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Mechanisms for Aerobic Degradation of Commercial Naphthenic Acids

Samuel Louis Muller
Clemson University, slmulle@clemson.edu

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MECHANISMS FOR AEROBIC DEGRADATION OF COMMERCIAL
NAPHTHENIC ACIDS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Hydrogeology

by
Samuel Louis Muller
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Accepted by:
James Castle, Committee Chair
John H. Rodgers, Jr.
Monique Haakensen

ABSTRACT

Naphthenic acids (NAs) are a class of thousands of carboxylic acids associated with petroleum degradation. They become dissolved in oil sands process waters (OSPW) during the bitumen extraction process, and the resulting process waters can elicit toxicity to aquatic organisms. NAs are weakly biodegradable, but have half-lives of months to years, making it difficult to treat NAs with bioremediation. Two methods for promoting aerobic degradation (cometabolism and mycoremediation) were investigated as proof of concept for effectiveness in degrading commercial NAs. A reciprocating reactor inoculated with a white rot fungus, *Pleurotus pulmonaris*, was built and compared to an uninoculated reactor to determine the effects of this fungus on NA degradation.

Inoculated reactors were more effective than uninoculated reactors in removing NAs, with zero-order half lives of 32 and 39 hours, respectively. This demonstrated the usefulness of both *P. pulmonaris* and a reciprocating reactor in promoting aerobic NA degradation. Cometabolic NA degradation using different substrates and substrate concentrations was investigated at bench scale. This study confirmed that cometabolic substrate addition increases NA removal rate in comparison to unamended degradation. It also showed that the concentration ratio of substrate to NAs affects the removal rate of NAs. This has important implications to the design of a constructed wetland treatment system for ecological risk mitigation of OSPW, where wetland detritus may serve as a cometabolic substrate to promote NA degradation.

DEDICATION

This thesis is dedicated to the Chattooga River. Completion of this thesis would not have been possible if not for the restorative touch of its waters. May it ever flow freely.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. James Castle for encouraging me to follow my interests throughout my research. His guidance helped shape me as a scientist. I also thank my committee members, Dr. John Rodgers Jr. and Dr. Monique Haakensen for providing insight into other disciplines and pushing me to strive for excellence. Additionally, I would like to thank the fellow members of the OSPW group for their assistance and feedback throughout the completion of this research.

Outside of the university, I received love and encouragement throughout the completion of this thesis. My girlfriend, Megan Chase, supported me through my graduate studies unfailingly. To my parents and twin sister, thank you for all of your love.

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CHAPTER ONE

INTRODUCTION

With global oil demand rising, non-conventional oil reserves are being relied upon increasingly to fulfill the world's need for petroleum (International Energy Agency 2014). The Athabasca Basin in northeastern Alberta, Canada contains the largest deposit of oil sands in the world, with proven reserves of approximately 169 billion barrels (Alberta Energy 2014). Oil sands are a biodegraded energy deposit containing bitumen, a highly viscous petroleum form that cannot be extracted with conventional oil production techniques (Alberta Government 2014). Currently, production is from shallow deposits that are surface mined, and an area of approximately 767 km^2 has been disturbed by oil sands production to date (Alberta Government 2014). Bitumen is traditionally extracted from the oil sands through a caustic hot water extraction using sodium hydroxide (Allen 2008). The resulting process water, which is acutely toxic to aquatic organisms, contains salts, trace metals, and organic compounds (Allen 2008). Oil producers in the Athabasca region operate under a zero-discharge policy, so the volume of accumulated process water continues to increase.

OSPW

Oil sands production requires a large volume of water to extract the bitumen from sands of the McMurray deposit. On average, 3 barrels of water are used to process one barrel of oil (Allen 2008, Quagraine et al. 2005). 2007 estimates place the volume of stored process water in the Athabasca region to be approximately 700 million m^3 (Dominski 2007). The water that accumulates on oil sands leases is known as oil sands

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process affected water (OSPW), which includes relatively clean water to highly toxic tailings pond water (Allen 2008). 80-95% of water used is recycled in the extraction process, and concentrations of constituents of concern (COC) increase with each reuse (Allen 2008).

In comparison to regional surface water, OSPW is enriched in salts, metals, residual hydrocarbons, and a complex mixture of carboxylic acids known as naphthenic acids (Allen 2008). OSPW has a pH of 8.0-8.4 and total dissolved solids concentrations in the moderately brackish range (200-2500 mg*L^{-1}) (Allen 2008). Dominant dissolved solids include sodium (a product of the caustic extraction), bicarbonate, chloride, and sulfate (Allen 2008). Trace metals in OSPW include aluminum, cadmium, chromium, copper, iron, lead, molybdenum, titanium, vanadium, and zinc (Mackinnon and Boerger 1986). Historical data on OSPW indicate that some tailings ponds contain trace metals at concentrations above Canadian Council of Ministers for the Environment (CCME) water quality guidelines for chronic effects to aquatic organisms (Allen 2008). However, the constituent of OSPW causing the greatest concern in terms of toxicity is the naphthenic acid fraction (Allen 2008).

Naphthenic Acids

Naphthenic acids (NAs) are a group of cyclic and aliphatic carboxylic acids with the general formula $C_nH_{2n+z}O_2$, where n is the carbon number, between 8 and 30, and Z is zero or a negative even integer describing the hydrogen deficiency (Clemente et al. 2004). NAs occur naturally in oil sands as a degradation product of crude oil (Headley and McMartin 2004). They are non-volatile and behave as surfactants (American

Petroleum Institute 2003). Water solubility increases with pH, and OSPW can contain up to 120 mg*L^{-1} NAs at a pH of 8-8.4 (Whitby 2010). OSPW tends to contain acyclic and high carbon number NAs that are recalcitrant to biodegradation (Lai et al. 1996, Clemente et al. 2004, Han et al. 2009, Herman et al. 1994).

Of the constituents found in OSPW, NAs are of most concern to regulators and oil producers. The toxicity of OSPW is attributed primarily to the extractable organic acid fraction, which is dominated by NAs (Allen 2008, Mackinnon and Boerger 1986). Commercial NAs are acutely toxic to fish at a concentration of 2.5-5 mg*L^{-1} (Swigert et al. 2015). Microorganisms cannot readily mineralize NAs, especially acyclic and high carbon number molecules that are most common in OSPW (Clemente et al. 2004, Whitby 2010).

Toxicity testing was a crucial component of this research. With the complicated nature of NA mixtures, various analytical techniques can result in different measured NA concentrations for the same sample. Due to the limitations of quantitative NA analysis, a change in acute toxicity after treatment can be used to confirm mitigation of risk to receiving aquatic systems.

Numerous studies have investigated microbial degradation of OSPW NAs, finding half-lives ranging from months to years (Han et al. 2009, Headley and McMartin 2004, Scott et al. 2005, Whitby 2010), which is too slow for efficient treatment of stored OSPW. This lack of success with in-situ bioremediation of OSPW, coupled with an increasing volume of stored water, requires a novel approach for treating the large volume of water as quickly as possible.

With the known challenges of NA bioremediation and an increasing social pressure to treat waters contaminated with NAs, strategies for increasing NA degradation rate are needed. The research presented in this thesis investigated two potential methods for increasing aerobic degradation rates for NAs: mycoremediation and cometabolism. The major objectives of this research were:

   1. Determine the rate and extent of removal of commercial NAs by aerobic cometabolism.

   2. Determine the rate and extent of removal of commercial NAs by a reciprocating mycoreactor.

1. Determine the rate and extent of removal of commercial NAs by aerobic cometabolism.

Three carbon sources were tested for the ability to promote NA degradation by cometabolism. Toxicity testing was used to confirm NA removal. The effect of substrate concentration on NA removal was also investigated.

1. Determine the rate and extent of removal of commercial NAs by a reciprocating mycoreactor.

Mycoremediation is a biotransformation process using fungal metabolism. White-rot fungi in particular show promise in the transformation of persistent organic molecules (Bhattacharya et al., 2014; Moreira et al., 2003; Okparanma et al., 2011; Pointing, 2001). A reciprocating mycoreactor was built to promote aerobic degradation of NAs by *Pleurotus pulmonaris*. NA concentrations were measured to determine rate and extent of

removal. Change in acute toxicity to a sentinel species was used to confirm analytical results and mitigation of ecological risk.

Thesis organization

This thesis is organized into four chapters, including an Introduction (Chapter 1) and Conclusions (Chapter 4). The two body chapters are:

Chapter 2: Effects of cometabolic substrates on commercial (Fluka) naphthenic acid degradation

Chapter 3: Mycoremediation of commercial (Fluka) naphthenic acids using a reciprocating reactor

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CHAPTER TWO

EFFECTS OF COMETABOLIC SUBSTRATES ON COMMERCIAL (FLUKA)

NAPHTHENIC ACID DEGRADATION

ABSTRACT

Cometabolism was investigated in bench-scale experiments as a potential removal process for naphthenic acids (NAs), which are a complex mixture of carboxylic acids produced during petroleum degradation and extraction. Due to their water solubility and slow biodegradation rates, NAs are persistent when dissolved in petroleum process water. Cometabolism, degradation of a recalcitrant constituent in the presence of an energy substrate, increases degradation rates for many complex organic compounds. Three substrates (wheat hay, corn syrup, and biofermentation product (Diamond V XPC™)) were compared to determine effectiveness for NA cometabolism. Changes in NA concentration with time were measured using an HPLC derivatization method. Because NA degradation decreases aquatic toxicity, acute toxicity testing with fathead minnows (*Pimephales promelas)* was used to assess decreases in NA concentration. Biofermentation product was the most effective cometabolic substrate, with acute toxicity eliminated after 12 days of treatment. Four concentrations of biofermentation product (0.5, 0.25, 0.1 and 0.05 g/L) were tested to determine the effect of substrate concentration on NA removal. Biofermentation product treatments of 0.5 and 0.25 g/L eliminated acute toxicity after 12 and 14 days of treatment, respectively, while concentrations of 0.1 and 0.05 g/L did not eliminate toxicity after 20 days of treatment. These results demonstrate that cometabolism is a promising NA treatment process and that substrate type and

concentration affect NA cometabolic degradation. Costs of cometabolic treatment, as well as influence of treatment on biogeochemical conditions, should be considered for pilot scale testing with OSPW NAs.

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1. INTRODUCTION

Naphthenic acids (NAs) are a group of cyclic and aliphatic carboxylic acids with the general formula $C_nH_{2n+z}O_2$, where n is the carbon number, between 8 and 30, and Z is zero or a negative even integer describing the hydrogen deficiency (Clemente et al., 2004). NAs occur naturally in petroleum as a degradation product of crude oil (Headley and McMartin, 2004). They are non-volatile and behave as surfactants (API, 2012).

NAs are a concern for petroleum producers. During processing of certain types of petroleum, including oil sands, NAs dissolve into oil sands process-affected waters (OSPW). These energy-derived NAs are corrosive to refinery pipelines and equipment and acutely toxic to aquatic organisms (Schramm et al., 2000). Due to toxicity and challenges in reuse of NA-contaminated water, petroleum producers need a treatment pathway to remove NAs from process water. The physical properties of NAs complicate their removal from water. NAs tend to remain in the water column due to their aqueous solubility. They do not sorb well to organic matter or minerals (Schramm et al., 2000). Low vapor pressure precludes volatilization to the atmosphere (API, 2012). NAs are weakly biodegradable, but half-lives in process water can range from years to decades (Whitby, 2010).

