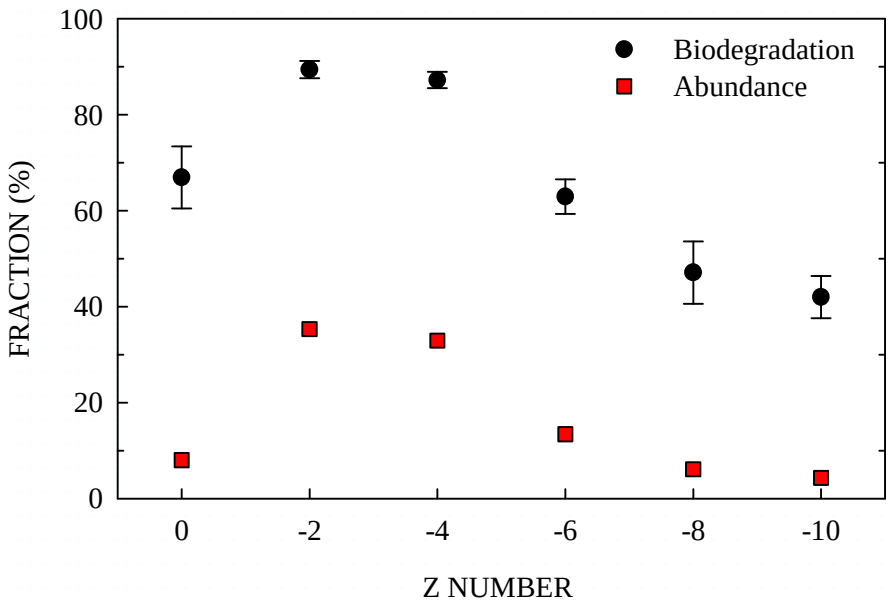


Figure 7.14. NA biodegradation (%) as a function of *Z* number in four NA-enriched cultures amended with different initial NA concentrations and NA relative abundance (%) in the TCI mixture according to *Z* number (Error bars represent mean values ± one standard deviation, *n* = 4; biotransformation assay III).

7.3.5 Biotransformation Assay IV

Previous studies have shown that a fraction of NAs in many oil sands processing wastewater and commercial NA mixtures is persistent, even over long incubation periods (Han et al., 2008; Quagraine et al., 2005a). The fraction of un-degraded NAs is poorly characterized and the reason for their recalcitrance is not well understood. To further investigate the cause of the residual NAs remaining in the NA-enriched culture, a portion of this culture was fed five times without culture wasting while monitoring the total NA concentration before and after feeding. Figure 7.15 shows that the residual NAs did not accumulate with each feeding cycle and the mean total and liquid-phase residual NA concentrations were 76.5±6.8 and 37.1±10.7 mg/L, respectively, regardless of the concentration of the initial feed, which ranged from 190 to 270 mg NA/L. These results differ from those obtained in the biotransformation assay III (Section 7.3.4), in which the residual NA concentration increased with increasing NA feed concentration. In the previous bioassay (biotransformation assay III), the biomass was washed until the residual NA concentration was below 3 mg/L, while in the current bioassay (biotransformation assay IV), the biomass was loaded with residual NAs from previous culture feedings. Prior NA accumulation resulted in a steady-state solid-phase (i.e., biomass) NA concentration that did not change with varying initial NA feed concentrations.

Both the total and liquid-phase residual NA concentrations were relatively constant before each feeding cycle, indicating no NAs accumulated in the liquid or solid-phase (i.e., biomass). Using the NA isotherm with the NA-enriched biomass (Chapter 5. Section 5.3.2), the equilibrium liquid-phase concentration of 37.1 mg/L corresponds to a

solid-phase concentration of 157.7 mg NA/g VSS, which is slightly higher than the measured concentration of 131.3 mg NA/g VSS in the biotransformation assay IV. The difference in the estimated and measured solid-phase NA concentrations is attributed to the isotherm being calculated using the non-biodegraded NA mixture, while the experimental results in this bioassay were measured based on the partitioning behavior of the residual fraction of NAs after biodegradation by the NA-enriched culture. Thus, the liquid-phase residual NA concentration is close to equilibrium with the biomass-associated NAs.

The congener NA distribution is shown in Figure 7.16. The weighted average molecular weight, carbon number and Z number (Table 7.5) indicate that the NA distribution was the same at the beginning and the end of the incubation period, and there was no accumulation of a specific group of individual NA compounds. Thus, it is concluded that the residual NAs remaining after each feeding cycle are not inherently recalcitrant, but rather are not degraded at such low individual NA concentrations. The NA-enriched culture is a mixed culture containing many different NA-degrading bacteria. It is not known if the NA-enriched culture degrades the NA mixture as a single biomass or if different species have different NA-degrading capabilities dependent on structure or groups of similar NA structures. The TCI NA mixture consists of an undetermined high number of NAs with different structures; therefore, while the total NA concentration may be significant, the concentration of individual NAs or groups of similar NAs may be below the minimum substrate concentration (S_{min}), below which the mixed culture or individual bacteria can no longer maintain a steady-state biomass concentration (Rittmann and McCarty, 2001), leading to a residual NA concentration. These results are

supported by those reported in Section 7.3.4, Figure 7.14, in which the NAs with higher ring number are in a lower abundance in the TCI NA mixture than other NAs. Thus, NAs with higher ring number are degraded to a lesser extent as a result of low individual or group concentrations and not because of molecular recalcitrance. To determine if the residual NA concentration would decrease further with extended incubation, the NA-enriched culture was left un-fed for 27 days. During this incubation period, the residual NA concentration remained constant within 42 and 57 mg NA/L (Figure 7.17), confirming that the residual NAs could not be further biodegraded.

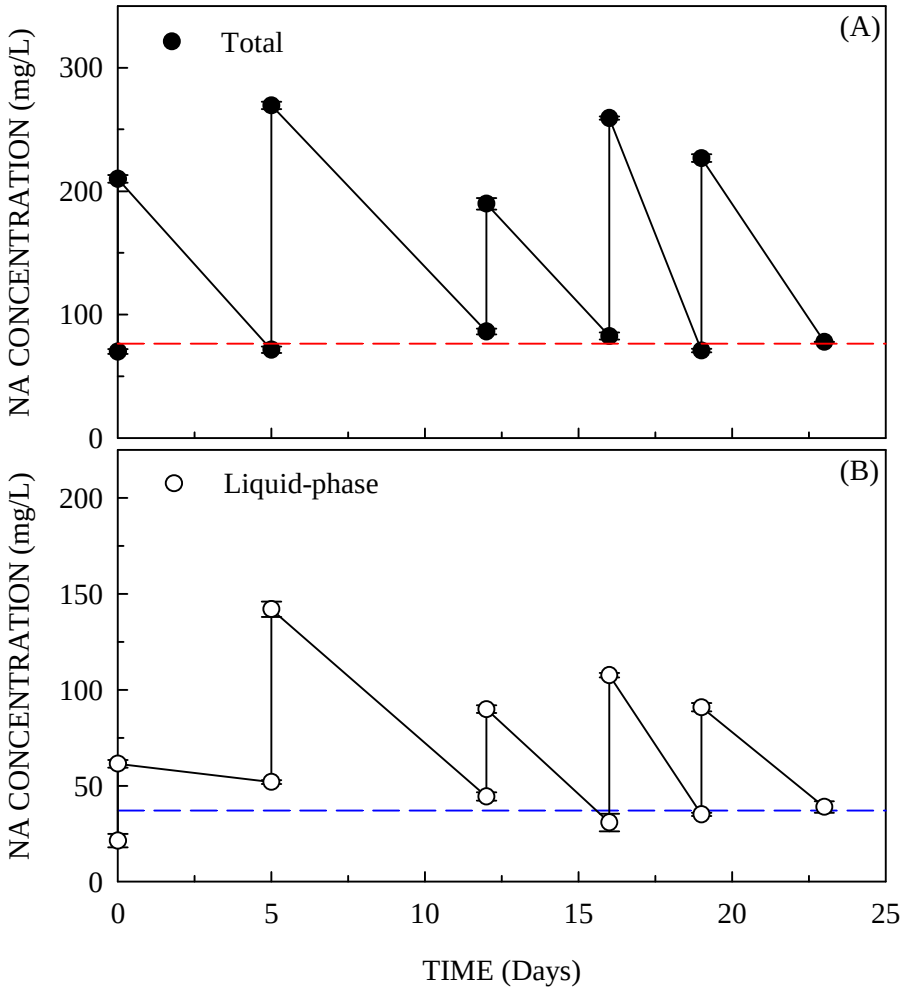


Figure 7.15. Total (A) and liquid-phase (B) NA concentration in the NA-enriched culture before and after five consecutive NA amendments without culture wasting (Error bars represent mean values ± one standard deviation, n = 3; dashed lines represent mean total and liquid-phase residual NA concentrations; biotransformation assay IV).

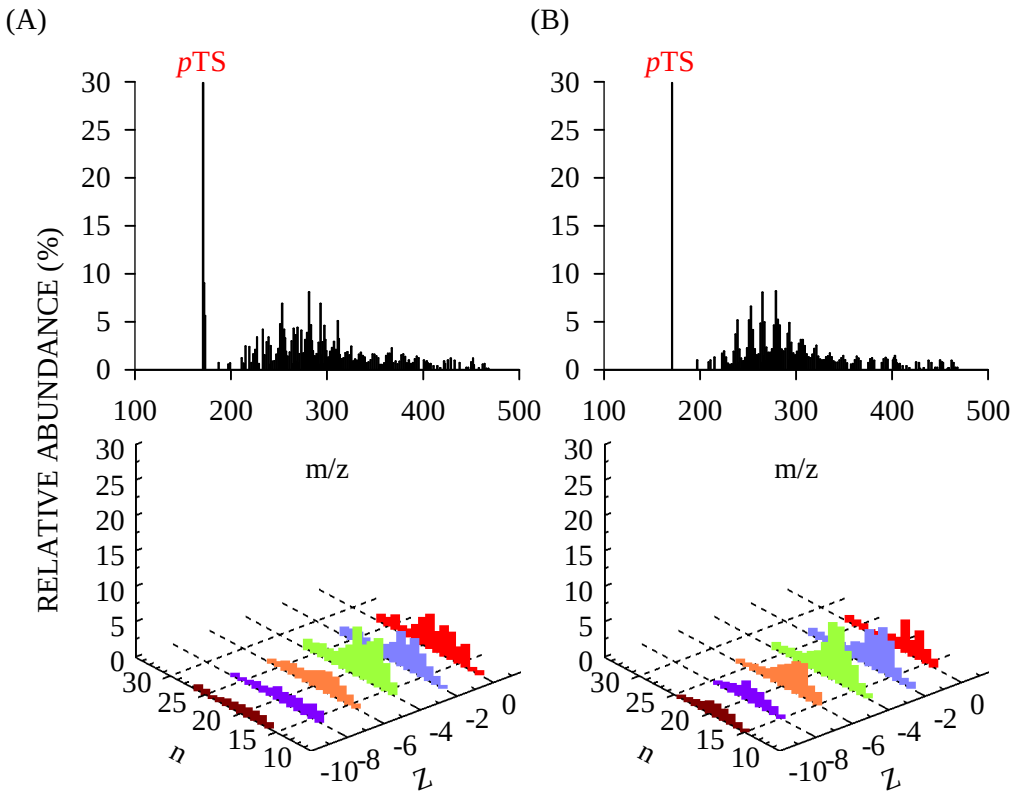


Figure 7.16. Total congener NA distribution of residual NAs on day 0 (A) and day 23 (B) after 5 successive feeding cycles without wasting (biotransformation assay IV; see Figure 7.15 for culture feeding cycles).

Table 7.5. Weighted average carbon number, *Z* number, molecular weight and formula of residual NAs on day 0 (A) and day 23 (B) after 5 successive feeding cycles without wasting (biotransformation assay IV; see Figure 7.15 for culture feeding cycles).