NA degradation rates are influenced by several factors, including dissolved oxygen availlability, microbial speciation, and molecular structure. Because aerobic degradation of NAs occurs more rapidly than anaerobic degradation, availability of dissolved oxygen has a strong influence on degradation rates (Del Rio et al., 2006; Herman et al., 1994). Nutrient availability affects NA degradation rates, as addition of phosphate can enhance NA degradation (Lai et al., 1996). However, most studies on NA degradation used NAs as the sole carbon source for microbial organisms (Del Rio et al., 2006; Whitby, 2010). NAs are only weakly biodegradable, and the molecular weight and structure impede their ability to support microbial growth (Han et al., 2008).

Cometabolism, the transformation of non-growth supporting constituents (e.g. NAs) in the presence of an energy substrate, can be utilized as a removal pathway for constituents of concern that are recalcitrant to unamended biodegradation (Hazen, 2010). The addition of an energy substrate increases biological activity when compared to aerobic or anaerobic degradation without added substrate (Arp et al., 2001). As microbes utilize nonspecific oxygenase enzymes to metabolize the energy substrate, the cometabolic substrate is oxidized concurrently (Chang and Alvarez-Cohen, 1995). Cometabolism is most useful for transforming organic compounds that do not directly support microbial growth (Hazen, 2010). Recalcitrant organic compounds such as pesticides, chlorinated solvents, and polycyclic aromatic hydrocarbons (PAHs) have been removed successfully from water using sucrose and other organic matter as cometabolic substrates (Baboshin et al., 2003; Chang and Alvarez-Cohen, 1995; Eggers et al., 2008; Hovarth, 1972). Although it has been proposed that readily available organic matter may

enhance NA degradation (Whitby, 2010), the effect of organic matter addition on degradation rate has not been investigated. Understanding the effects of adding an organic carbon source on NA degradation may contribute to development of efficient bioremediation strategies for NAs.

The goal of this research was to determine if commercial NAs can be effectively removed through aerobic cometabolic treatment. Quantitative NA analysis and toxicity bio-assays were utilized to determine the most effective of three substrates (corn syrup, biofermentation product, and hay) for ability to promote NA removal (Figure 2.1). The objectives were to 1) determine rate and extent of NA removal for three cometabolic substrates, 2) measure change in acute toxicity due to NA cometabolism, and 3) determine the effects of cometabolic substrate concentration on NA removal.

Although increased cyclicity of energy-derived NAs (when compared to commercial NAs) results in longer degradation half-lives for energy-derived NAs (Han et al., 2008; Scott et al., 2005), commercial NAs have been used for previous degradation and toxicity studies (Clemente et al., 2004; Melvin et al., 2013; Scott et al., 2005; Swigert et al., 2015). Commercially available NAs provide a reproducible and repeatable source of the most toxic NA fraction (Marentette et al., 2015) and can justify further investigation of cometabolism using more compositionally complex and aged (energy-derived) NAs that are more resistant to aerobic degradation.

2. METHODS

2.1 Experimental design and explanatory parameters

Experiments were conducted in a climate-controlled greenhouse at Clemson University (Clemson, SC). Twelve 18.9 L polyethylene buckets were used as experimental vessels. Each bucket was filled with 16 L of municipal tap water and buffered with sodium bicarbonate (certified ACS grade) at a concentration of 1 g/L to resist pH change and maintain NA solubility. Fluka NAs (CAS 1338-24-5) (Sigma Aldrich, St. Louis, MO) were added to each bucket to achieve a nominal concentration of 50 mg/L by stirring with an electric drill paint mixer. This concentration is near the aqueous solubility of Fluka NAs (Table 2.1) and allowed for measurement of degradation through three concentration half-lives.

Three carbon sources, selected for availability and prior performance, were tested in triplicate. These substrates were biofermentation product (Diamond V XPC™, Diamond V Mills, Cedar Rapids, IA), corn syrup (Karo™ brand), and hay (wheat, obtained locally in Clemson, SC). Biofermentation product was previously demonstrated as effective in increasing removal efficiencies of selenium and arsenic in a pilot-scale constructed wetland treatment system (CWTS) (Spacil et al., 2011). Corn syrup is an inexpensive and readily available substrate effective in treating halomethane concentrations otherwise considered too high for biological treatment (Shan et al., 2010). Hay is an inexpensive and widely available substrate and has been demonstrated to increase COC removal efficiencies from soil (Shahsavari et al., 2013). Each substrate was

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tested in triplicate at a nominal concentration of 0.5 g/L. This concentration was chosen because substrate concentration should exceed COC concentration in cometabolism, as substrate oxidation rates are greater than contaminant oxidation rates (Arp et al., 2001). 0.5 g of OsmoCote® Plus Flower and Vegetable Smart-Release® plant food (Scotts, Marysville, OH) was added to each corn syrup bucket to supply macro- and micronutrients. Biofermentation product did not require nutrient amendments (Table 2.2). An untreated control, with no added substrates, was also tested in triplicate, with 50 mg/L of Fluka NAs added to municipal tap water buffered with 1 g/L sodium bicarbonate. Buckets were aerated using an air pump and air stones to maintain aerobic conditions. Buckets were covered with polyethylene lids to prevent evaporation.

Samples were collected in 500 mL polyethylene bottles from each bucket at 2 day intervals for 20 days. Explanatory parameters were measured to confirm that conditions for aerobic degradation (dissolved oxygen >2 mg/L, ORP > 100 mV) existed during the experiment. Water temperature, pH, alkalinity, hardness, dissolved oxygen concentration (DO), and reduction/oxidation potential (ORP) were measured at each sample collection (Table 2.3).

2.2 Quantitative NA analysis

NA analysis was performed with a Dionex UltiMate 3000 HPLC system following a derivatization procedure (Yen et al., 2004) modified from a method used to measure short and long chain fatty acids as 2-nitrophenylhydrazides (Miwa et al., 1985). This method requires derivatization of fatty acids as 2-nitrophenylhydrazide HCl in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl because fatty acids do

not show useful absorption in the visible or UV spectrums (Miwa et al., 1985). The method detection limit is 5 mg/L (Yen et al., 2004). A standard curve was prepared using nominal NA concentrations of 0 to 80 mg/L using the same commercial NAs. A sample with a nominal NA concentration of 50 mg/L was analyzed in triplicate, and precision of the method is ±6 mg/L.

Because this derivatization method detects all fatty acids present in a sample, a control for fatty acids other than NAs was incorporated to determine influence of substrate addition on measured fatty acid concentrations. Each cometabolic substrate was added to a borosilicate glass jar containing 0.5 L of Nano-pure water buffered with 0.5 g of $NaHCO_3$. Jars were aerated for 20 days, and samples were collected at 2-day intervals using a syringe and stored in 20 mL glass vials under refrigeration. Samples were measured for total fatty acids following the above HPLC method. These measured concentrations were subtracted from total fatty acid concentrations from treated samples to determine if NA concentration could be measured in treated samples.

Removal rate coefficients were calculated with a kinetic model offering the best fit to the data. Rate coefficients of removal were calculated by zero-order kinetics:

$$k = -(C_t/t) + C_0 \qquad \qquad \text{Equation 1}$$

where t is time of measurement (hours), k is the zero-order rate constant $(hours^{-1})$, C_0 is NA concentration at t=0 (mg/L), and C_t is NA concentration at time=t (Fetter, 1999).

2.3 Sorption to solid substrates

The extent of removal attributed to adsorption to solid substrates (biofermentation product and hay) was measured. 0.25 g of each substrate was added to a separate borosilicate glass jar containing 0.5 L of Nano-pure water to which 0.5 g of $NaHCO_3$ had been added. The jars were autoclaved at 135°C for 45 minutes for sterilization. Samples were collected with a sterile syringe at 6 hour intervals for 1 day and after 72 hours, and NA concentrations were measured. A sample was collected before substrate addition to establish an initial NA concentration.

2.4 Toxicity

Acute toxicity testing was used as an additional line of evidence for NA removal. Using a sensitive sentinel species, *Pimephales promelas,* cometabolic substrates were assessed for their ability to alter toxicity of commercial NAs. The effect of cometabolism of NAs on survival of *P. promelas* was evaluated in 96-h static/nonrenewal toxicity tests conducted using 200 mL of each sample collected, following USEPA freshwater toxicity testing protocol with (n=30) organisms per exposure (USEPA, 2002). *Pimephales promelas* was cultured at Clemson University's Aquatic Animal Research Laboratory based on USEPA (2002) methods. Test organisms were ≤ 24 h old at the initiation of each experiment. All experiments were conducted in light- and temperature-controlled incubators at 23±2°C with a 16 h light/8 h dark photoperiod. Nano-pure water containing $NaHCO_3$ at a concentration of 1 g/L was used as the unmanipulated control. Statistically significant differences in survival between treatments and controls were determined by analysis of variance with Tukey's post-test using Statistical Analysis System (SAS, Cary, NC). In addition, potential toxicity associated directly with cometabolic substrates was

measured using a static nonrenewal test, with *P. promelas* exposed to each substrate at a concentration of 0.5 g/L. These organisms were not exposed to NAs.

2.5 Effects of cometabolic substrate concentration on NA degradation

The most efficacious substrate in mitigating effluent toxicity, based on the shortest time to eliminate acute toxicity, was selected to determine if cometabolic substrate concentration affects NA removal rates. The same experimental design as used for comparison of cometabolic substrates was used to test 4 concentrations (0.05 g/L, 0.1 g/L, 0.25 g/L, 0.5 g/L) of the same cometabolic substrate. NA concentration and change in toxicity were measured following the methods described in sections 2.2 and 2.4.

3. RESULTS

3.1 Explanatory parameters

Aerobic conditions were maintained throughout the experiment for all measurements and replicates (Table 2.4), with DO ranging from 8.24 to 9.22 mg/L. ORP remained positive (318-501 mV) in all buckets throughout the experiment. For all buckets containing cometabolic substrates, pH increased throughout the experiment (Table 2.4). Alkalinity increased concurrently with pH in all buckets.

3.2 NA concentrations and effects of cometabolic substrates on HPLC analysis

The measured mean initial NA concentration was 48 mg/L, with a range of 41 mg/L to 53 mg/L among all buckets. Cometabolic substrates did not appear to affect initial NA concentrations, as measured initial concentrations between treated buckets and untreated controls were within the method precision (Figure 2.2). Measured NA concentrations for biofermentation product and corn syrup treatments increased with time

during the first 8-10 days (Figure 2.2), which most likely resulted from microbially-associated fatty acids produced in these samples. This is supported by measurement of apparent NA concentrations in samples from buckets containing biofermentation product and corn syrup to which no NAs were added (Figure 2.3). Subtraction of substrate-associated fatty acid concentrations from total concentrations (fatty acids + NAs) in NA degradation samples was not valid, as it indicated toxic NA concentrations in samples eliciting no acute toxicity (Figure 2.4). However, a decrease in apparent NA concentrations for corn syrup and biofermentation product treatments from day 10 to 20 indicate that NA degradation was occurring in these treatments, which was confirmed through toxicity tests (Figures 2.2 and 2.4).

Because hay in water not containing NAs did not result in measurable fatty acids during this testing period (Figure 2.3), NA degradation with hay as a cometabolic substrate could be measured using the HPLC method. With the addition of hay, mean NA concentration decreased from 49 to 9 mg/L, with a zero-order removal rate of 1.8 mg $L^{-1}d^{-1}$, half-life of 13 days, and removal efficiency of 82%. NA degradation rates for corn syrup and biofermentation product could not be determined due to the influence of fatty acids on HPLC analysis. In the untreated control, mean NA concentrations decreased from 47 to 42 mg/L after 20 days, which was within the method detection limits.