Sample	*n*	*Z*	MW	Formula
Day 0	18.7	-4.9	288.6	$C_{18.7}H_{32.5}O_2$
Day 23	18.6	-4.7	287.2	$C_{18.6}H_{32.4}O_2$

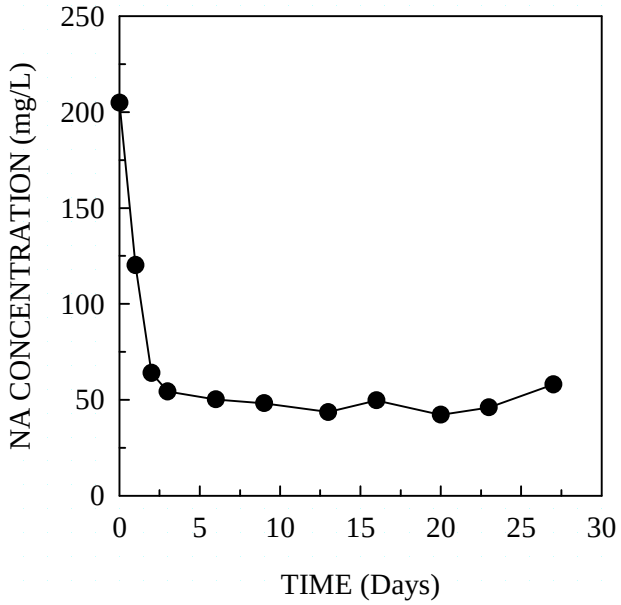


Figure 7.17. Total NA concentration in NA-enriched culture incubated unfed for 27 days.

7.3.6 Biotransformation Assay V

To investigate the extent of NA mineralization to CO_2, a closed bottle, aerobic biotransformation assay was conducted with the NA-enriched culture. Figure 7.18A and B show the total and soluble NA concentrations and seed-corrected O_2 consumption and CO_2 production throughout the 6-day incubation period. Based on carbon balance calculations, at the end of the 6-day incubation period, 85% of NAs had been biotransformed, of which, approximately 44% of the NA-carbon was utilized for biomass

growth, and 28.5% was mineralized to CO_2 (Figure 7.18C). The remaining 27.5% of biotransformed NA-carbon was likely partially oxidized to biotransformation intermediates, containing additional carboxylic groups and additional oxygen atoms. Previous studies have shown that oxidized NAs are found in both refinery and commercial mixtures as a result of partial aerobic biodegradation of NAs (Grewer et al., 2010; Han et al., 2008; Johnson et al., 2011). Similar to our previous bioassays, the lower MW NAs were preferentially degraded, resulting in an increase in the weighted average MW, carbon number and *Z* number. The extent of mineralization by the NA-enriched culture was generally lower than reported in another study, in which more than 90% of two different NA mixtures were biotransformed and in both cases, approximately 60% of the carbon was detected as CO_2 (Clemente et al., 2004). Also, Herman et al. (1994) found that 50% of a commercial NA mixture was completely mineralized. One study by Clemente et al. (2003) showed a lower extent of NA mineralization, in which only a 30% decrease in NA concentration was observed with 13% of the carbon detected as CO_2. In our study, the total NAs biotransformed represented 71% of the initial total COD and, accounting for biomass growth, the measured total COD decreased by 45% during the incubation period, indicating that a NA fraction was biotransformed to more oxidized intermediates (Figure 7.19). Analytical limitations prevented the identification of oxidized biotransformation intermediates due to the lack of sensitivity of the MS, the complexity of the NA mixture and the relatively low individual NA concentrations within this mixture.

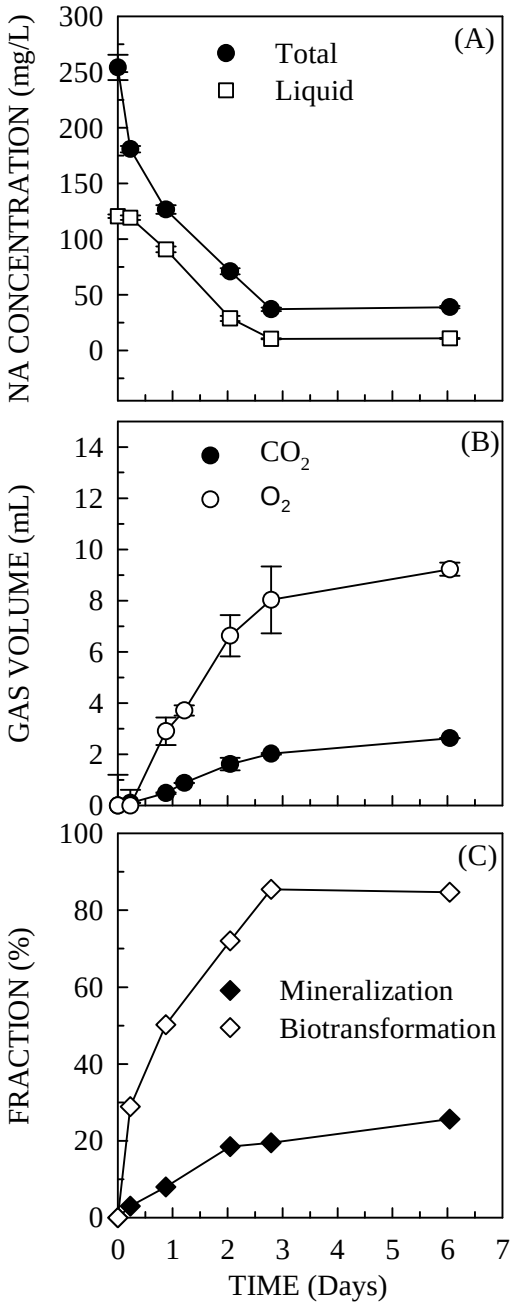


Figure 7.18. NA concentration (A), seed-corrected carbon dioxide production and oxygen consumption (B) and fraction of NA mineralization and biotransformation (C) (Error bars represent mean values ± one standard deviation, n = 3; biotransformation assay V).

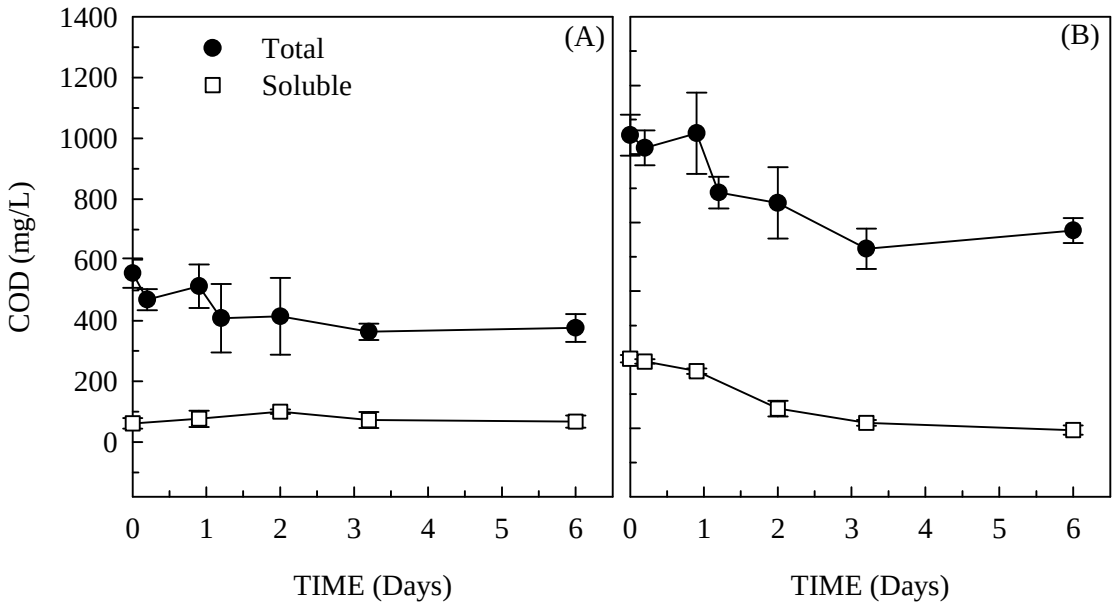


Figure 7.19. Total and soluble COD in seed control (A) and NA-amended (B) closed, aerobic culture series (Error bars represent mean values ± one standard deviation, $n = 3$; biotransformation assay V).

7.3.7 Biotransformation Assay VI

The initial refinery mixed liquor bacteria were capable of NA degradation (see Section 7.3.1.2, above); however, the un-amended and NA-amended cultures developed from the NA-degrading inocula were not capable of NA degradation (see Section 7.3.2, above). To investigate the conditions and timeframe under which the NA-degrading biomass loses its biodegradation capability, a batch assay was conducted with the cultures developed from the NA-enriched culture fed synthetic wastewater only. This batch assay lasted 21 and the NA data are shown in Figure 7.20. The culture fed with NAs and synthetic wastewater did not lose its biotransformation capability, regardless of the NA to synthetic WW ratio, which was decreased from 1 to 0.25 to 0.075 (Figure 7.8). On the other hand, when the culture fed only synthetic WW was amended with NAs at the end of a feeding cycle on day 14, it did not degrade NAs during the 21-day incubation period, indicating that this culture lost its ability to degrade NAs in 14 days or less when maintained with a NA-free feed (Figure 7.20). The loss of biotransformation capability was faster for the NA-enriched biomass than it was for the refinery mixed liquor biomass, which at 3 months of development without NAs was still capable of NA degradation when no other carbon source was provided (see Section 7.3.2, above). This is likely because the refinery mixed liquor biomass was much more diverse than the NA-enriched culture biomass, which had been developed with an NA-only feed for over 3 years.

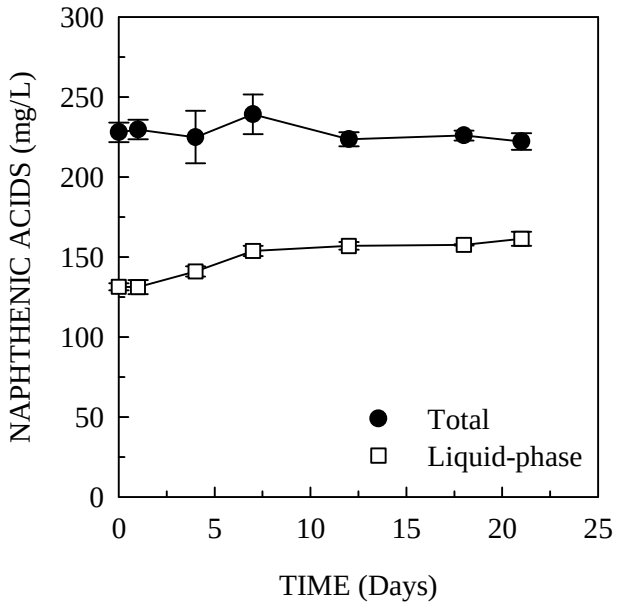


Figure 7.20. Total and liquid-phase NA concentrations in batch biotransformation assay using NA-enriched side culture fed only synthetic wastewater (Error bars represent mean values ± one standard deviation, n = 3; biotransformation assay VI).

7.3.8 Microbial Community Analysis

To investigate the differences in microbial community structure between the NA-enriched and two side cultures developed with a feed of NAs and synthetic wastewater and synthetic wastewater only (see Section 7.2.1, above), DNA was extracted from each culture biomass and 112 clones were randomly selected from each sample. Table 7.6 summarizes the phylogenetic relationships and Figure 7.21 shows the position of the phylotypes in the phylogenetic tree for the NA-enriched and the two side cultures. Of the six major groups identified in the NA-enriched culture, 80% belonged to the *γ-proteobacteria* class. The majority of the NA-enriched culture bacteria were uncultured species, closely related to known NA-degraders, such as *Pseudomonas putida* and *Pseudomonas florescens*, and other known hydrocarbon degrading bacteria found in oil sands process water and oil contaminated sites, such as *Aeromonas* and *Acinetobacter* (Del Rio et al., 2006; Herman et al., 1993; Plaza et al., 2008). Of the groups identified in the two wastewater-fed side cultures, the most abundant bacteria identified were most closely related to the *Methylophilus* and *Methylobacillus* genera, which are known methanol utilizing bacteria and accounted for 61 and 63% of the bacteria identified in the NAs and synthetic wastewater fed and synthetic wastewater only fed side cultures, respectively (Jenkins et al., 1987; Osaka et al., 2006). This group of bacteria was likely enriched as a result of the methanol present in the synthetic wastewater, which is required to dissolve low solubility compounds such as phenol and naphthalene. Bacteria closely related to the *Xanthomonas* genus, which are known petroleum hydrocarbon degraders, were also abundant in the two wastewater fed side cultures and accounted for 16 and 30% in the NAs and synthetic wastewater fed and synthetic wastewater-only fed cultures,

respectively (Plaza et al., 2008). Both wastewater fed cultures were found to contain a small percentage of *γ-proteobacteria* similar to the NA-enriched culture and known NA-degraders; however, the culture fed wastewater and NAs contained 4% *γ-proteobacteria,* while the wastewater-only fed culture contained less than 1%. The slightly higher abundance of probable NA-degraders in the NAs and wastewater-fed culture is most likely the reason this culture maintained its ability to degrade NAs even after NAs were not included in its feed, while the wastewater-only fed culture did not. These results indicate that known NA-degrading bacterial species are not abundant in the wastewater fed cultures, especially the culture fed only wastewater without NAs. The population of NA degraders was rapidly lost within 2 weeks feeding only wastewater (see Section 7.3.7, above) and thus, the wastewater-only fed culture quickly lost its ability to degrade NAs.