3.3 Sorption to solid substrates

No measurable concentration decrease occurred by sorption to hay or biofermentation product after 72 hours, with NA concentrations ranging from 46-54 mg/L for all samples (Figure 2.5). These concentrations are within the precision of the

analytical method, indicating that measurable concentrations of NAs likely did not sorb to solid substrates.

3.4 Toxicity of NA-containing effluent

All three cometabolic substrates resulted in an eventual decrease in toxicity of NAs (Table 2.5, Figure 2.6). Initial water samples elicited 100% mortality to *P. promelas*. Water containing biofermentation product showed a decrease in toxicity after ten days. Organisms showed 100% survival after 12 days of treatment, except for 80% survival in one of three experimental buckets. When corn syrup was used as the cometabolic substrate, samples treated for 14 days resulted in 20-40% survival of test organisms, and 100% survival was observed in all samples from 18 days of treatment. Hay was the least effective substrate in altering acute toxicity, with 50% survival of the test organism after 20 days, likely due to the substrate recalcitrance. The untreated control showed no measurable change in toxicity throughout the experiment. Cometabolic substrates alone did not elicit acute toxicity to *P. promelas*.

3.5 Effects of changing cometabolic substrate concentration on NA degradation

Biofermentation product concentration influenced the removal of commercial NAs. Toxicity results for concentrations of 0.5 g/L and 0.25 g/L differed little, with both concentrations eliminating acute toxicity after 14 days (Table 2.6). 0.1 g/L of substrate was less effective than either 0.25 or 0.5 g/L in mitigating toxicity, as this concentration did not eliminate acute toxicity after 20 days (Figure 2.7). No measurable change in toxicity to the sentinel organism occurred over 20 days of treatment with a cometabolic

substrate concentration of 0.05 g/L, where substrate concentration equaled NA concentration.

4. DISCUSSION

Fluka NAs are structurally simple in comparison to OSPW-derived NAs, with a smaller proportion of molecules containing aromatic rings (Marentette et al., 2015). Because NA structure affects biodegradability (Han et al., 2008), it is likely that energy-derived NA removal rates would differ from commercial NA removal rates. However, with limited availability of energy production-derived NAs, commercial NAs were used as a repeatable surrogate for preliminary study of cometabolism of OSPW NAs. Therefore, toxicity results cannot be applied directly to OSPW NAs, but they serve as evidence to support additional experiments to determine rate and extent of removal of OSPW NAs by cometabolism, as well as the lowest effective substrate concentration for NA degradation.

Actual concentration of NAs in samples containing biofermentation product and corn syrup could not be measured accurately using HPLC analysis due to microbially-associated fatty acids produced during substrate metabolism. Measured NA concentrations from a single time point varied from 84 to 109 mg/L. Although the 96-h LC50 for *P. promelas* with Fluka NAs is 1.9 mg/L (Kinley, 2015), NA concentrations up to 54 mg/L were measured in samples eliciting no acute toxicity (Figures 2.2 and 2.6). Hay added to water alone did not produce measurable amounts of fatty acids (Figure 2.3), indicating that measured NA concentrations from hay samples are likely accurate. Toxicity results supported measured NA concentration decreases in hay-treated water. As

the substrates did not elicit toxicity to *P. promelas,* changes in toxicity can be attributed to NA removal rather than substrate consumption. This also indicates that fatty acids produced during aerobic degradation of NAs and cometabolic substrates do not elicit toxicity to the test organism. In untreated controls, NA concentrations did not change significantly (based on method precision) over 20 days, with no decrease in toxicity to *P. promelas*. This contrasts with cometabolic treatment, where addition of each cometabolic substrate resulted in measurable changes in effluent toxicity (Figure 2.3). This demonstrates that addition of the cometabolic substrates tested can promote NA removal in comparison to unamended water. It also highlights the usefulness of toxicity in verifying NA removal, as absence of acute toxicity would indicate NA concentrations below published LC50 values for that organism.

Results from toxicity bio-assays demonstrate that the biofermentation product concentration affected aqueous NA removal. There was no significant difference in toxicity at any time point between substrate concentrations of 0.5 and 0.25 g/L (α=0.05), suggesting the occurrence of a point of diminishing returns when adding additional cometabolic substrate. Determining the minimum substrate concentration (0.1 to 0.25 g/L in this case) required to remove NAs at acceptable rates can be used to decrease costs associated with cometabolic bioremediation. There was no measurable change in toxicity over 20 days of treatment when substrate concentration equaled NA concentration, which indicates that the biofermentation product concentration must exceed NA concentration (in mg/L) in order to establish a robust microbial community that can effectively degrade NAs.

Although hay was a less effective substrate than biofermentation product at the bench scale, it may be a better substrate for field-scale cometabolic NA treatment. Treatment with hay resulted in a decrease in NA concentrations and acute toxicity, demonstrating that hay is an effective substrate for NA degradation. Hay is less expensive and more widely available than biofermentation product. It is also more recalcitrant than biofermentation product or corn syrup, which could avoid high biological oxygen demand (BOD) and anaerobic conditions caused by addition of biofermentation product or corn syrup.

Results of this experiment demonstrate that substrate addition alters toxicity of NAs by increasing NA removal rates. The inability to measure NA concentration in treated samples (except for hay) highlights the need for appropriate analytical techniques when measuring microbial NA degradation, particularly when NAs are not the sole carbon source in water.

5. CONCLUSIONS

This research demonstrates that cometabolism increases rate of NA degradation when compared to aerobic degradation with NAs as the sole carbon source. Both substrate type and concentration affected removal rates. Cometabolism mitigated acute toxicity to aquatic organisms. This research highlights the value of toxicity testing as an analytical method for assessing NA removal. When quantitative analysis is unreliable for measuring degradation, toxicity testing can indicate whether treated water will adversely affect receiving systems. The overall costs of cometabolic remediation should be

considered for implementation with OSPW NAs, as costs of effective substrate

concentrations at bench-scale when large treatment volumes are involved.

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Table 2.1: Physical and chemical properties of naphthenic acids

Parameter	General Characteristic	Reference
Identification[a]	1338-24-5 (CAS No)	Sigma-Aldrich (2014)
Color[a]	Pale yellow, dark amber	Sigma-Aldrich (2014)
Physical State[a]	Viscous liquid	Sigma-Aldrich (2014)
Molecular weight[b]	140-450 amu	Brient et al. (1995)
Water solubility[a]	88.1 mg/L at pH 7.5	API (2012)
Vapor pressure[a]	1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C	API (2012)
Log K_{OW} [c]	˜4 at pH 1	Schramm et al. (2000)
	˜2.4 at pH 7	Schramm et al. (2000)
	< 0.1 at pH 10	Schramm et al. (2000)
Density[s]	0.92 g/mL	Sigma-Aldrich (2014)
Viscosity[a]	22 mm^2/s	Sigma-Aldrich (2014)
pKa[b]	5 to 6	Brient et al. (1995)

[a]Alkylated cyclopentane carboxylic acids (mixture)

[b]Average molecular weight for refined naphthenic acids

[c]Weathered naphthenic acid mixture; for oil sands process water (OSPW) NAs

Table 2.2: Mass percentages of C, N, and P for tested cometabolic substrates

Substrate	% Carbon	% Nitrogen	% Phosphorus	C:N Ratio
Biofermentation product[a]	40.38	2.52	0.44	16:1
Corn syrup[b]	32	0	0	nd[d]
Hay[c]	30-35	0.4	0.38	90:1

[a] Diamond V (2012)
[b] Corn Refiners Assn (2006)
[c] Philipp and Jennings (2007)
[d] Not determined

Table 2.3: Methods for measuring explanatory parameters.

Parameter	Method	Method Detection Limit
Temperature	Direct instrumentation: YSI Model 52 (APHA 2005)[a]	0.5° C
pH	Direct Instrumentation: Orion Model 420A (APHA 2005)[a]	0.01 SU
Conductivity	Direct Instrumentation: YSI Model 30 (APHA 2005)[b]	0.1 μS/cm
Alkalinity	Standard Methods: 2320B (APHA 2005)[b]	2 mg/L as $CaCO_3$
Hardness	Standard Methods: 2340C (APHA 2005)[b]	2 mg/L as $CaCO_3$
ORP	Standard Methods: 2580 (APHA 2005)[a]	1 mV
DO[c]	Direct Instrumentation: YSI Model 52 (APHA 2005)[a]	0.1 mg/L

[a] Measured in-situ in each bucket at each sample collection, at a depth of 5 cm

[b] Measured in laboratory after sample collection

[c] Dissolved oxygen

Table 2.4: Explanatory parameters measured in experimental vessels for cometabolic substrates and untreated control.

Parameter	Initial conditions[a]	Biofermentation product[b]	Corn syrup[b]	Hay[b]	Untreated control[b]
Temperature (° C)	20.2-20.9	19.3-23.2	19.6-23.1	19.5-22.8	19.1-22.5
pH (SU)	8.31-8.43	8.35-9.19	8.38-9.12	8.37-9.05	8.35-8.47
Alkalinity (mg/L as $CaCO_3$)	760-800	540-820	644-808	536-784	644-720
Hardness (mg/L as $CaCO_3$)	72-90	68-92	82-92	72-104	74-96
DO (mg/L)	8.38-8.75	8.31-9.01	8.24-8.78	8.36-9.02	8.35-9.22
ORP (mV)	381-404	318-454	345-501	352-480	372-420

[a]Range among 12 buckets prior to adding substrates
[b]Range among 3 replicates throughout duration of experiment (10 sample intervals)

Table 2.5: Summary of 96-h acute toxicity tests for comparison of cometabolic substrates. Percent survival is mean of 3 samples, with a total of 30 organisms per exposure.

Treatment duration (days)	Biofermentation product	Corn syrup	Hay	Untreated control
	% Survival, mean			
0	0	0	0	0
2	0	0	0	0
4	0	0	0	0
6	0	0	0	0
8	0	0	0	0
10	47	0	0	0
12	93	0	0	0
14	100	0	30	0
16	100	23	30	0
18	100	30	100	0
20	100	33	100	0

Table 2.6: Summary of 96-h acute toxicity tests for comparison of biofermentation product concentrations. Percent survival is mean of 3 samples, with a total of 30 organisms per exposure.

Treatment duration (days)	Substrate concentrations			
	0.5 g/L	0.25 g/L	0.1 g/L	0.05 g/L
	% Survival, mean			
0	0	0	0	0
2	0	0	0	0
4	0	0	0	0
6	0	0	0	0
8	0	0	0	0
10	53	30	0	0
12	100	80	0	0
14	100	100	0	0
16	100	100	0	0
18	100	100	17	0
20	100	100	40	0

Figure 2.1: Conceptual model for investigating cometabolic remediation of commercial NAs.

Question: which substrate promotes fastest NA removal rate?

Toxicity bio-assays: Which substrate results in the fastest elimination of acute toxicity?

Quantitative analysis: Which substrate results in the fastest NA removal rate?

Test acute toxicity of collected samples at 2-day intervals with 96-h *P. promelas* tests

Measure total NA concentration at 2-d intervals with HPLC derivatization method

Influences on analysis: Sorption and fatty acids

Verify substrates do not elicit toxicity in absence of NAs

Measure fatty acids produced aerobic degradation of substrates

Test sorption to solid substrates under sterile conditions

Sorption to bucket: untreated control

Calculate NA removal rate for each substrate, accounting for influencing factors

Most effective substrate: fastest removal rate or earliest elimination of acute toxicity

Figure 2.2: Measured NA concentrations for cometabolic degradation. Measured initial concentrations were within method precision limits of ±6 mg/L. Increase in NA concentration with time for the first 8-10 days for corn syrup and biofermentation product indicates that aerobic metabolism of these substrates interferes with HPLC NA analysis. Toxicity testing was used instead to compare substrates for NA removal. Trend line drawn through mean value (n=3). Bars represent range of 3 samples at each data point.
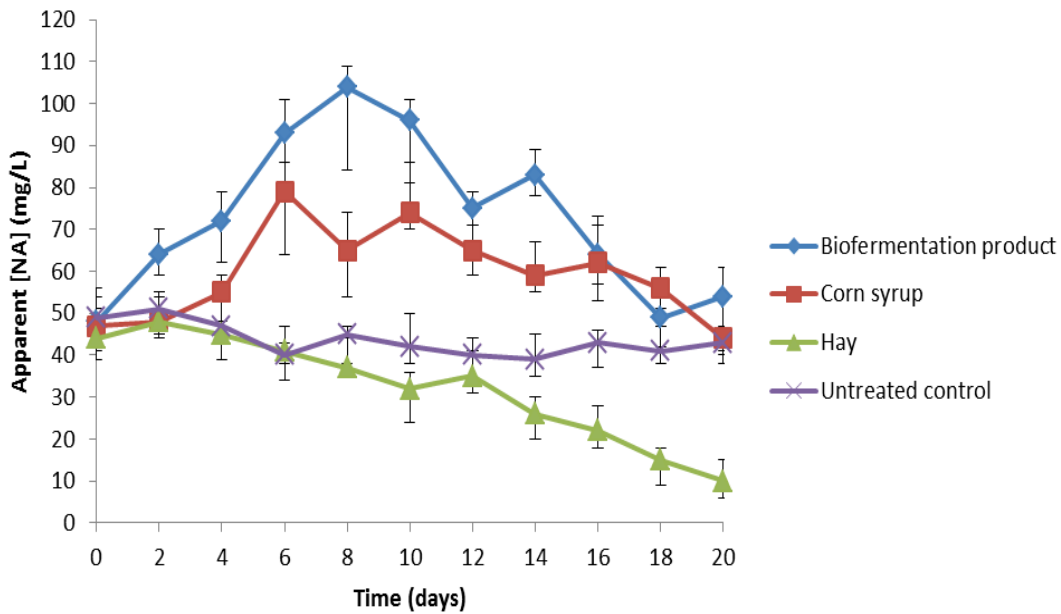
Figure 2.3: Measured NA concentrations when substrates were added to water in absence of NAs. Increases in measured NA concentrations for biofermentation product and corn syrup were attributed to production of fatty acids during aerobic metabolism of these substrates.
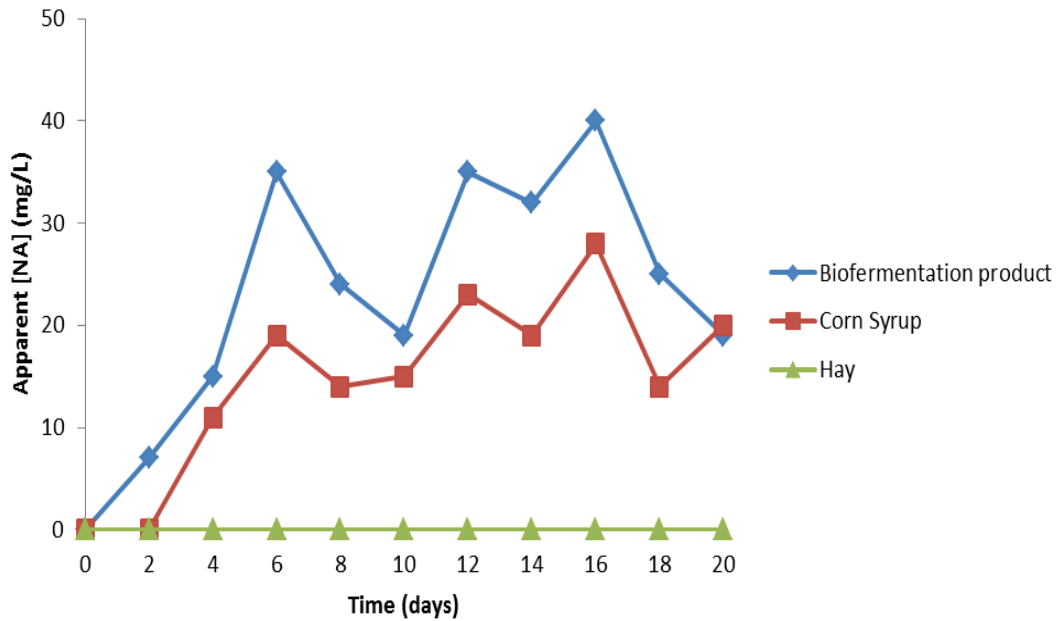
Figure 2.4: Subtraction of measured substrate-associated fatty acids from total measured NA concentrations for cometabolic degradation of NAs. Samples treated with biofermentation product and corn syrup show toxic NA concentrations for samples where acute toxicity was eliminated (day 12 for biofermentation product, and day 18 for corn syrup). NA concentrations in hay treatments were supported by toxicity testing.
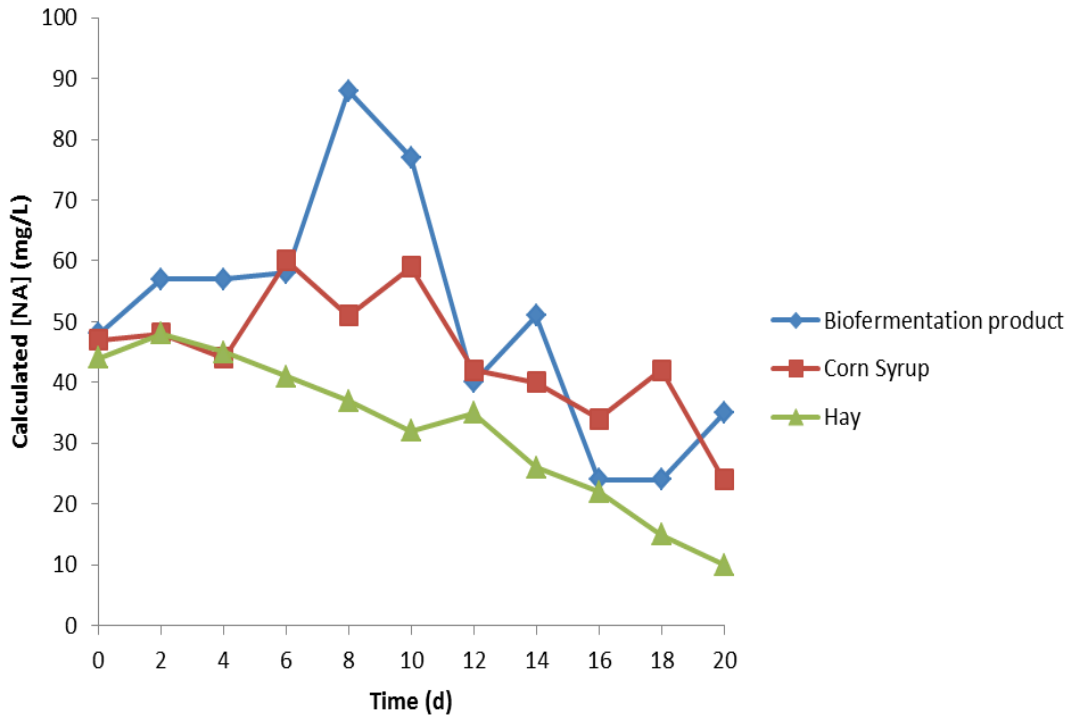
Figure 2.5: Results of sterile sorption tests for solid substrates. Concentration changes remaining within method precision limits (±6 mg/L).
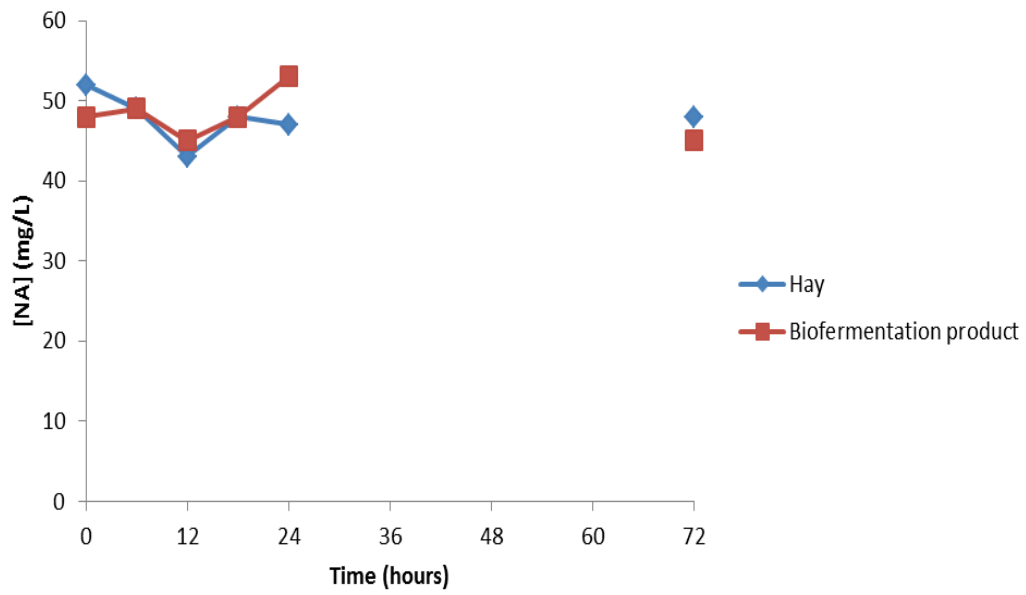
Figure 2.6: Changes in acute toxicity to *P. promelas* due to cometabolism of NAs, with biofermentation product showing the earliest measured response in toxicity. No measurable change in toxicity occurred in untreated controls. Trend line drawn through mean value at each data point. Bars represent range of survival in treatments (n=10 organisms, three replicates per treatment).
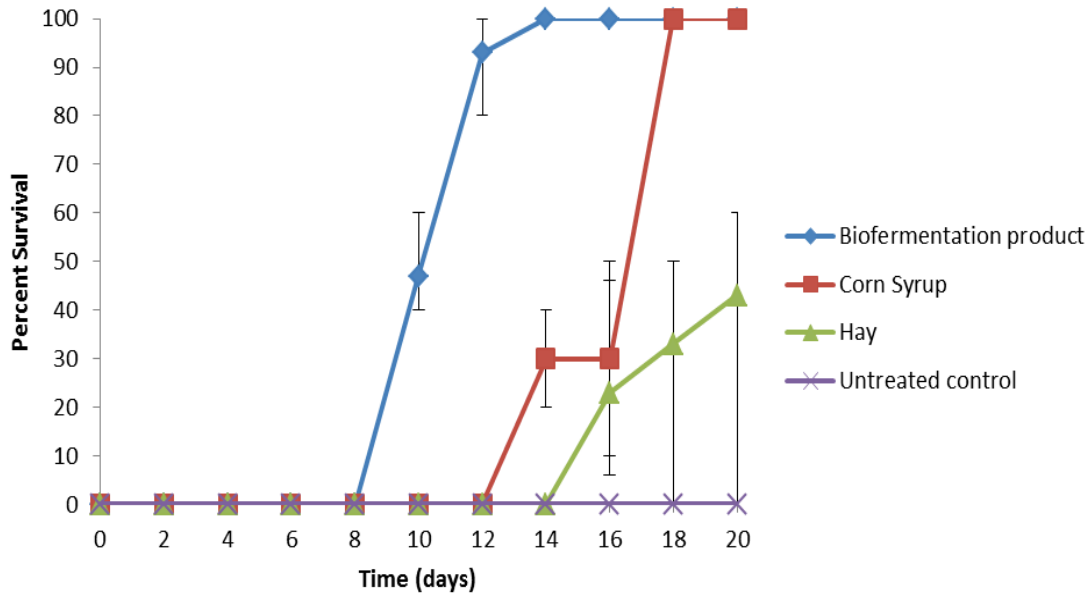
Figure 2.7: Efficacy of different concentrations of biofermentation product for mitigating acute toxicity to *P. promelas*. Bars represent range of survival in replicates (n=10 organisms, three replicates per treatment).
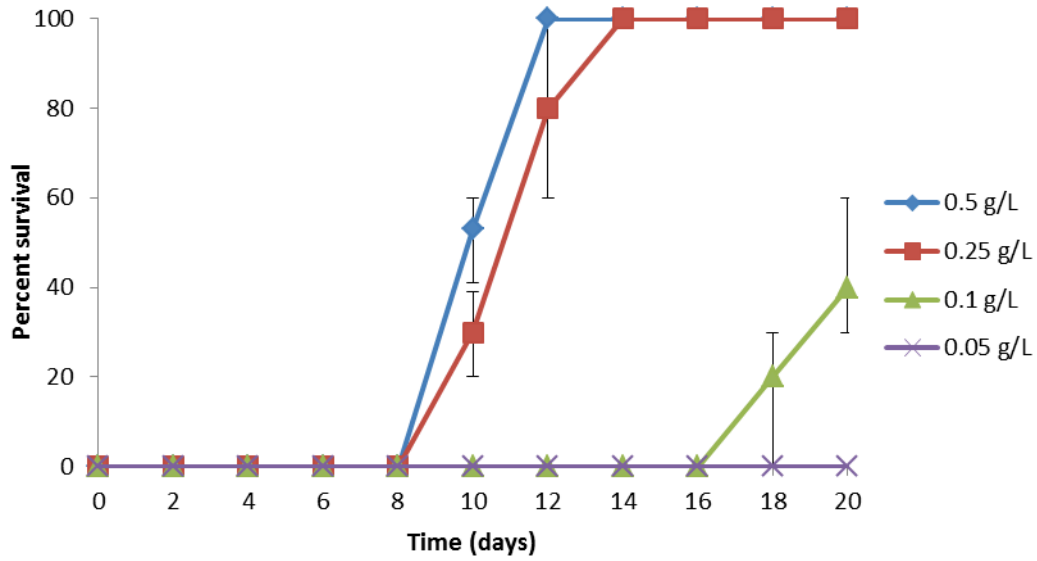
CHAPTER THREE