Table 7.6. Phylogenetic relationship of microorganisms detected in the NA-enriched culture and NA-enriched side cultures fed synthetic wastewater and NAs and wastewater only.

Culture	Phylotype name	# of clones	Closest Species/Clone type in GeneBank	Similarity (%)	Taxonomy
NA-Enriched	NA-1	28	Uncultured γ-*proteobacterium* clone CM38F12	99	Class: γ-*proteobacteria*
	NA-2	50	Uncultured γ-*proteobacterium* clone 58	99	Class: γ-*proteobacteria*
	NA-3	4	*Sphingopyxis sp.*	97	Genus: *Sphingopyxis*
	NA-4	12	Uncultured *bacterium* clone ncd2676e10c1	99	Domain: *Bacteria*
	NA-5	4	*Nitrospira sp.*	99	Genus: *Nitrospira*
Synthetic wastewater and NAs	WWNA-1	66	*Methylobacillus sp.*	98	*Genus: Methylobacillus*
	WWNA-2	17	*Xanthomonas sp.*	98	Genus: *Xanthomonas*
	WWNA-3	19	Uncultured *bacterium* clone P50-15	96	Domain: *Bacteria*
	WWNA-4	4	*Pseudomonas putida*	96	Genus: *Pseudomonas*
	WWNA-5	2	Uncultured *Acidobacteria bacterium* clone Alchichica_AQ2_2_1B_112	98	Class: *Acidobacteria*
	WWNA-6	1	*Rhizobium sp.*	96	Genus: *Rhizobium*
Synthetic wastewater only	WW-1	63	*Methylobacillus sp.*	91	*Genus: Methylobacillus*
	WW-2	30	*Xanthomonas sp.*	97	Genus: *Xanthomonas*
	WW-3	4	Uncultured *bacterium* clone G3	95	Domain: *Bacteria*
	WW-4	3	*Acidovorax sp.*	94	Genus: *Acidovorax*
	WW-5	1	*Pseudomonas sp.*	94	Genus: *Pseudomonas*

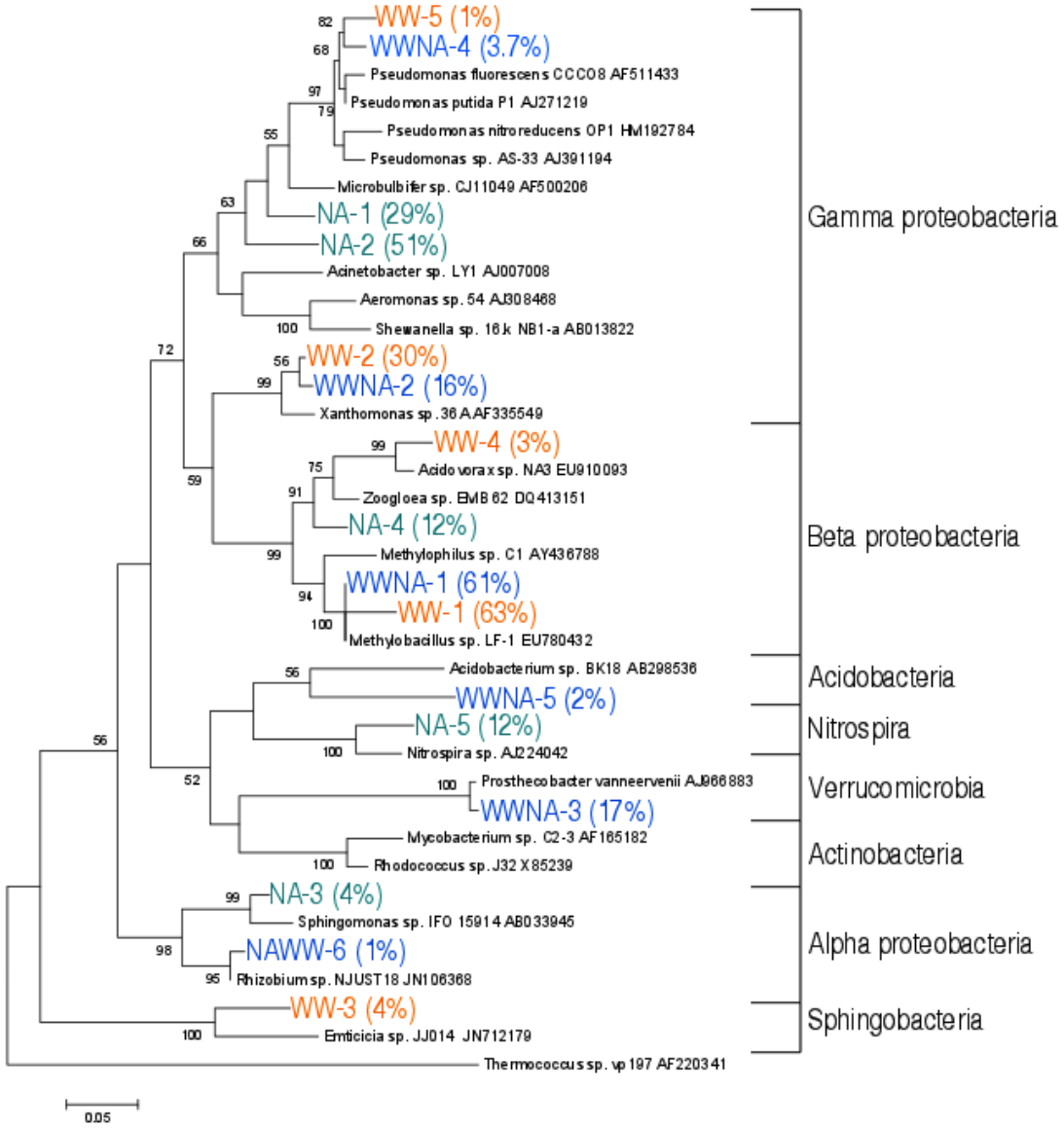


Figure 7.21. Phylogenetic tree of bacterial populations identified in the NA-enriched culture (green, NA), and two side cultures fed synthetic wastewater and NAs (blue, NAWW) and wastewater only (orange, WW) constructed by the neighbor-joining method based on the 16S rRNA gene sequences. Fraction of bacterial population indicated after bacterial name (%). Bootstrap values (>50%) from the analysis of 1000 replicates are shown at each node of the tree.

7.4 Summary

The commercial NA mixture used in this study was partially biodegraded by cultures developed with an oil refinery activated sludge inoculums. However, a fraction of the NA mixture was not biotransformed and remained as residual, more likely because of low bioavailability of the very low concentrations of individual NAs. NA biodegradation depended on the age of the cultures and the conditions under which the cultures were developed relative to NA exposure and availability of other, degradable carbon sources. Although a large portion of the commercial NA mixture was biotransformed, only a relatively small fraction was mineralized to CO_2. Further investigation into the biotransformation products is necessary as they may not be readily degradable, thus having the potential to accumulate and contribute to refinery wastewater and process water toxicity. Although it is extremely difficult to identify biotransformation intermediates for a complex NA mixture, use of model compounds in future biotransformation studies would allow identification of partially degraded or oxidized metabolites.

Refinery NAs are typically found at very low concentrations within a complex matrix of other hydrocarbons, which could limit the extent of NA biodegradation in refinery wastewater and process water treatment systems and thus result in the discharge of NA-bearing effluents with potential NA accumulation in the environment. Complete elimination of non-bioavailable, residual, and persistent NAs from refinery effluents may require the use of alternative, tertiary treatment processes, such as adsorption or advanced oxidation. Further understanding of the NA biotransformation process in terms of products formed as well as conditions affecting their biodegradation will help the

development of effective treatment processes, thus decreasing the potentially harmful effects of NAs on the environment.

CHAPTER 8

EFFECT OF STRUCTURE ON THE TOXICITY AND BIOTRANSFORMATION POTENTIAL OF MODEL NAPHTHENIC ACIDS

8.1 Introduction

NAs are the major organic constituent in crude oil that causes corrosion and toxicity problems in refineries and wastewater treatment systems. It is well known that the NA structure plays a significant role on biodegradability; however, the effects are not completely understood. For instance, Clemente et al. (2004) and Scott et al. (2005) demonstrated that NAs with lower carbon numbers (*n*) and less cyclization (lower *Z*) are more degradable than NAs having high *n* and *Z* values. Han et al. (2008) later stated that *n* does not affect the biodegradability of NAs. Moreover, Smith et al. (2008) indicated that branching of R substantially decreases NA biodegradability. In the current study (Chapter 7) and previously published literature results, less than 100% degradation/removal of NAs is reported in bioassays studying the biodegradation of NAs in commercial mixtures or crude oil extracts. As it is not completely understood which NA structures are recalcitrant, there is a need to further investigate the effect of NA structure on biodegradability.

NAs are among the most toxic components of refinery process waters and the degree of toxicity varies with the type and concentration of NAs; however, the chemical composition and complexity of the NA mixture may be a stronger determinant of toxicity. Crucial information on toxicity, especially structure-toxicity information for NAs is not widely available. Until now, all studies have derived their conclusions mainly

on the effect of molecular weight and cyclization on toxicity and biodegradation; however, there have been no reports on the effect of structural differences of NA isomers on biodegradation and toxicity.

The objectives of this research were to: a) assess the toxicity of individual model NAs using quantitative structure-activity relationships (QSAR) and compare with measured Microtox® acute toxicity; and b) determine the effect of NA structure on NA biotransformation potential through prediction of biodegradability and experimental biodegradation assays.

8.2 Materials and Methods

8.2.1 Model Naphthenic Acids

Ten carboxylic acids, selected as model NAs, were purchased from Sigma Aldrich (St. Louis, MO): octanoic acid, *n*-valproic acid, 2-ethylhexanoic acid, cyclohexylacetic acid, 1-methyl-1-cyclohexane carboxylic acid, 2-methyl-1-cyclohexane carboxylic acid, 3-methyl-1-cyclohexane carboxylic acid, 4-methyl-1-cyclohexane carboxylic acid, dicyclohexylacetic acid and 5β-cholanic acid. Stock solutions (5 to 10 g/L) of each model compound were made 1 N NaOH.

8.2.2 Quantitative Structure-Activity Relationships (QSAR)

Molecular structure and hydrophobicity (log K_{ow}) were used as the descriptors and toxicity and aerobic biodegradability probability were used as the end-points of the quantitative structure-activity relationship (QSAR) model developed. ChemDraw v.10 (Cambridge Soft Inc., Cambridge, MA) was used to construct molecular structures and to

obtain Simplified Molecular Input Line Entry System (SMILES) notations of representative NA molecules. The log K_{ow} value for each NA was calculated using ALOGPS 2.1 on-line software (http://www.vcclab.org/lab/alogps/, Virtual Computational Chemistry Laboratory, Munich, Germany) using the SMILES as input. ALOGPS is an advanced log K_{ow} prediction software that was developed with 12908 molecules from the PHYSPROP database using 75 E-state indices and calculates log K_{ow} of a molecule using 64 neural networks. Therefore, it is capable of predicting non-equal log K_{ow} for isomers, which is not possible with conventional software packages which perform log K_{ow} calculations using the group contribution method. The root mean squared error of the predictions is 0.35 and the standard mean error is 0.26 for a set of randomly selected 6500 compounds.