MYCOREMEDIATION OF COMMERCIAL (FLUKA) NAPHTHENIC ACIDS USING

A RECIPROCATING REACTOR

ABSTRACT

Mycoremediation was evaluated as a potential process for mitigating risk due to naphthenic acids (NAs) in petroleum-derived effluents. Reciprocating reactors with wood chips inoculated with white-rot fungus, *Pleurotus pulmonaris,* were compared to reactors with uninoculated wood chips to determine the effects of this white-rot fungi on commercial (Fluka) NA removal. In reactors inoculated with white-rot fungi, NA concentrations decreased from a mean initial concentration of 51 mg/L to non-detectable concentrations after 2 days of treatment, with a half-life of 32 hours and zero-order removal rate constant of 0.78 days^{-1}. NA removal rates by white-rot fungi equal or exceed rates by other aerobic processes such as aquatic microbe metabolism. Analytical results were confirmed with acute toxicity tests using a sensitive fish (*Pimephales promelas)*, with no measurable toxicity after 2 days of treatment. Complete mortality was observed for a control containing sterile wood chips, demonstrating effectiveness of reciprocating reactors in decreasing aqueous NA concentrations. Inoculated reactors were effective in decreasing NA concentrations, with rates exceeding those for uninoculated reactors, indicating potential for this technology to mitigate risks associated with NAs.

1. INTRODUCTION

Naphthenic acids (NAs) are a concern for many petroleum producers, including those processing oil sands deposits. NAs are a complex group of cyclic and aliphatic carboxylic acids with the general formula $C_nH_{2n+z}O_2$, where n is the carbon number, between 8 and 30, and Z is zero or a negative even integer describing the hydrogen deficiency (Headley and McMartin, 2004) (Table 3.1). NAs occur naturally in petroleum as a degradation product of crude oil (Allen, 2008). NAs are non-volatile and behave as surfactants (API, 2012), causing corrosion to refinery pipelines and equipment, and are acutely toxic to aquatic organisms when dissolved in oil sands process water (OSPW) (Schramm et al., 2000). Due to the toxicity and challenges in reuse of NA-contaminated water, petroleum producers are in need of a treatment pathway to remove NAs from process water. However, the structural composition and characteristics of NAs may complicate traditional remediation techniques. NAs are weakly biodegradable by aerobic and anaerobic microorganisms (Del Rio et al., 2006). However, this is a relatively slow process, with half-lives ranging from months to decades (Del Rio et al., 2006; Whitby, 2010). The limited biodegradability of NAs is due predominantly to molecular size and structure of the compound (Han et al., 2008; Holowenko et al., 2002; Scott et al., 2005). In particular, NAs with increased cyclicity and alkyl branching are most resistant to microbial degradation. Published OSPW-associated NA degradation rates are too slow to be considered in a bioremediation strategy for OSPW. Alternative treatment processes that may augment traditional biodegradation pathways need to be investigated for feasibility in mitigating ecological risks associated with NA-contaminated effluents.

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Mycoremediation is a biotransformation process using fungal metabolism. White-rot fungi in particular show promise in the transformation of persistent organic molecules (Bhattacharya et al., 2014; Moreira et al., 2003; Okparanma et al., 2011; Pointing, 2001). White-rot fungi are a non-taxonomic group of fungi defined by their unique ability to degrade lignin. Lignin is a complex cross-linked polymer that is resistant to degradation by nearly all organisms except white-rot fungi (Singh, 2006). These fungi utilize extracellular enzymes (e.g. manganese peroxidase, laccase, and lignin peroxidase) along with endogenously produced H_2O_2 to access wood polysaccharides locked within lignin complexes (Pointing, 2001). When coupled with endogenously produced H_2O_2, these enzymes are powerful oxidizers that carry out reactions that break carbon bonds in lignin (Pointing, 2001; Singh, 2006). These enzymes are nonspecific to any structure due to the complex nature of lignin, which gives white-rot fungi the ability to degrade organic compounds recalcitrant to microbial degradation (Migliori et al., 2012, Moreira et al., 2003, Pointing, 2001). These properties indicate a potential degradation mechanism for NAs.

Commercially available NAs (Fluka) were used to assess feasibility of mycoremediation for degradation of NAs. Although commercially available NAs are less structurally complex than energy-derived NAs, this NA preparation has been used previously for degradation and toxicity studies (Clemente et al., 2004; Melvin et al., 2013, Scott et al., 2005; Swigert et al., 2015). Commercially available NAs provide a reproducible and repeatable source of NAs for assessment of NA mycoremediation and

can be used as justification for further investigation of the degradation mechanism with more compositionally complex OSPW NAs.

To measure change in NA exposures, and mitigation of aquatic risks, toxicity testing was used in this experiment to verify changes in exposure of NAs by mycoremediation. Fathead minnow (*Pimephales promelas*) is sensitive to NA exposures, with 96-h LC_{50} to juvenile *P. promelas* of 5.6 mg/L (Swigert et al., 2015), and 7-d LC_{50} for larval *P. promelas* of 1.9 mg/L (Kinley, 2015). The change of toxicity to sentinel species can be used to support measured NA concentrations in determining the mitigation of risk to aquatic organisms.

The purpose of this research is to determine feasibility of white-rot fungal degradation with *Pleurotus pulmonaris* as a remediation strategy for NAs. A bioreactor to support white-rot fungal degradation was designed. The specific objectives of this study were to: 1) determine the rate and extent of removal of commercial (Fluka) NAs by white-rot fungal (*Pleurotus pulmonaris*) treatment, and 2) measure changes in toxicity following white-rot fungal treatments in terms of mortality with sentinel fish (*P. promelas*) in 96-hr static tests.

2. METHODS

2.1 Experimental Design

Experiments were conducted in a climate-controlled greenhouse at Clemson University (Clemson, SC). A bioreactor was built in triplicate to test the degradation of NAs by *P. pulmonaris* (Figures 3.1 and 3.2). Because white-rot lignin degradation is an obligately aerobic process (Pointing, 2001), a reciprocating reactor design was used. A

reciprocating reactor allows for periodic relief from submersion, which is crucial to survival of this non-aquatic fungi and maintenance of aerobic conditions in the reactor. A 37-L polyethylene bin was used for the reactor. 10 cm of gravel was added to the bottom of the reactor to isolate the substrate from any water remaining after reciprocation, and 30 L of inoculated mixed hardwood chips ranging from 1-5 cm were placed carefully in each reactor to minimize disturbance to the mycelia. The reciprocating design was accomplished by using FMI flow-metered pumps to remove water from the reactor every 6 h. Pumps were set at a rate of 500 ml/min, which drained the reactor in 30 minutes. An 18.9 L polyethylene bucket was used as a retention basin for each reactor. A float switch in the retention basin was used to trigger a FMI pump when the basin was full, pumping the water back into the reactor for another 6-h contact time. Reactors were exposed to sixteen 6-h contact times throughout the experiment.

Mixed hardwood chips (oak and poplar) (King Lumber, Liberty, SC) were used as the fungal substrate in the reactor. Wood chips were autoclaved before being inoculated with *Pleurotus pulmonaris* (Phoenix oyster mushroom) spawn obtained from Mushroom Mountain in Easley, SC. This species was chosen for its rapid substrate colonization and aggressive ligninolytic activity on a variety of woods (Cotter 2014). The inoculated wood chips were placed in sterilized polyethylene bags until the wood chips were fully colonized and secondary metabolites began to accumulate. These metabolites indicate the presence of desired ligninolytic enzymes (Moreia et al., 2003).

An uninoculated reactor was built in triplicate as a control for endemic microbial degradation and sorption occurring concurrently with *P. pulmonaris* degradation. The

uninoculated reactors used the same design as inoculated reactors, except the wood chips were neither sterilized nor inoculated with *P. pulmonaris*. Wood chips were soaked in water overnight before uninoculated reactor construction. At the end of the experiment, inoculated reactors were deconstructed to verify survival of mycelium through the experiment.

2.2 Water formulation

Fluka NAs (CAS 1338-24-5) (Sigma Aldrich, St. Louis, MO), a mixture of alkylated cyclopentane carboxylic acids, were added to municipal tap water to achieve a nominal concentration of 50 mg/L. Sodium bicarbonate (Certified ACS grade, Sigma Aldrich) was added to achieve a nominal concentration of 1 g/L to serve as a buffer to maintain pH above 7, ensuring NAs remained in the aqueous dissolved phase. 16 L of NA-containing water was added to each reactor.

2.3 Explanatory parameters

Alkalinity, conductivity, dissolved oxygen concentration (DO), hardness and reduction/oxidation potential (ORP) were measured as explanatory parameters to verify conditions needed for aerobic degradation (dissolved oxygen >2 mg/L, ORP > 100 mV) and NA solubility (Table 3.2). Explanatory parameters were measured at the beginning of the experiment and concurrently with each sample collection.

2.4 Sorption

Sorption of NAs to wood chip substrate was quantified to control for sorption to reactor substrates. 500 mL of autoclaved wood chips were added to a 1-L borosilicate glass jar in triplicate. Jars were filled with UV-sterilized Nano-pure water containing 1

g/L sodium bicarbonate and 50 mg/L commercial NAs. Water samples for quantitative NA analysis were collected from jars at 12-h intervals for 4 days using a sterile syringe. Change in NA concentration over time was measured to determine the magnitude of NA removal by sorption.

2.5 NA measurement and removal rate calculations

300 mL samples were collected in polyethylene bottles from each reactor at 12-hour intervals for 4 days for quantitative NA analysis and toxicity testing. Samples were stored at 4°C until analysis. NA analysis was performed using a Dionex UltiMate 3000 HPLC system, following a derivatization method (Yen et al., 2004) modified from an HPLC method for measuring short and long chain fatty acids as 2-nitrophenylhydrazides (Miwa et al., 1985). This method requires derivatization of fatty acids as 2-nitrophenylhydrazide HCl in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl, as fatty acids such as NAs do not show useful absorption in the visible or UV spectrums (Miwa et al., 1985). The detection limit for this method is 5 mg/L (Yen et al., 2004), with a measured precision of ±6 mg/L. A standard curve of commercial NAs from 0 to 80 mg/L was used to determine concentration from peak area. Analytical results were used to determine rate, extent, and efficiency of removal of NAs. Extent of removal is the final concentration of NAs post-treatment. Removal rate coefficients were calculated by zero-order kinetics:

$$k = -(C_t/t) + C_0 \hspace{4cm} \text{Equation 1}$$

where t is time of measurement (hours), k is the zero-order rate constant $(hours^{-1})$, C_0 is NA concentration at t=0 (mg/L), and C_t is NA concentration at time=t (Fetter, 1999).

2.6 Toxicity Testing and Statistical Analysis

Inoculated and uninoculated reactors were assessed for their ability to alter toxicity of commercial NAs to a sensitive sentinel species, *Pimephales promelas* (fathead minnow). *P. promelas* is sensitive to commercial NAs, with LC50 values ranging from 1.9-5.6 mg/L (Swigert et al., 2015; Kinley, 2015). The effects of biodegradation of NAs on survival of *P. promelas* was evaluated in 96-h static/non-renewal toxicity tests following a USEPA freshwater toxicity testing protocol with (n=30) organisms per exposure (USEPA, 2002). *P. promelas* was cultured at Clemson University's Aquatic Animal Research Laboratory. Test organisms were ≤ 24 h old at the initiation of each experiment. All experiments were conducted in light- and temperature-controlled incubators at 23±2°C with a 16 h light/8 h dark photoperiod. Nano-pure water containing 1 g/L $NaHCO_3$ and hardwood chip leachate was used as a control. Normally distributed, homogeneous data were analyzed by one-way analysis of variance (ANOVA). Differences among treatments were identified by follow-up pairwise comparisons and contrasts using linear models. Differences were considered significant at $p \leq 0.05$ (JMP v11; SAS Institute Inc., Cary, NC, USA).

3. RESULTS

3.1 Explanatory Parameters

Values for explanatory parameters were similar between inoculated and uninoculated reactors (Table 3.3). ORP remained above +275 mV for all measurements in both inoculated and uninoculated reactors (Figure 3.3), demonstrating the ability of the

reciprocating reactor to maintain aerobic conditions. ORP was not measured in sterile sorption controls. This was supported by DO remaining at or above 7.81 mg/L at all time points for all replicates. Alkalinity and pH decreased with time in all reactors, with greater decreases measured in inoculated than in uninoculated reactors (Table 3.4). As the only difference between inoculated and uninoculated reactors was inoculation with *P. pulmonaris,* this difference suggests that the fungi increased acidity of the water. This is likely a result of acidification caused by white-rot fungal enzymes (Singh, 2006).

3.2 Sorption

In the sterile sorption control, NA concentration decreased from 49 mg/L to 43 mg/L (Figure 3.4). This concentration change is within the precision of the analytical method, indicating that sorption to wood chips did not contribute to NA removal. NA concentrations between reactors and the sterile sorption control were significantly different for all time points after initiation of the experiment.

3.3 NA measurement and removal rate calculations

Initial NA concentrations ranged from 46-54 mg/L. All inoculated reactors decreased NA concentration to non-detect by 2.5 days, for a removal efficiency >90% based on method detection limits of 5 mg/L (Figure 3.4). Uninoculated reactors removed NAs to non-detectable concentrations by 3.5 days. Significant differences in NA concentrations between inoculated and uninoculated reactors were observed from 12 to 72 hours (p<0.0001). Reaction rates were determined using data from time=0 through the first time point for which NA concentrations were non-detectable. Zero-order (linear) kinetics were the best fit, with r^2 values of 0.948 and 0.995 using mean values (n=3) for

inoculated and uninoculated reactors, respectively (Table 3.5). Observed half-lives for NAs were 32 hours for inoculated reactors and 39 hours for uninoculated reactors. Although the overall removal rate (from t=0 until NAs were non-detectable) for inoculated reactors (0.78 d^{-1}) was greater than for uninoculated reactors (0.65 d^{-1}), NA removal rates for both reactors were approximately equal between 12 and 60 hours following a lag time (time required for removal to begin) of 12 hours (Figure 3.4). This indicates that inoculated reactors may decrease lag time for NA removal to begin in comparison to uninoculated reactors, but removal rates were similar after the lag time.

3.4 Toxicity testing

Acute toxicity testing using fish confirmed NA removal within treatments. At test initiation, all samples elicited complete mortality to *P. promelas,* indicating that undegraded Fluka NAs are toxic to test organisms. After 60 hours of treatment in inoculated reactors, toxicity was eliminated, whereas 100% survival was not observed for uninoculated reactors until 72 hours of treatment (Table 3.6, Figure 3.5). Toxicity of water treated by inoculated and uninoculated reactors was significantly different at 48 and 60 hours, with p<0.0001. No measurable change in acute toxicity was observed for the sterile sorption control.

4. DISCUSSION

This bench-scale study demonstrates potential for mycoremediation as a removal pathway for recalcitrant organic constituents. Naphthenic acid half-lives of 25 hours for inoculated reactors are less than or equal to half-lives of 1 to 8 days for microbial degradation of Fluka NAs (Han et al. 2008), indicating that aerobic degradation by *P.*

pulmonaris is a relatively fast NA removal pathway. Toxicity testing showed that inoculated reactors were able to degrade NAs to non-toxic concentrations without production of toxic byproducts. However, further testing is required to determine removal rates and extents for constituents not tested in these experiments. Fluka NAs are structurally simple in comparison to OSPW-derived NAs, with a smaller proportion of molecules containing aromatic rings (Marentette et al., 2015). As NA structure affects biodegradability (Han et al., 2008; Scott et al., 2005), it is likely that OSPW NA removal rate and extent would be different from results observed here. Because availability of OSPW derived NAs is limited, commercial NAs were used as a surrogate for preliminary investigation of the ability of *P. pulmonaris* to degrade NA compounds.

Results indicate that toxicity testing is useful for monitoring NA degradation. Whole-effluent toxicity testing with sensitive sentinel species is a useful indicator of potential effects to receiving aquatic systems, and can verify quantitative analytical results when toxicological endpoints are known. Because the selected test organisms are sensitive to a wide range of toxic substances, toxicity bioassays can additionally reveal the presence of toxic substances or degradation byproducts that are either unknown or not quantified by analysis. The lack of acute toxicity in samples in which NA concentration was below the detection limit of 5 mg/L adds a metric to indicate NA removal. This demonstrates that no acutely toxic metabolites/byproducts were produced during degradation. The robustness and flexibility of *P. pulmonaris* allow it to be utilized in a variety of remediation scenarios in which mycoremediation is a potential treatment process.

Performance of *P. pulmonaris* in these experiments indicates that it is a suitable species for mycoremediation. Mycelial coverage of wood chips did not decrease noticeably after reactors were used for treatment, demonstrating that *P. pulmonaris* tolerates submersion without negative effects on mycelial survival. *P. pulmonaris* is known for fast growth and aggressive ligninolytic activity (Cotter, 2014), which is the driving force of white-rot mycoremediation. *P. pulmonaris* is one of only a few white-rot fungi that readily consumes both hardwood and softwood, widespread in temperate forests throughout the world, and is able to survive in a wide range of conditions (UTK, 2015). Non-detectable NA concentrations were measured after 2.5 days of treatment with inoculated reactors, while uninoculated reactors required 3.5 days of treatment to reach this endpoint. As the only difference between the reactors was the inoculated presence of *P. pulmonaris,* this demonstrates the effectiveness of this species in increasing commercial NA removal rates.

NA concentration is a descriptive parameter that encompasses potentially thousands of different compounds. Because molecular structure influences biodegradation rates (Han et al., 2008), bulk composition of Fluka NAs will change with time as more degradable NAs are preferentially removed. Application of rate laws is dependent on the data points used to calculate rates. In this study, coefficients were calculated to describe change in concentration with time to an endpoint defined as NA analysis method detection limits. Using zero-order kinetics, observed half-lives for NAs were shorter for inoculated reactors than for uninoculated reactors. With a different endpoint, analytical method, or NA mixture, rate coefficients may diverge. Therefore,

specific half-lives and reaction rates should be applied to other situations and NA mixtures with caution, and application of rate laws to NAs should be considered situationally. Calculating rate coefficients offers valuable information for scaling up design of bench-scale remediation systems. Carefully applied kinetics that consider properties of the NA mixture and an environmentally relevant endpoint can be used to optimize contact time in NA bioremediation system design.

5. CONCLUSIONS

This experiment demonstrates that a white-rot fungus, *P. pulmonari,s* in a reciprocating reactor increases removal rate and decreases toxicity of commercial NAs. The unique metabolic processes of *P. pulmonaris* may increase viability of bioremediation for treatment of otherwise recalcitrant organic compounds. Additionally, it highlights the potential of *P. pulmonaris* for use in bioremediation, as it was shown to tolerate repeated submersion. This research demonstrates the value of toxicity testing for assessing NA removal. Although removal rates calculated in this investigation cannot be applied directly to OSPW NAs, they indicate potential utility of *P. pulmonaris* to mitigate ecological risks associated with NAs in OSPW. Further investigation would determine if reciprocating reactors with or without *P. pulmonaris* inoculation are effective in removing OSPW NAs at rates exceeding those observed for aerobic degradation by aquatic microorganisms.