ECOSAR ™ (EPISuite v4.0, US EPA), which is a generic model designed to estimate the toxicity of a wide range of compounds, was used to calculate acute *Daphnia magna* LC_{50} toxicity values for the selected model NA compounds. Log K_{ow} calculated using ALOGPS and SMILES were used as input. The ECOSAR model estimates toxicity using log K_{ow} (i.e., hydrophobicity) and chemical structure based on the structural similarities to other experimentally tested chemicals. The BIOWIN v4.01 model 5 (MITI linear biodegradation probability model) was used to estimate the biodegradation probability of the selected NA structures under aerobic conditions (EPISuite v4.0, US EPA). The BIOWIN model estimates the biodegradation probability based on NA structure using a fragment-based method.

Using SMILES as input, the OECD (Organization for Economic Co-operation and Development) QSAR Toolbox Version 2.3 (OECD, 2012) was used to predict metabolites and assemble possible biodegradation pathway(s) for select model NAs.

8.2.3 Biotransformation Assays

To assess the biotransformation potential of the ten model NAs, aerobic batch biotransformation assays were performed. The NA-enriched culture (see Section 7.2.1, above) was washed three times with 10 mM phosphate buffer as described in Chapter 7, Section 7.3.1 until the residual NA concentration was less than 3 mg/L and then re-suspended in aerobic culture media amended with 20 mg NH_4^+-N/L. Portions of 100-mL of the re-suspended culture were added to ten 250-mL Erlenmeyer flasks and each model compound was added at an initial concentration of approximately 50 mg/L and the pH was adjusted to 7 using 1N HCl. A control series was also set up in the same manner using only aerobic culture media containing 20 mg NH_4^+-N/L and each model NA at 50 mg/L. The initial biomass concentration was 305±19 mg VSS/L. The flasks were loosely capped and agitated on an orbital shaker at 190 rpm at room temperature (22 to 24°C). Total NAs and pH were measured throughout the incubation period.

To investigate the extent of NA mineralization to CO_2, a closed bottle, aerobic biotransformation assay was conducted with the NA-enriched culture (as described in the fifth biotransformation assay in Chapter 7, Section 7.2.2, above) to determine to what extent two model compounds, valproic acid (Z = 0) and cyclohexylacetic acid (Z = -2), were mineralized to CO_2. The NA-enriched culture biomass was washed as described in Chapter 7, Section 7.2.2, re-suspended in aerobic culture media and amended with 20

NH_4^+-N/L. The washed culture was then divided into three culture series as follows: two sacrificial culture series were set up in 25-mL serum bottles amended with 15 mL NA-enriched culture with 50 mg NA/L of each model NA; two sacrificial control culture series were set up with 50 mg/L of each model NA and 200 mg/L sodium azide to inhibit any biological activity; and a control seed culture series was also set up with only the NA-enriched culture and no NAs. All three culture series were sealed with Teflon-lined stoppers and aluminum crimps and 2 mL pure O_2 was injected into the headspace containing air to maintain a positive pressure and to ensure oxygen was in excess. The initial biomass in all serum bottles was 219±5 mg VSS/L. The culture series were incubated at room temperature (22 to 24°C), agitated on an orbital shaker at 190 rpm. On day 0, 1, 2, 3 and 7, each culture series was sacrificially sampled for CO_2, O_2, total NAs, soluble COD, VFAs, organic acids, pH and other possible intermediates using LC/MS scanning from m/z range of 30 to 1000 in both positive and negative ESI mode. On each sampling day, two NA-amended and two NA-free seed culture bottles were acidified with 1 mL of 6 N H_2SO_4, agitated for 4 hours, and the headspace CO_2 concentration was measured in triplicate. Prior to opening for liquid analysis, the headspace O_2 concentration in the un-acidified serum bottles was quantified. The sodium azide-amended series were sampled for all of the above-mentioned parameters at the end of the 7 day incubation period. The pH was maintained in between 6.5 and 7 in all culture series. The extent of NA mineralization was determined based on carbon mass balance calculations. The biomass incorporated carbon in the NA-amended culture series was determined assuming $C_5H_7O_2N$ as the biomass molecular formula.

8.2.4 Batch NA Biotransformation Kinetics and Parameter Estimation

For this work, Monod kinetics were used to describe substrate (i.e., NA) utilization and microbial growth for each individual model NA and the following differential equations were used:

$$\frac{dS_{NA}}{dt} = -\left(\frac{k_{NA}\ S_{NA}}{K_{SNA}+S_{NA}}\right) X_{NA} \quad (8.1)$$

$$\frac{dX_{NA}}{dt} = \left(\frac{Y_{NA}\ k_{NA}\ S_{NA}}{K_{SNA}+S_{NA}}\right) X_{NA} - bX_{NA} \quad (8.2)$$

where $S_{NA,}$ and X_{NA} are model NA and biomass concentrations (mg NA/L and mg VSS/L, respectively); t is time (days); k_{NA} is the model NA maximum specific biodegradation rate (mg NA/mgVSS•day); K_{SNA} is the model NA half-saturation constant (mg NA/L); Y_{NA} is the theoretical yield coefficient (mg VSS/mg NA); and b is the microbial decay coefficient (day^{-1}).

Based on Gibbs' free energy values calculated in this work (see Section 8.3.2, below) and bioenergetic calculations, the yield coefficient for the aerobic degradation of the model NAs was calculated as follows (Rittmann and McCarty, 2001):

$$\mathrm{A} = \frac{-\frac{\Delta G_p}{\varepsilon n} + \frac{\Delta G_c}{\varepsilon}}{\varepsilon \Delta G_r} \quad (8.3)$$

$$\mathrm{Y} = \frac{5.65}{8(1+\mathrm{A})} \quad (8.4)$$

where A is the electron equivalents (e^- eq) of model NA converted to energy per e^- eq cells synthesized (ignoring decay); ε is the efficiency of energy transfer, to or from energy carrier (ATP); ΔG_p (kJ/e^- eq) is the free energy required to convert the model NA to pyruvate $(= \Delta G^{o'}_{pyruvate} - \Delta G^{o'}_{NA})$; ΔG_c (kJ/e^- eq-cells) is the free energy required to

convert pyruvate and ammonia to cells and ΔG_r (kJ/ e^- eq) is the free energy released per e^- eq substrate converted for respiration $(= \Delta G_{O_2}^{o'} - \Delta G_{NA}^{o'})$.

Using equations 8.3 and 8.4, the yield coefficient was estimated to be 0.43 mg VSS/mg NA-COD (= 1.04 mg VSS/mg NA). The initial biomass concentration, X_{NA}, measured at the start of the bioassay was 305 mg VSS/L. The decay rate values for heterotrophs are generally in the range of 0.05 to 0.15 day^{-1} (Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). Varying the decay did not significantly affect the estimated maximum specific biodegradation rate, $k_{NA,}$ and thus, a microorganism decay rate of 0.1 day^{-1} was chosen and kept constant for all simulations.

The K_{SNA} value was varied within the range of 1 to 400 and the effect on the model fit (root mean square deviation, RMSD) to the experimental data was not significant; therefore, based on the simulation results and reported literature values for K_s (estimated based on NA biodegradation kinetics; Paslawski et al., 2009), a value of 350 mg/L was chosen and kept constant in all simulations. Parameter estimation was conducted as described in Chapter 6, Section 6.2.2, above using Berkeley Madonna Software Version 8.3 (Macey and Oster, 2006). Lag periods observed in biodegradation were ignored when estimating NA biodegradation kinetics. All parameter values used to calculate the yield coefficient and biodegradation rates are summarized in Table 8.1.

Table 8.1. Estimated and literature values used to calculate the microbial yield coefficient and estimate the biodegradation kinetics of the model NAs.

Parameter	**Value**
$\Delta G^{o\prime}_{pyruvate}$ (kJ/e$^-$ eq)	35.1
$\Delta G^{o\prime}_{NA}$ (kJ/e$^-$ eq)[a]	28.7
$\Delta G^{o\prime}_{O_2}$ (kJ/e$^-$ eq)	-78.7
ΔG_p (kJ/e$^-$ eq)	6.4
ΔG_c (kJ/e$^-$ eq)	18.8
ΔG_r (kJ/e$^-$ eq)	-107.4
n	1
ε	0.6
A (e$^-$ eq NA to energy/e$^-$ eq VSS synthesized)	0.65
Y (mg VSS/mg NA-COD)	0.43
Y (mg VSS/mg NA)	1.04
$X_{N,\ initial}$ (mg VSS/L)	305
b (day^{-1})	0.1
K_{SNA} (mg NA/L)	350

[a] Mean Gibb's free energy value for all model NAs

8.3 Results and Discussion

8.3.1 Properties of Model Compounds

The log K_{ow} values were estimated for each model compound and the results are shown in Figure 8.1A, 8.1B and 8.1C as a function of carbon number, *Z* number and molecular weight, respectively. The NA hydrophobicity increases with increasing carbon number; the hydrophobicity of monocyclic NAs is less than the hydrophobicity of acyclic NAs having the same carbon number (Figure 8.1B). The estimated log K_{ow} values ranged from 2.14 to 5.45 and estimated pK_a values were very similar for all NAs in the range of 4.7 to 4.82 (Table 8.2).

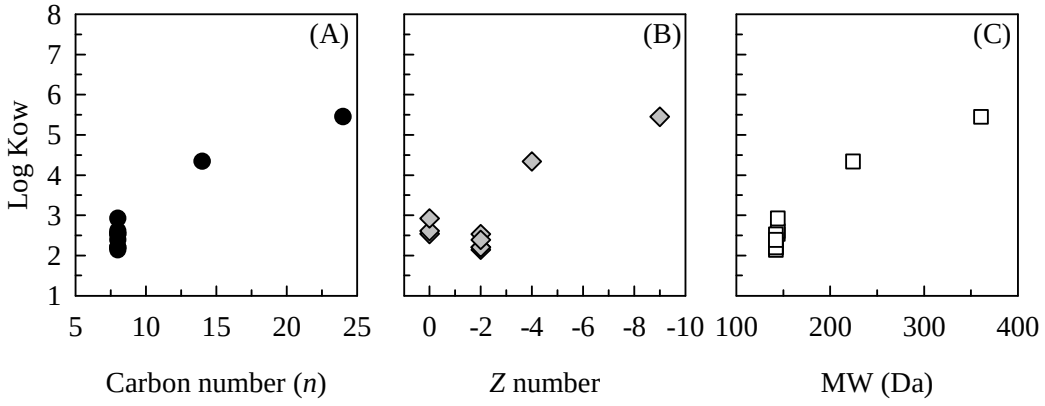


Figure 8.1. Estimated Log K_{ow} as a function of carbon number (A), Z number (B) and molecular weight (C) for ten model NAs.

8.3.2 NA Toxicity

The structural variations of NA molecules include the degree of cyclization (*Z*), location of branch (R) on a ring, and branching. The effect of these structural variations on toxicity was assessed for eight model NAs with 8 carbons and *Z* numbers of 0 and -2 and two additional higher MW model NAs with 14 carbons and *Z* = -4 and 24 carbons and *Z* = -8. The LC_{50} toxicity value to *Daphnia magna* was estimated using the EPISUITE ECOSAR model and compared to measured Microtox® acute EC_{50} toxicity values for the ten model compounds studied and the results are shown in Figure 8.2A and 8.2B, respectively. Figure 8.3A and B show that the toxicity of the lower MW (*n* = 8), small NA molecules does not vary much with *Z* number; however, they are less toxic than the larger, more hydrophobic NA molecules. The QSAR predicted LC_{50} values indicate that the cyclic NAs are less toxic than the acyclic NAs of the same MW; however, the measured Microtox® acute toxicity values indicated the opposite. Figure 8.4 shows the predicted and measured toxicity for each model NA and indicates that the LC_{50} and EC_{50} values cannot be directly compared since the predicted toxicity is for *Daphnia magna* and the measured toxicity is for *Vibrio fischeri*; however, the observed trend is the same for both organisms. Figure 8.5 shows a correlation (r^2=0.475) between the predicted and measured toxicity values. A strong correlation cannot be made, likely due to the difference in test species for predicted and measured toxicity values.

The results of both the QSAR and measured Microtox® acute toxicity suggest that the toxicity of NAs is influenced by their hydrophobicity, agreeing with previously published toxicity studies (Frank et al., 2009; Frank et al, 2010; Jones et al., 2011;

Whitby, 2010). Due to the surfactant properties of NAs, cell narcosis is the major mode of action (Frank et al., 2009; Frank et al., 2010; Jones et al., 2011; Zhang et al., 2011)

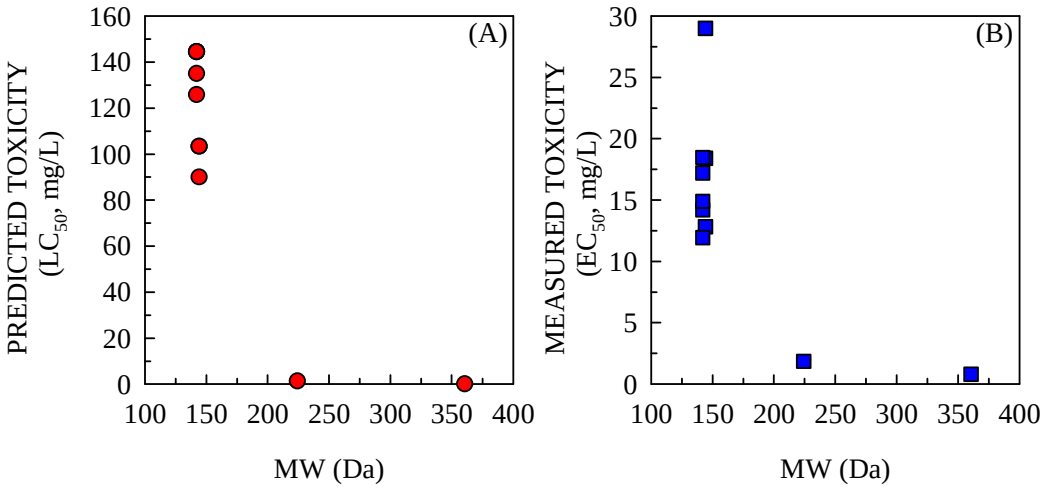


Figure 8.2. Effect of molecular weight on EPISUITE predicted acute *Daphnia magna* (LC_{50}) toxicity (A) and measured Microtox® acute (EC_{50}) toxicity (B) values for ten model NAs.

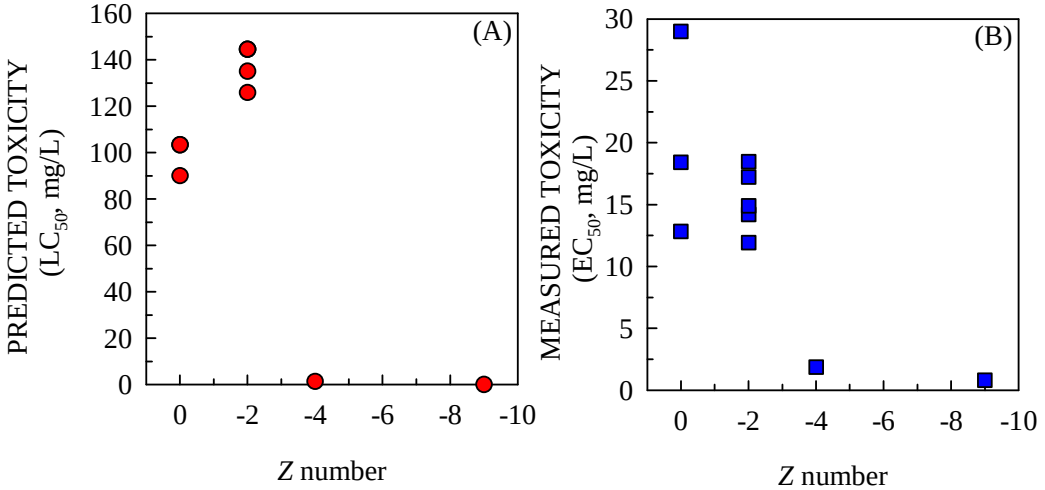


Figure 8.3. Effect of *Z* number on EPISUITE predicted acute *Daphnia magna* (LC_{50}) toxicity (A) and measured Microtox® acute (EC_{50}) toxicity (B) values for ten model NAs.

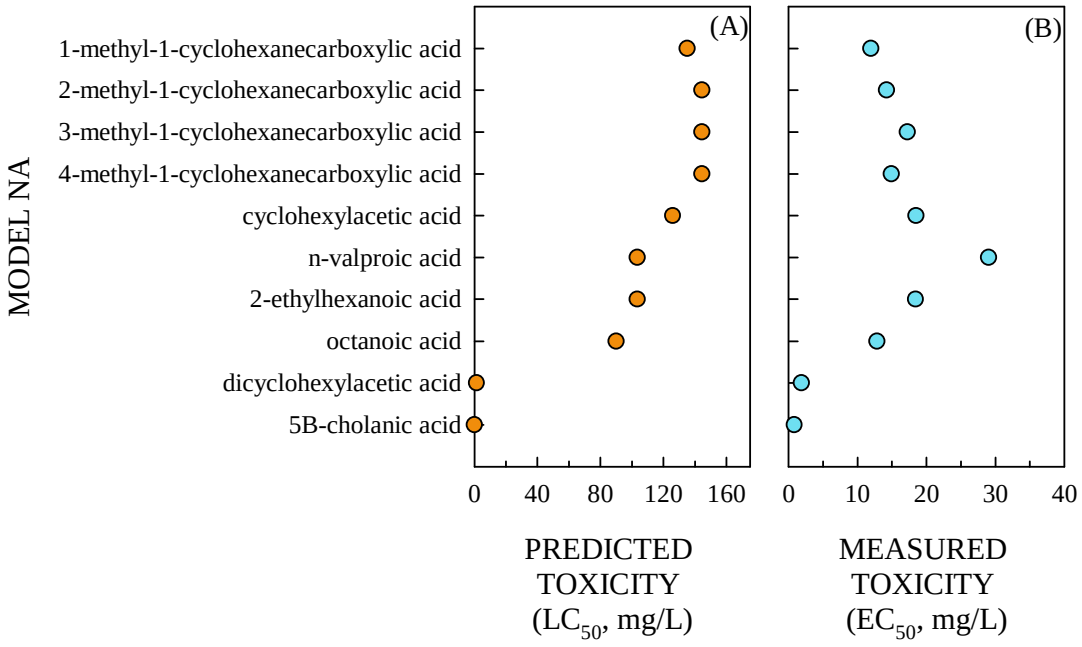


Figure 8.4. Predicted toxicity to *Daphnia magna* (A) and measured toxicity to *Vibrio fischeri* (B) for each model NA tested.

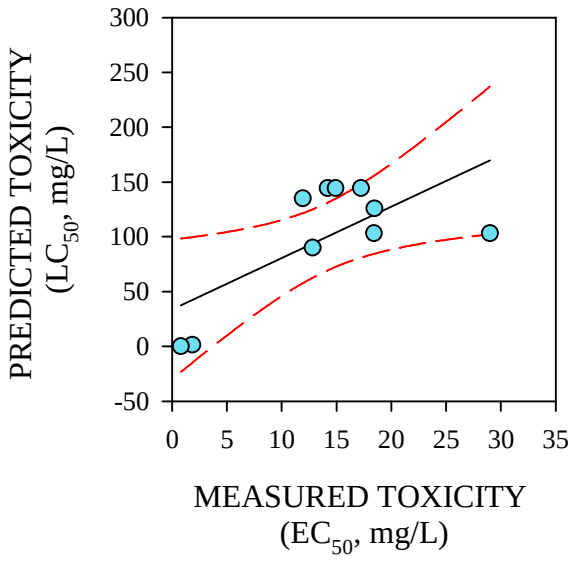


Figure 8.5. Correlation between predicted toxicity to *Daphnia magna* and measured toxicity to *Vibrio fischeri* for each model NA tested ($r^2 = 0.475$).

8.3.3 Probability of NA Biodegradation

The effect of NA structure on aerobic biodegradability was predicted based on Gibb's free energy values and BIOWIN v4.01 model 5 (MITI linear biodegradation probability model) and compared to the results of experimental biodegradation assays. The standard Gibb's free energies of various model NAs were calculated as described in Chapter 6, Section 6.2.1, above and the reduction and oxidation reactions are shown in Table 8.3 and Table 8.4, respectively. The Gibb's free energy of reduction reaction for model NAs with Z = 0, -2, -4 and -8 were estimated to be 28.7, 28.7, 29.0 and 26.6 kJ/eeq, respectively. The free energy of NA oxidation was between -4726.8 and 14003.8 kJ/mol NA for all model NAs. Therefore, biodegradation of all NAs is energetically feasible under aerobic conditions and complete NA mineralization should yield an average energy of 107.06 kJ per electron equivalent.

The aerobic biodegradation probability predicted by the BIOWIN model for each model NA is summarized in Table 8.2. The results indicate that the aerobic biodegradation potential is highest for acyclic NAs such as octanoic, valproic and 2-ethylhexanoic acid, and lowest for the highest MW model NA, 5β-cholanic acid. The aerobic biodegradability is very similar for all model NAs with 8 carbon atoms; however, the probability of aerobic biodegradation is significantly lower for 1-methyl-1-cyclohexylcarboxylic acid, which contains a quaternary carbon atom (Table 8.2).

Table 8.2. Characteristics of model NAs.

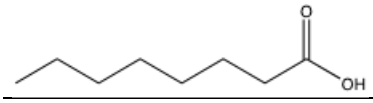
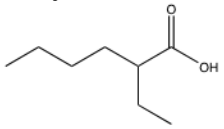
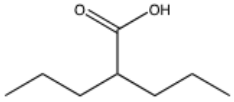
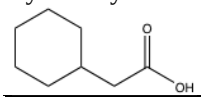
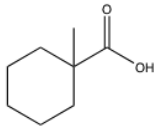
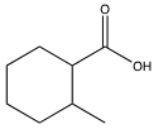
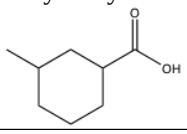
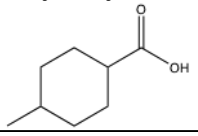
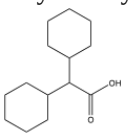
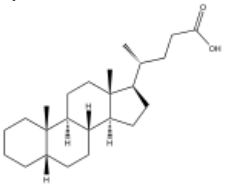
Model NA	*n*	*Z*	MW	Log K_{ow}	pK_a	Aerobic Biodegradability Probability
Octanoic acid 	8	0	144.2	2.94	4.75	0.86
2-ethylhexanoic acid 	8	0	144.2	2.61	4.75	0.86
n-valproic acid 	8	0	144.2	2.54	4.75	0.86
Cyclohexylacetic acid 	8	-2	142.2	2.53	4.72	0.75
1-methyl-1-cyclohexanecarboxylic acid 	8	-2	142.2	2.14	4.7	0.57
2-methyl-1-cyclohexanecarboxylic acid 	8	-2	142.2	2.2	4.82	0.75
3-methyl-1-cyclohexanecarboxylic acid 	8	-2	142.2	2.2	4.82	0.75
4-methyl-1-cyclohexanecarboxylic acid 	8	-2	142.2	2.39	4.82	0.75
Dicyclohexyl acetic acid 	14	-4	224.2	4.34	4.7	0.71
5β-cholanic acid 	24	-8	360.6	5.45	4.75	0.28

Table 8.3. NA half-reactions and their Gibb's free energy at pH 7 and 298 ^{o}K.

Reduction Reactions	$\Delta G'^{o}$ **(kJ/e$^-$ eq)**
C = 8, Z = 0 (valproic acid, 2-ethylhexanoic acid, octanoic acid)	
$\frac{8}{44}CO_2+H^++e^-\leftrightarrow\frac{1}{44}C_8H_{16}O_2+\frac{14}{44}H_2O$	28.7
C = 8, Z = -2 (cyclohexylacetic acid, methyl cyclohexane carboxylic acid)	
$\frac{8}{42}CO_2+H^++e^-\leftrightarrow\frac{1}{42}C_8H_{14}O_2+\frac{14}{42}H_2O$	28.7
C = 14, Z = -4 (dicyclohexylacetic acid)	
$\frac{14}{76}CO_2+H^++e^-\leftrightarrow\frac{1}{76}C_{14}H_{24}O_2+\frac{26}{76}H_2O$	29.0
C = 24, Z = -8 (5β-cholanic acid)	
$\frac{24}{134}CO_2+H^++e^-\leftrightarrow\frac{1}{134}C_{24}H_{40}O_2+\frac{46}{134}H_2O$	26.6

Table 8.4. NA oxidation reactions and their Gibb's free energy at pH 7 and 298 ^{o}K.