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Table 3.1: Physical and chemical characteristics of Fluka naphthenic acid (Sigma-Aldrich)

Parameter	General Characteristic	Reference
Identification[a]	1338-24-5 (CAS No)	Sigma-Aldrich (2014)
Color[a]	Pale yellow, dark amber	Sigma-Aldrich (2014)
Physical state[a]	Viscous liquid	Sigma-Aldrich (2014)
Molecular weight[b]	140-450 amu	Brient et al. (1995)
Water solubility[a]	88.1 mg/L at pH 7.5	API (2012)
Vapor pressure[a]	1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C	API (2012)
Log K_{OW} [c]	˜4 at pH 1	Schramm (2000)
	˜2.4 at pH 7	Schramm (2000)
	< 0.1 at pH 10	Schramm (2000)
Density[a]	0.92 g/mL	Sigma-Aldrich (2014)
Viscosity[a]	22 mm^2/s	Sigma-Aldrich (2014)
pKa[c]	5 to 6	Brient et al. (1995)

[a]Alkylated cyclopentane carboxylic acids (mixture)

[b]Average molecular weight for refined naphthenic acids

[c]Weathered naphthenic acid mixture; for oil sands process water (OSPW) NAs

Table 3.2: Explanatory parameter measurement methods.

Parameter	Method	Method Detection Limit
Temperature	Direct instrumentation: YSI Model 52 (APHA, 2005)[a]	0.5° C
pH	Direct Instrumentation: Orion Model 420A (APHA, 2005)[a]	0.01 SU
Conductivity	Direct Instrumentation: YSI Model 30 (APHA, 2005)[b]	0.1 µS/cm
Alkalinity	Standard Methods: 2320B (APHA, 2005)[b]	2 mg/L as $CaCO_3$
Hardness	Standard Methods: 2340C (APHA, 2005)[b]	2 mg/L as $CaCO_3$
Dissolved oxygen	Direct Instrumentation: YSI Model 52 (APHA, 2005)[a]	0.1 mg/L
ORP	Standard Methods: 2580 (APHA, 2005)[a]	1 mV
NA quantitative analysis	HPLC derivatization (Yen et al., 2004)	5 mg/L
Toxicity	USEPA freshwater toxicity testing protocol (USEPA, 2002)	Not applicable

[a]Measured in-situ at each sample collection

[b]Measured in laboratory after sample collection

Table 3.3: Explanatory parameters, initial conditions and range (n=3) throughout experiment.

Parameter	Initial condition[a]	Inoculated reactors, range[b]	Uninoculated reactors, range[b]
pH (SU)	8.30-8.42	7.35-8.36	7.42-8.44
Alkalinity (mg/L as $CaCO_3$)	910-950	210-940	240-925
Hardness (mg/L as $CaCO_3$)	72-84	60-82	64-78
Conductivity (μS/cm)	920-965	445-1020	486-980
Dissolved oxygen (mg/L)	8.04-8.38	7.65-8.48	7.81-8.27
Temperature (°C)	18.4-19.2	16.5-20.2	16.6-20.2
ORP (mV)	284-305	277-317	302-323

[a]Range of measurements from all replicates (inoculated and uninoculated reactors) at time=0, before water contacted reactors

[b]Range of values for all replicates over 8 sample collection periods (12 to 96 hours of treatment)

Table 3.4: Comparison of mean (and range; n=3) for initial and final pH and alkalinity for inoculated and uninoculated reactors.

Parameter	Inoculated reactors		Uninoculated reactors	
	Initial (range)	Final (range)	Initial (range)	Final (range)
pH (SU)	8.41 (8.37-8.45)	7.42 (7.38-7.46)	8.42 (8.39-8.46)	7.52 (7.45-7.57)
Alkalinity (mg/L as $CaCO_3$)	930 (860-980)	310 (270-340)	925 (880-940)	501 (470-530)

Table 3.5: Mean removal rate coefficients and extents for inoculated reactors, uninoculated reactors, and sterile sorption control.

Parameter	Treatment		
	Inoculated reactors	Uninoculated reactors	Sterile sorption control
Mean initial [NA], mg/L (range[a])	51 (46-53)	50 (47-54)	49 (47-51)
Removal extent, mg/L	BDL[b]	BDL[b]	43 (41-44)
Removal efficiency, %	>90	>90	12
Rate equation	$C = -0.78t + 45$	$C = -0.65t + 50$	nd[c]
R^2	0.9479	0.9954	nd[c]
Rate coefficient (h^{-1})	0.78	0.65	nd[c]
$t_{0.5}$ (h)	32	39	nd[c]

[a]n=3

[b]Below method detection limit (5 mg/L)

[c]Not determined due to concentration change less than 3 half-lives

Table 3.6: Summary of mean (n=3) NA concentrations and 96-h survival for *P. promelas* for inoculated reactors, uninoculated reactors, and sterile sorption control.

Treatment duration (hours)	Inoculated reactors		Uninoculated reactors		Sorption test	
	[NA] (mg/L)	% survival	[NA] (mg/L)	% survival	[NA] (mean, mg/L)	% survival (mean)
0	51	0	50	0	49	0
12	32	0	43	0	49	-
24	23	0	34	0	46	0
36	15	0	26	0	48	-
48	8	46.7	18	0	45	-
60	2	100	11	3.3	42	-
72	BDL[a]	100	4	100	43	0
84	BDL[a]	100	BDL[a]	100	42	-
96	BDL[a]	100	BDL[a]	100	43	-

[a]Below method detection limit (5 mg/L)

Toxicity tests for sorption test waters only performed for t=0, 24, 72 hours

Figure 3.1: Schematic of reciprocating reactor design. Water is contained in reactor for 6-h contact time. Reactor is drained to the retention basin over a 30-min period. When retention basin is full, a float switch triggers the pump to refill the reactor. Uninoculated reactors use identical construction, but wood chips were neither sterilized nor inoculated with *P. pulmonaris*.
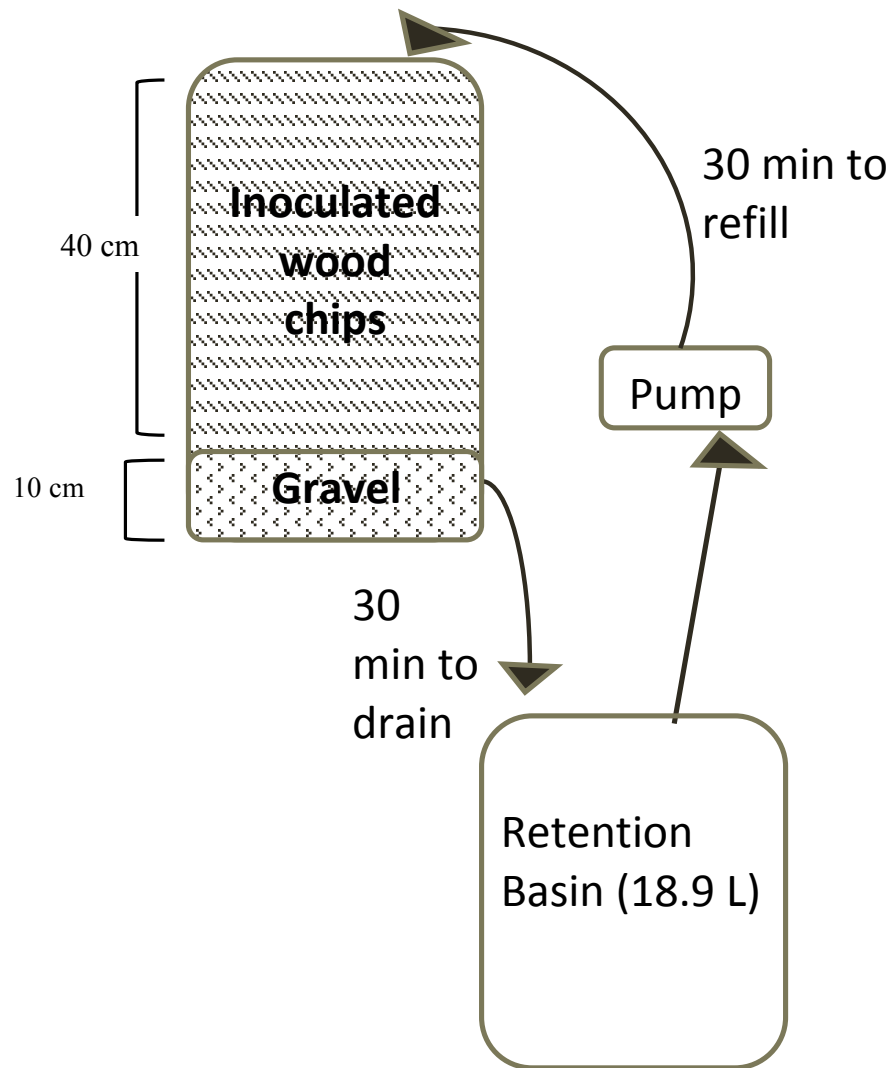
Figure 3.2: Reciprocating reactors. Large bins at top of photo are reactors. Buckets serve as retention basins. Pumps are timed to start draining reactors after 6-h contact time. When reactors are fully drained, float switches in retention basins trigger pumps to refill reactors through tubing for another 6-h contact time.
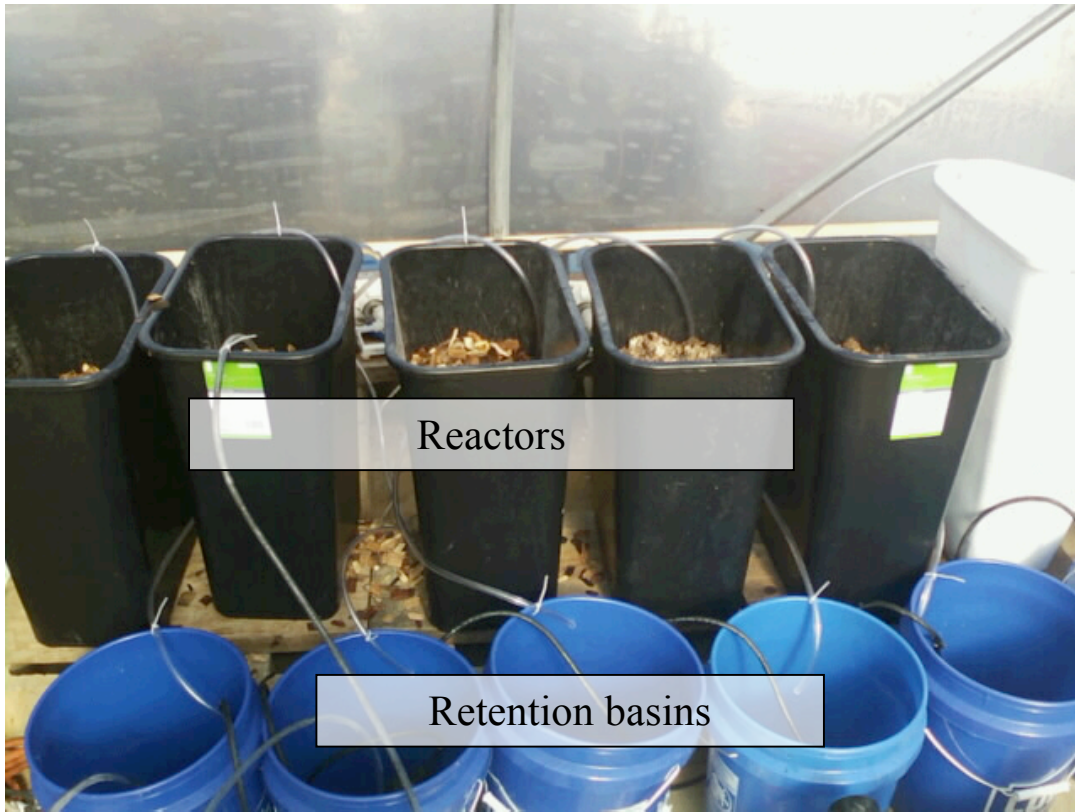
Figure 3.3: Decreasing ORP throughout a reactor cycle, with positive (oxidizing) conditions maintained. Each point represents a mean of three replicates.
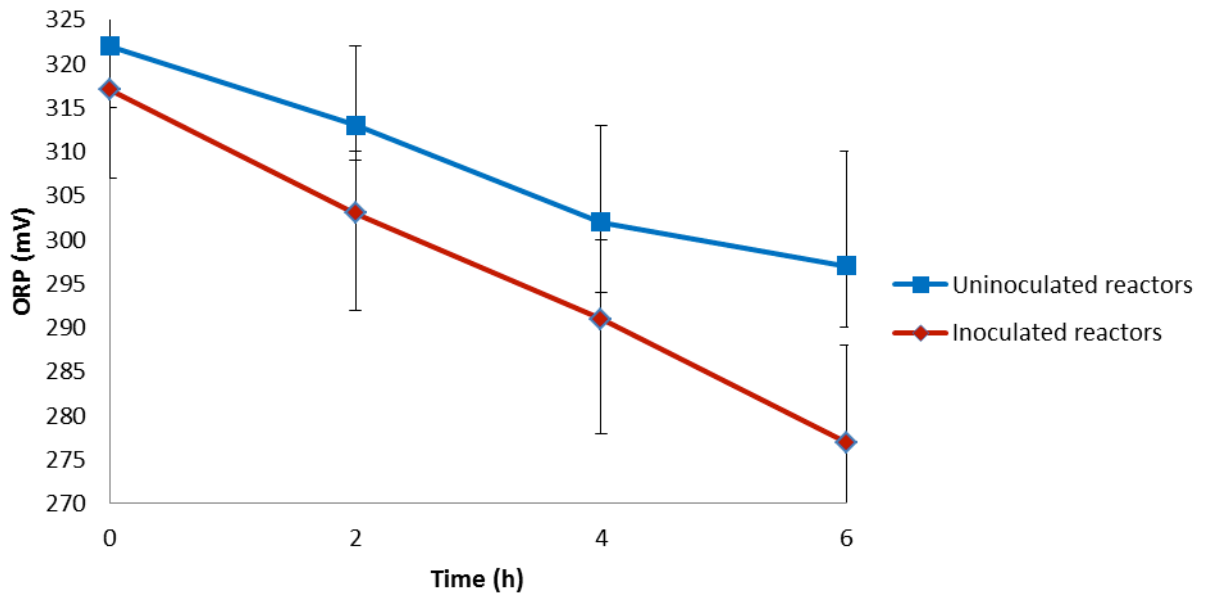
Figure 3.4: Change in NA concentration for inoculated reactors, uninoculated reactors, and sterile sorption control. Trend lines are for mean (n=3) value. Bars represent minimum and maximum values. Inoculated reactors showed the fastest overall removal rate for NAs. No removal was attributed to sorption.
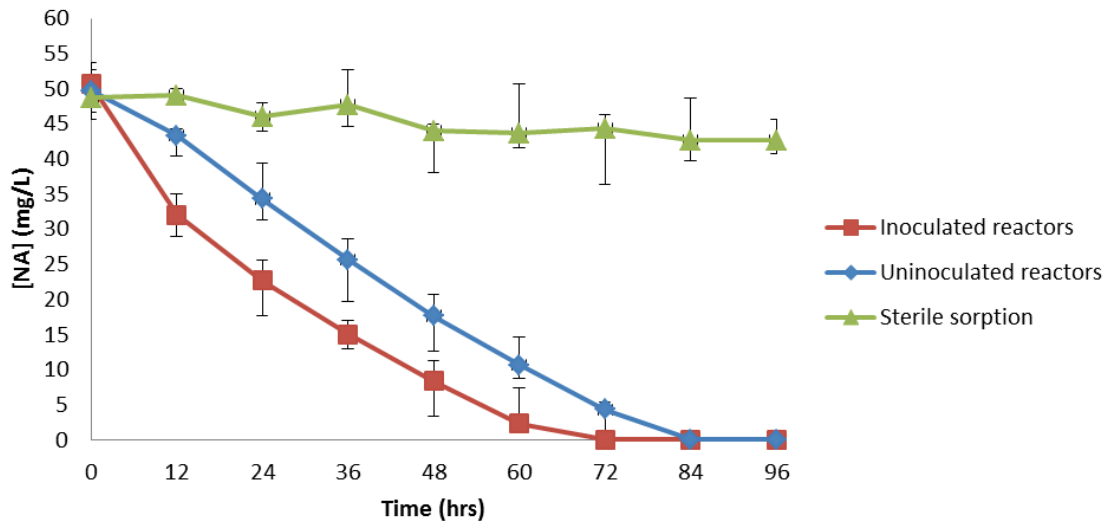
Figure 3.5: Change in acute toxicity to *P. promelas* for inoculated and uninoculated reactors. Inoculated reactors eliminated toxicity in all samples after 60 hours of treatment. Trend lines are for mean (n=3) values at each data point. Bars represent minimum and maximum values.