Oxidation Reactions	$\Delta G'^{o}$ **(kJ/mol NA)**
C = 8, Z = 0 (valproic acid, 2-ethylhexanoic acid, octanoic acid)	
$C_8H_{16}O_2+11\ O_2\leftrightarrow 8\ CO_2+8\ H_2O$	-4726.8
C = 8, Z = -2 (cyclohexylacetic acid, methyl cyclohexane carboxylic acid)	
$C_8H_{14}O_2+10.5\ O_2\leftrightarrow 8\ CO_2+7\ H_2O$	-4513.5
C = 14, Z = -4 (dicyclohexylacetic acid)	
$C_{14}H_{24}O_2+19\ O_2\leftrightarrow 14\ CO_2+12\ H_2O$	-8183.9
C = 24, Z = -8 (5β-cholanic acid)	
$C_{24}H_{40}O_2+33\ O_2\leftrightarrow 24\ CO_2+20\ H_2O$	-14003.6

8.3.4 NA Biotransformation Potential

Biodegradation assays were set up to investigate the biotransformation potential of ten model NAs by the NA-enriched culture. Time course data for all 8-carbon model NAs tested are shown in Figure 8.4. Figure 8.6 shows that 7 of the 8 eight-carbon model NAs were biodegraded by the NA-enriched culture in less than 4 days. Only one model NA, 1-methyl-1-cyclohexane carboxylic acid, was not biodegraded throughout the 7-day incubation period. This model NA was likely recalcitrant due to the quaternary carbon present in this compound. None of the other model NAs tested has a quaternary carbon. These results indicate that the first step in the degradation of the seven biodegradable NAs is likely α-oxidation (Figure 8.7); however, in the case of 1-methyl-1-cyclohexane carboxylic acid, the quaternary carbon cannot be oxidized and biodegradation does not proceed. The results support the QSAR predictions that this compound is less biodegradable than the other eight-carbon model NAs.

The biodegradation kinetics of the model NAs were simulated using equations 8.3 and 8.4 and the estimated biodegradation rates and RMSD values are summarized in Table 8.5. The measured and simulated NA concentrations are shown in Figure 8.8. Of the seven model NAs that were biodegraded by the NA-enriched culture, octanoic acid and 2-ethylhexanoic acid were degraded the fastest, within 1 day or less, followed by 2-methyl-1-cyclohexanecarboxylic acid, 3-methyl-1-cyclohexane carboxylic acid and 4-methyl-1-cyclohexane carboxylic acid (Table 8.5). Lag periods were observed in the degradation of the two branched, acyclic model NAs (2-ethylhexanoic and valproic acids), contradicting the QSAR prediction that all acyclic NAs are the most biodegradable; however, the lag period was ignored when estimating the model NA

biodegradation rates (Figure 8.8). The lag period for 2-ethylhexanoic acid was approximately 1 day, upon which degradation proceeded rapidly. The lag period observed for valproic acid was shorter than 2-ethylhexanoic acid; however, degradation was much slower once valproic acid degradation proceeded. All compounds (excluding 1-methyl-1-hexylcarboxylic acid) were degraded within four days of incubation.

In addition to the eight lower MW model NAs, the biotransformation potential of two higher MW, more complex NAs, dicyclohexylacetic acid and 5β-cholanic acid, was investigated. Both compounds were recalcitrant and were not degraded during an extended, 18-day incubation (Figure 8.9). These compounds are more complex and have a higher cyclization and MW; thus, they are more representative of NAs found in refinery wastewater or oil sands process water. These results agree with previous bioassays of this study (Chapter 7) and previously reported literature data that higher MW NAs with increased cyclization are more recalcitrant than the lower MW, less complex NAs (Biryukova et al., 2007; Clemente et al., 2004; Han et al., 2008; Holowenko et al., 2002; Scott et al., 2005; Watson et al., 2002).

Table 8.5. Estimated k_{NA} (mg NA/mg VSS-day) and RMSD values for the aerobic biodegradation of the selected model NAs by the NA-enriched culture.

Model NA	$\boldsymbol{k_{NA}}$	**RMSD**
Octanoic acid	4.05	7.3
2-ethylhexanoic acid	2.76	2.4
n-valproic acid	1.41	2.7
Cyclohexylacetic acid	1.65	5.6
1-methyl-1-cyclohexane carboxylic acid	0.01	0.9
2-methyl-1-cyclohexane carboxylic acid	2.23	3.1
3-methyl-1-cyclohexane carboxylic acid	2.24	2.2
4-methyl-1-cyclohexane carboxylic acid	2.21	2.9

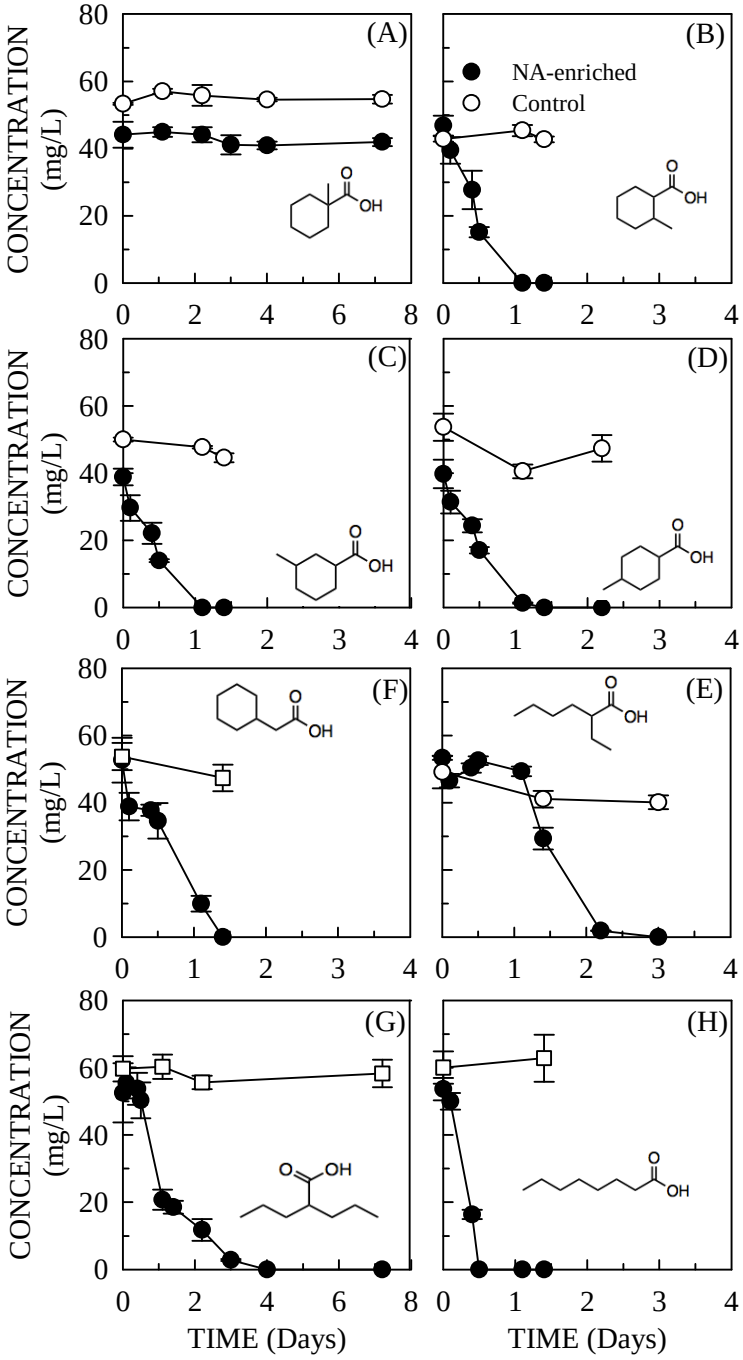


Figure 8.6. Aerobic biotransformation of 8-carbon isomer model NA compounds by the NA-enriched culture series containing the following model NAs: 1-methyl-1-cyclohexane carboxylic acid (A), 2-methyl-1-cyclohexane carboxylic acid (B), 3-methyl-1-cyclohexane carboxylic acid (C), 4-methyl-1-cyclohexane carboxylic acid (D), cyclohexylacetic acid (E), 2-ethylhexanoic acid (F), valproic acid (G) and octanoic acids (H). (Error bars represent mean values ± one standard deviation, n = 3).

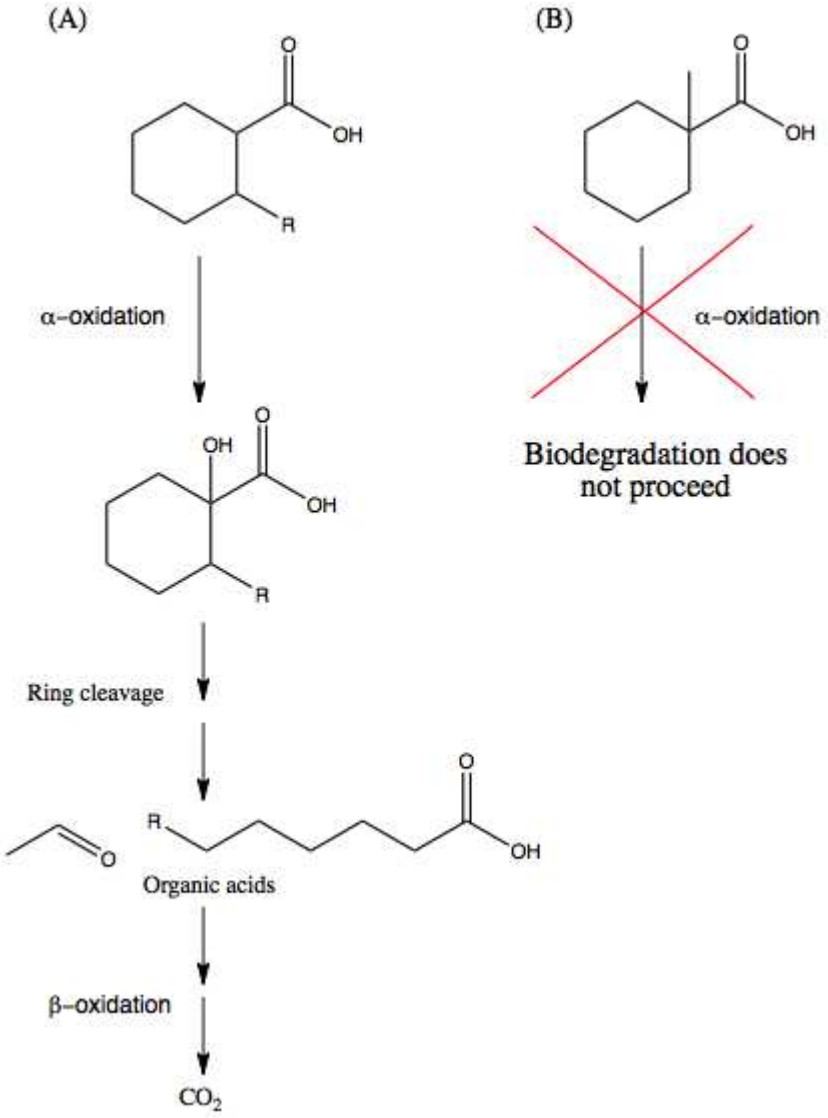


Figure 8.7. Proposed α- and β-oxidation pathways for cyclohexylacetic acid (OECD Toolbox, 2012).

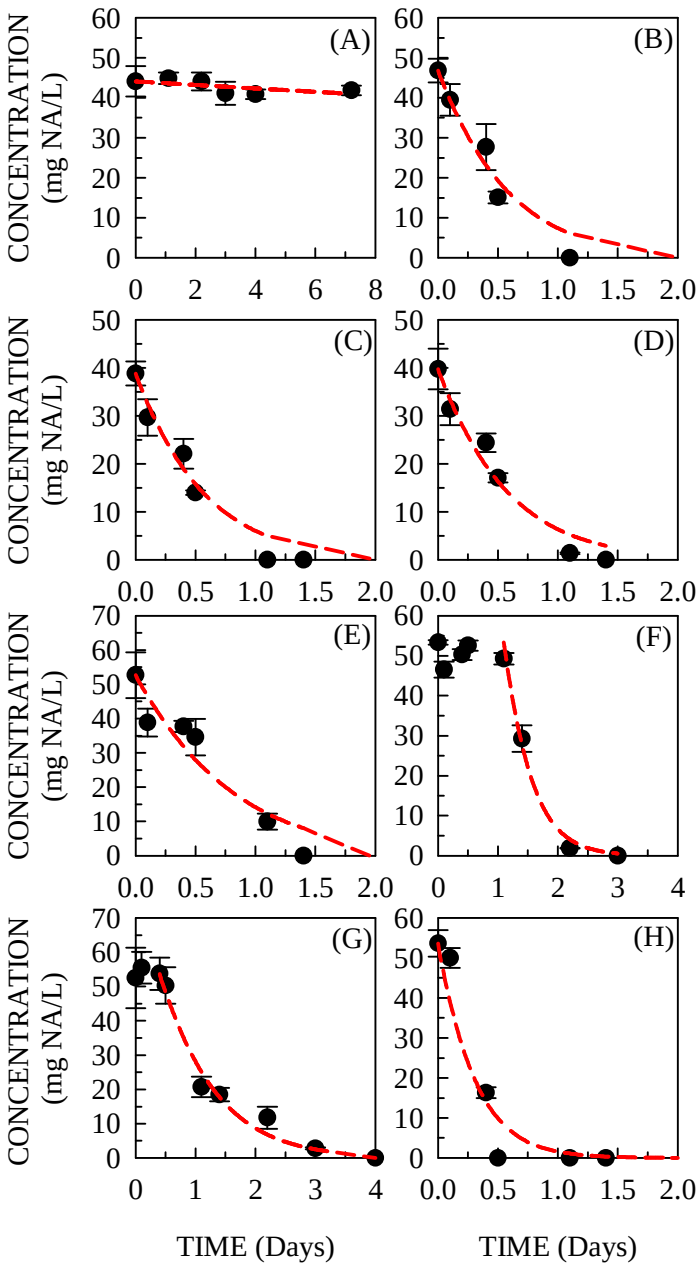


Figure 8.8. Measured (symbols) and simulated (dashed lines) NA concentrations in the NA-enriched culture series containing the following model NAs: 1-methyl-1-cyclohexane carboxylic acid (A), 2-methyl-1-cyclohexane carboxylic acid (B), 3-methyl-1-cyclohexane carboxylic acid (C), 4-methyl-1-cyclohexane carboxylic acid (D), cyclohexylacetic acid (E), 2-ethylhexanoic acid (F), valproic acid (G) and octanoic acids (H).

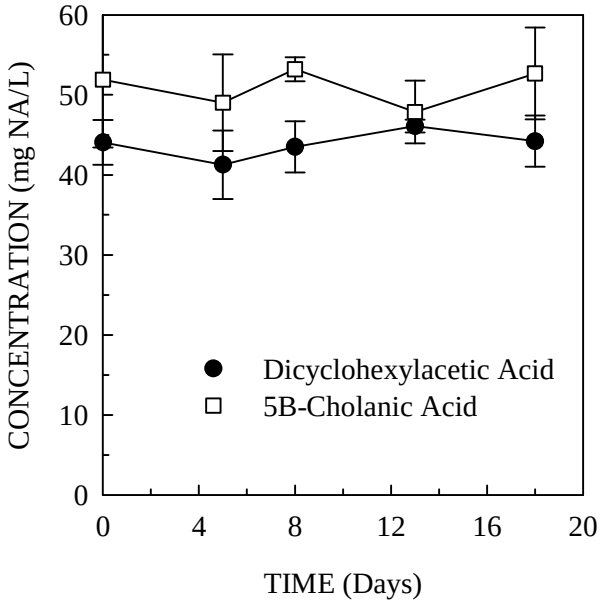


Figure 8.9. Dicyclohexylacetic acid and 5β-cholanic acid concentrations over the 18-days incubation using the NA-enriched culture (Error bars represent mean values ± one standard deviation, $n = 3$).

8.3.5 NA Mineralization Potential

To investigate to what extent the biodegradable model NAs are mineralized to CO_2, a closed bottle, aerobic biodegradation assay was set up using two model NAs, valproic acid and cyclohexylacetic acid. The NA concentration, carbon dioxide production, oxygen consumption and soluble COD for both model compounds and their respective controls are shown in Figure 8.10 and Figure 8.11. Similar to the previous biotransformation assay, valproic acid degradation was slower than cyclohexylcarboxylic acid, which is also indicated by both slower oxygen consumption and carbon dioxide production. The results of this study show that 100% of the two model NAs were

biotransformed and completely mineralized to carbon dioxide, with 21 and 35% of valproic and cyclohexylacetic acid utilized for biomass growth, respectively. VFAs, organic acids or intermediates were not detected in any of the samples analyzed throughout the incubation period. These results do not agree with the results of the mineralization of the NA mixture (Chapter 7, Section 7.3.6) in which 85% of the mixture was biotransformed but only 44% was mineralized to CO_2. The observed difference is likely due to the fact that the NA-enriched culture is capable of completely mineralizing the lower MW NAs and can only partially oxidize or not biotransform the higher MW, more complex NAs.

Although metabolites were not detected, the aerobic degradation pathway was predicted based on the model NA structure, previous studies, predictions of the OECD QSAR toolbox software and the measured final product, carbon dioxide. The proposed aerobic degradation pathways for the selected two model NAs are shown in Figure 8.12 and 8.13, respectively. Similar to other studies, the most likely degradation pathway is *β*-oxidation, in combination with *α*- or *ω*-oxidation. The microbial attack of the acyclic NA, *n*-valproic acid, is likely initiated by oxidation of the β- or ω- carbon. Both pathways result in the formation of smaller organic acids which are mineralized to CO_2 (Figure 8.12). The structure and predicted metabolites of the cyclic NA, cyclohexylacetic acid, indicate that the initial step of biodegradation is likely oxidation of the α-carbon, loss of one carbon and followed by the oxidation of the β-carbon resulting in ring cleavage, formation of smaller organic acids and finally mineralization to CO_2 (Figure 8.13).

Table 8.6. Carbon balance for closed bottle, aerobic biodegradation of valproic acid and cyclohexylacetic acid by the NA-enriched culture.

Carbon Component	Valproic Acid		Cyclohexylacetic Acid	
	mmol C	% of total carbon added	mmol C	% of total carbon added
NA Degradation	0.0416	100	0.0422	100
CO_2 Production	0.0315	75.9	0.0295	69.9
Biomass Growth	0.0086	20.6	0.0146	34.6

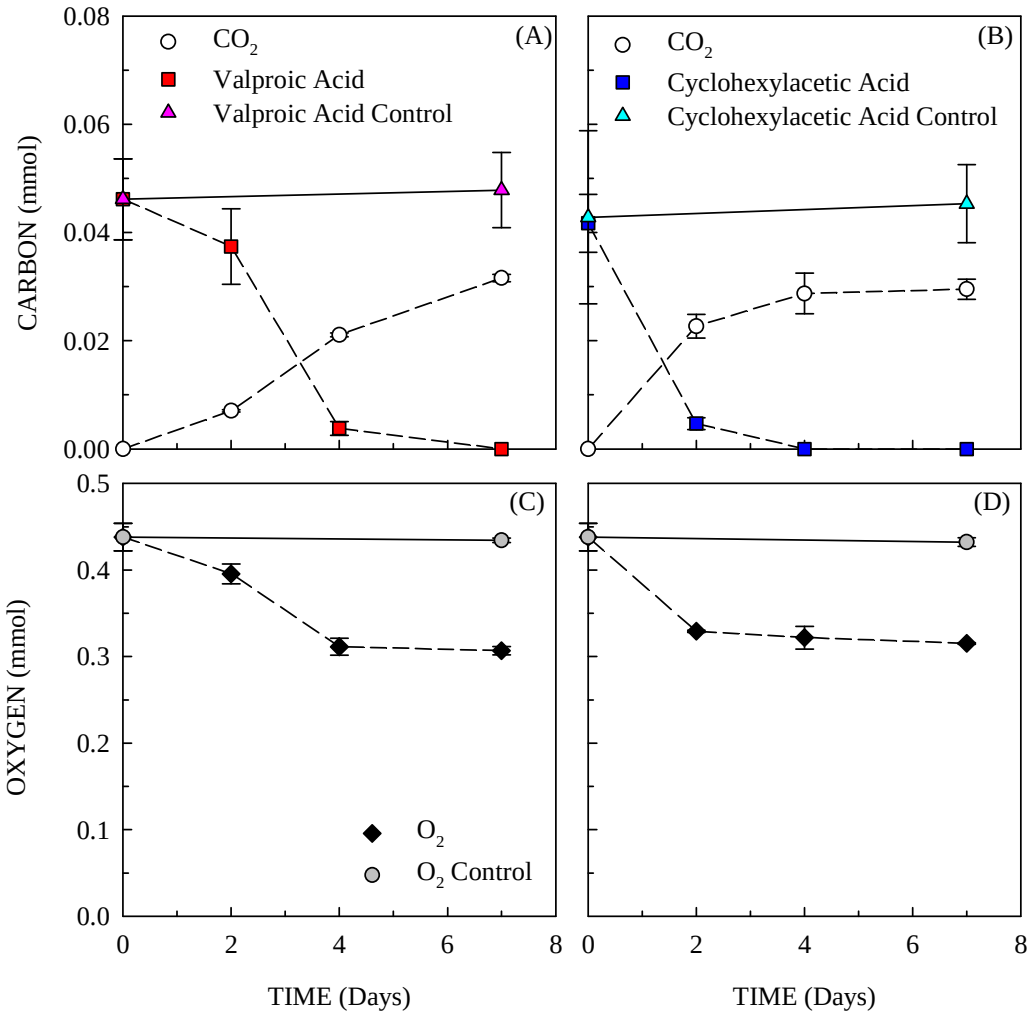


Figure 8.10. NA degradation, carbon dioxide production and oxygen consumption in valproic acid (A, C) and cyclohexylacetic acid (B, D) closed bottle, aerobic NA-enriched culture series (Error bars represent mean values ± one standard deviation, $n = 2$).

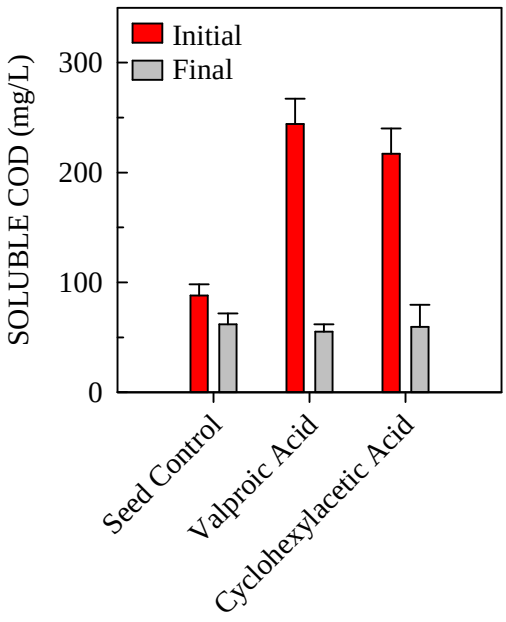


Figure 8.11. Initial and final soluble COD in seed control, valproic acid and cyclohexylacetic acid series in the closed bottle, aerobic model NA biotransformation assay (Error bars represent mean values ± one standard deviation, n = 3).

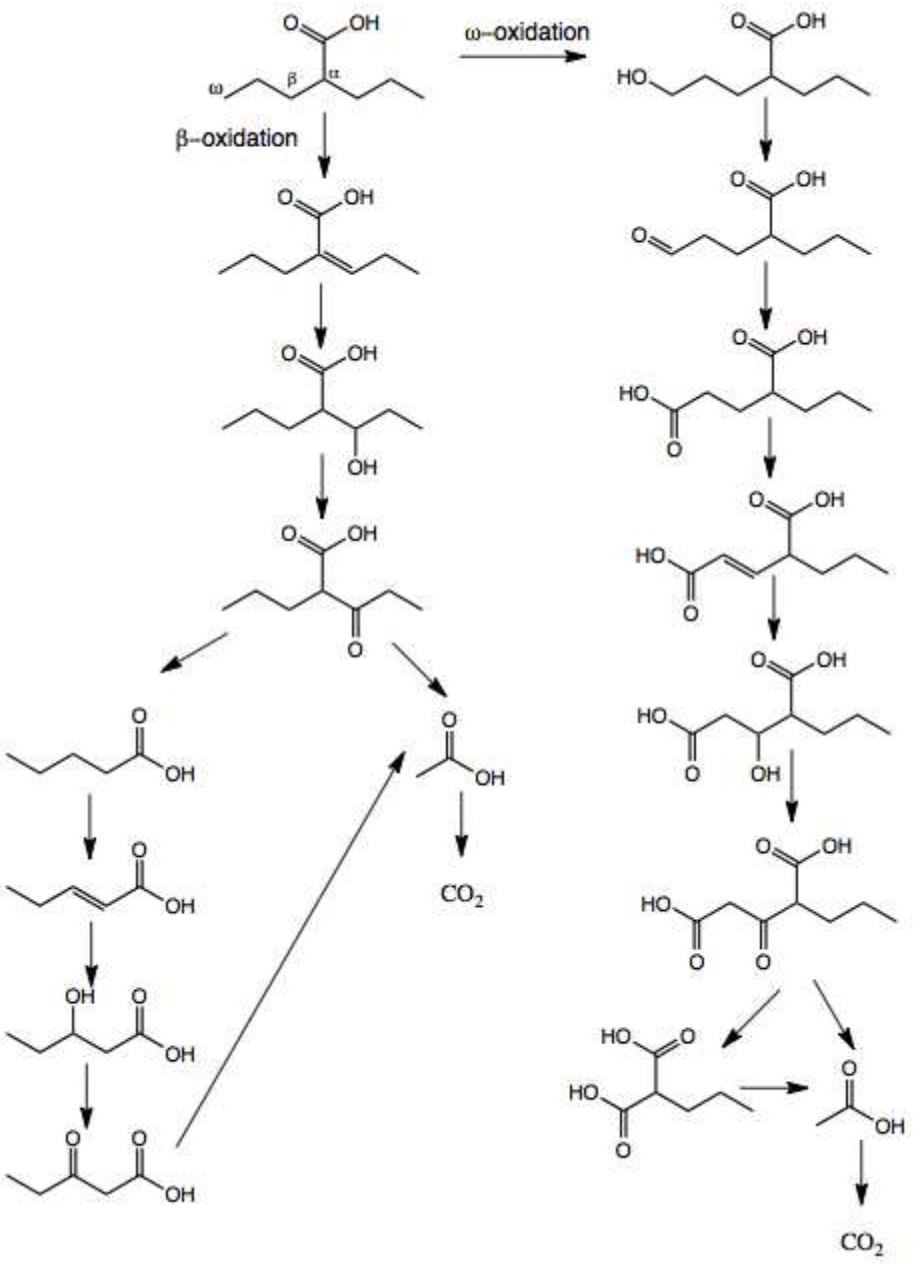


Figure 8.12. Proposed β- and ω-oxidation pathways for valproic acid (OECD Toolbox, 2012).

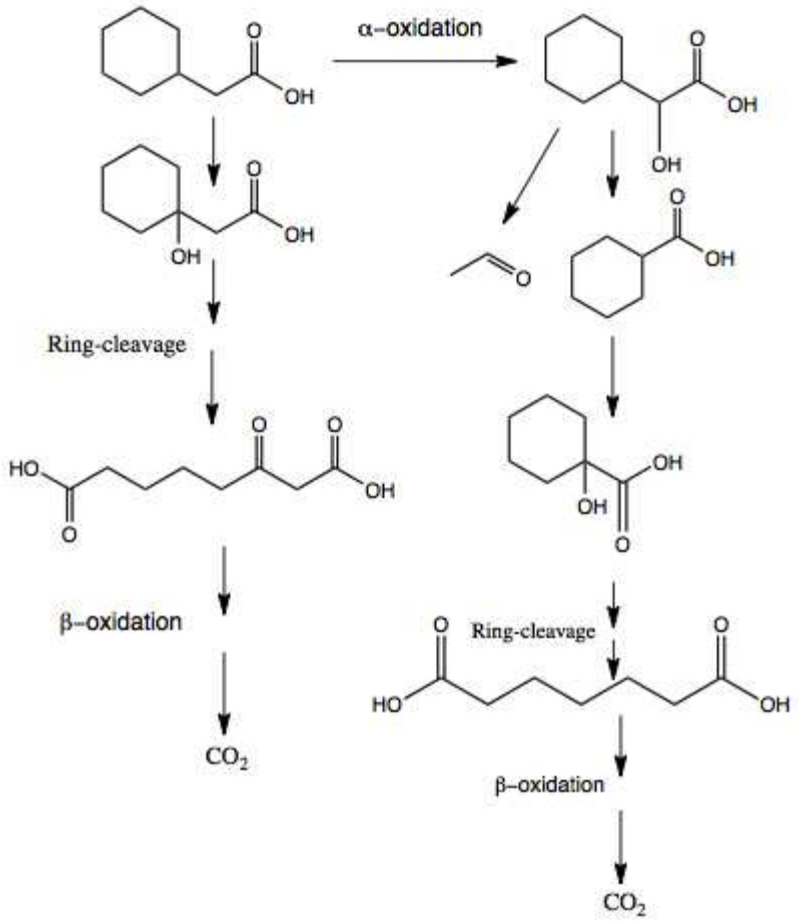


Figure 8.13. Proposed α- and β-oxidation pathways for cyclohexylacetic acid (OECD Toolbox, 2012).

8.4 Summary

Although previous studies have investigated the effect of NA structure on toxicity and biodegradability, the effect of structure within a group of NA isomers has not been previously investigated. Although significant differences were not observed in the predicted and measured toxicity between the eight NA isomers used in this study, the NA structure had a significant effect on biodegradability. Branched model NAs exhibited lag periods and slower degradation rate than non-branched or simple cyclic model NAs. The presence of a quaternary carbon even in a low MW NA results in molecular recalcitrance.

The results of this study agree with previous studies and indicate that the higher MW, more hydrophobic NAs are more toxic and recalcitrant. The results also indicate that complex structures, such as quaternary carbon-bearing NAs, increase recalcitrance, even in low MW NAs. The quaternary carbon as well as an increase in MW and cyclization significantly increased the recalcitrance of the studied model NAs. The recalcitrance of the higher MW as well as quaternary carbon-bearing NAs provide further insight into the results on the biodegradation of a complex NA mixture (see Chapter 7) and confirm that the residual NAs observed in the NA-enriched culture effluent is likely the result of molecular recalcitrance in addition to NA bioavailability.

Given the complexity of NA distribution in refinery wastewaters and the limited availability of representative individual NAs in the market, developing structure-activity relationships to understand the fate and effect of NAs in biological treatment systems and the environment, as well as possible complications resulting from the reuse of NA-bearing treated water in the refineries, is crucial. Such a methodology will be beneficial

in understanding the results obtained from bioassays and in developing treatment options for NA-bearing refinery wastewater.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

This study assessed the fate, toxicity and biotransformation potential of naphthenic acids through a series of toxicity, inhibition and biotransformation assays. Biotransformation potential and inhibition of NAs was investigated under aerobic, anoxic and fermentative/anaerobic conditions.

A NA mixture, representative of NAs found in refinery wastewater streams, was used in biotransformation and inhibition assays. Individual model NAs were also selected to investigate the effect of NA structure on both biotransformation and toxicity. NA hydrophobicity and structure were used to predict the NA biotransformation potential and toxicity and the predictions were compared to the results of experimental toxicity and biotransformation assays. Biotransformation of NAs is thermodynamically feasible under aerobic, anoxic and fermentative/anaerobic conditions; however, only select NAs were biodegraded/biotransformed under aerobic conditions. The following conclusions can be drawn based on the results of this research:

(1) The desalter brine is the main source of NAs in refinery wastewater; however, NAs are ubiquitous in refinery wastewater treatment systems (i.e., influent, mixed liquor and effluent).

(2) Activated sludge units of crude oil refineries are effective in lowering the effluent NA concentrations as well as the effluent toxicity; however, it was not possible to correlate Microtox® acute toxicity values to NA concentrations in such complex

samples. Other components contribute significantly to the measured toxicity and thus, the measured toxicity largely depends on the wastewater composition.

(3) Desorption and biodegradation of chronically-sorbed NAs in refinery mixed liquors were limited, resulting in sludge with elevated NA concentrations as compared to influent NA levels, indicating that lower refinery effluent concentrations are due to both biodegradation and adsorption to biosolids. High NA concentrations measured on the biosolids indicate that treatment of refinery biosolids is required and important for the reduction of NAs.

(4) NAs were found to accumulate on the biosolids and partition to both artificial and natural adsorptive media. Therefore, NAs are likely to be found in anoxic or anaerobic environments; however, they are not degraded under these conditions and could potentially accumulate to toxic levels in these environments.

(5) The commercial NA mixture used in this study was partially biodegraded by cultures developed with oil refinery activated sludge inoculum. However, a fraction of the NA mixture was not biotransformed and remained as residual as a result of low individual NA concentrations below the minimum substrate concentration at which they are no longer degraded. The results indicate that biodegradation alone will not completely remove NAs and combination with tertiary physical/chemical treatment methods is required for complete NA removal from refinery effluents.

(6) Although a large portion of the commercial NA mixture was biotransformed, only a small fraction was mineralized to CO_2. The un-mineralized NAs were likely biotransformed to more oxidized metabolites.

(7) NA structure had a significant effect on biodegradability. Branched model NAs exhibited lag periods in batch bioassays and lower degradation rate than non-branched or simple cyclic model NAs. The presence of a quaternary carbon even in a low MW NA resulted in recalcitrance. Larger MW NAs with increased cyclization were also recalcitrant.

9.2 Recommendations

This research is a comprehensive study of NAs from the source (crude oil) to the refinery effluent and the results have provided information on the physical, chemical and biological processes that determine the fate and effect of NAs in engineered and natural systems. The results of this research can be used to improve NA-bearing wastewater treatment and disposal as well as provide guidelines and recommendations for future practices and/or regulations.

Given the complexity of NA distribution in refinery wastewaters and the limited availability of representative individual NAs in the market, developing structure-activity relationships to understand the fate and effect of NAs in biological treatment systems and the environment, as well as possible complications resulting from the reuse of NA-bearing treated water in the refineries, is crucial. Identification of more complex and representative NA structures in refinery wastewater and oil sands tailings ponds through advanced analytical methods will allow for more relevant structure-activity studies to be conducted.

Multiple studies, in addition to this one, have indicated that NAs are not completely degraded and likely biotransformed to more oxidized products. Further

understanding of the NA biotransformation process in terms of products formed as well as conditions affecting their biodegradation will help the development of effective treatment processes, thus decreasing the potentially harmful effects of NAs on the environment. In addition, further investigation into the biotransformation products is necessary as they may not be readily degradable, thus having the potential to accumulate and contribute to refinery wastewater and process water toxicity. Although it is extremely difficult to identify biotransformation intermediates for a complex NA mixture, advancement in analytical techniques and use of model compounds in future biotransformation studies would allow identification and analysis of partially degraded or oxidized metabolites. Advanced molecular biology tools can be used to confirm the NA degradation pathway through use of NA-degrading isolates and various model NAs. Identification of NA-degrading organisms as well as genes and enzymes involved in NA biotransformation can provide insight into the factors causing NA recalcitrance.

Complete elimination of non-bioavailable, residual, and persistent NAs from refinery effluents may require the use of alternative, tertiary treatment processes, such as adsorption or advanced oxidation. Activated carbon, especially PAC , is complementary to microbial biodegradation and a combination of physico-chemical and biological processes may be necessary to achieve the complete removal of NAs from the petroleum refineries effluents. The use of a PAC filter or post treatment for removal of recalcitrant NAs should be investigated through small scale continuous flow systems that can provide systematic information about the use of different adsorptive media as both pre- and post-treatment to refinery activated sludge units.

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