CHAPTER FOUR

SUMMARY AND CONCLUSION

NAs are a primary constituent of concern in OSPW that may elicit toxicity to aquatic organisms in receiving systems (Whitby, 2010). Aerobic biodegradation is a potential treatment pathway for NAs, but it can be a slow process. The objective of this research was to investigate methods to increase the rate of aerobic NA degradation. The major objectives were:

1. Determine the effects of cometabolic substrates on aerobic NA degradation.

2. Determine the rate and extent of removal of commercial NAs by a reciprocating mycoreactor.

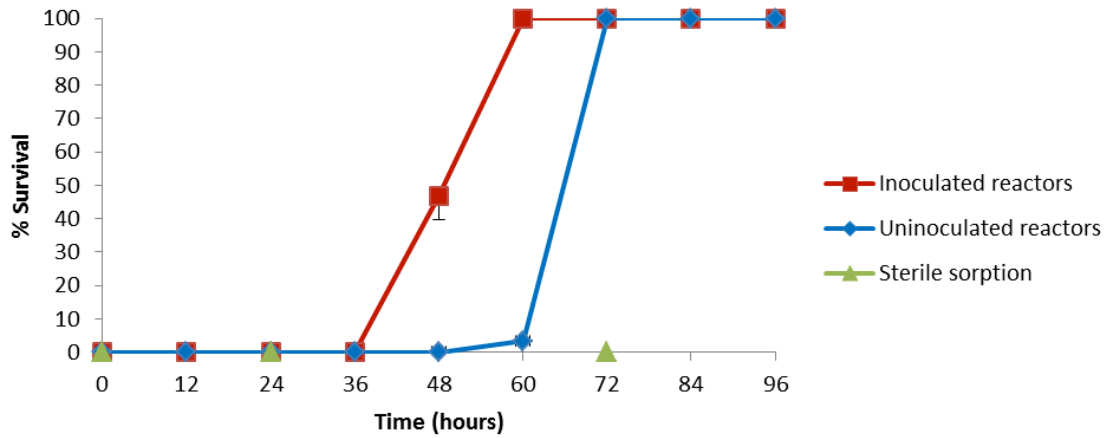**1. Determine the effects of cometabolic substrates on aerobic NA degradation.**

The purpose of this study was to determine the effects of three cometabolic substrates on NA degradation. The specific objectives were to 1) measure change in acute toxicity due to NA cometabolism, and 2) determine the effects of different concentrations of cometabolic substrate on NA removal. Of the three tested substrates (biofermentation product, corn syrup, and hay), biofermentation product was demonstrated to alter toxicity in the least amount of time, with test organisms showing no acute response after 12 days of treatment. The results of this study indicate that the presence and type of organic matter can influence NA degradation, which may allow for greater degradation rates than would be seen when NAs are the sole carbon source. This has important implications to a CWTS, where detritus may function similarly to hay as a cometabolic substrate,

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promoting NA degradation. It was also demonstrated that substrate concentration influences rate of NA cometabolism. A biofermentation product:NA ratio of 10:1 was not significantly more effective than a ratio of 5:1, indicating that there is a point of diminishing returns when adding additional biofermentation product.

Cometabolism experiments revealed an advantage of toxicity testing for evaluating NA degradation. In samples where organic matter influenced measured NA concentrations, toxicity testing was used to confirm NA removal. Toxicity testing should be integrated into NA degradation monitoring. Both the operational definition of NAs and the analytical method used can influence measured NA concentrations in a sample (Scott et al., 2008). As a result, quantitative analysis may be insufficient to determine if water will pose ecological risk to receiving systems. Toxicity testing with a sentinel species coupled with quantitative analysis offers a more complete representation of ecological risk than quantitative analysis alone, and both metrics could be useful to regulators when determining if treated water is safe for discharge.

2. Determine the rate and extent of removal of commercial NAs by a reciprocating mycoreactor

The purpose of this study was to investigate the use of white-rot fungi for NA bioremediation. The specific objectives were to 1) determine the rate and extent of removal of commercial (Fluka) NAs by white-rot fungal (*Pleurotus pulmonaris*) degradation, and 2) measure the changes in toxicity following treatments in terms of mortality with sentinel fish (*Pimephales promelas*) in 96-hr static tests. A reciprocating reactor was built to allow for periodic relief from submersion for the terrestrial fungus *P.*

pulmonaris. NA half-lives were 25.4 hours for inoculated reactors and 38.5 hours for control reactors not inoculated with *P. pulmonari*s. Both inoculated and uninoculated reactors removed NAs to non-detect concentrations (<5 mg/L) for a removal efficiency >90%. These results indicate that the presence of *P. pulmonaris* can increase the overall rate of NA removal. Quantitative NA analysis was confirmed with toxicity testing, which provided evidence of ecological risk mitigation by reciprocating mycoreactor. In addition, this study demonstrated that *P. pulmonaris* can tolerate sixteen 6-h periods of submersion when used in a reciprocating reactor.

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APPENDICES

METHOD FOR MEASURING GENERAL WATER QUALITY PARAMETERS: pH, DISSOLVED OXYGEN, CONDUCTIVITY, TEMPERATURE, ALKALINITY, AND HARDNESS

Jeff Schwindaman, Brenda M. Johnson, Laura E. Ober, John H. Rodgers, Jr.

1.0 OBJECTIVE

The purpose of this protocol is to measure various general water quality parameters. Parameters such as pH, dissolved oxygen (DO), conductivity, temperature, alkalinity, and hardness are fundamental water quality parameters and are necessary for all water chemistry related studies.

2.0 HEALTH AND SAFETY

Proper lab attire, including scrubs, lab coat, gloves and safety glasses must be worn at all times.

3.0 PERSONAL/TRAINING/RESPONSIBILITIES

Any graduate research assistant familiar with the equipment and laboratory techniques and trained in this and referenced SOPs may perform this procedure.

4.0 REQUIRED MATERIALS

4.1 Reagents

Reagent:	Test:
Milli-Q water	all tests
pH buffers (4,7,&10)	pH, alkalinity
0.02 N standard sulfuric acid solution (H_2SO_4)	alkalinity
Eriochrome Black T indicator	hardness
Standard EDTA titrant (0.01M, 0.02N)	hardness
Buffer solution (Reference Standard Methods 2340C)	hardness

4.2 Supplies

Supply:	Test:
Graduated cylinder	alkalinity, hardness
100-mL beakers	all tests

Magnetic stir bar
alkalinity, hardness
50-mL burette and stand
alkalinity, hardness

4.3 Equipment

Orion-model A325 pH Meter
Orion-model 420A pH Meter
YSI 55Dissolved Oxygen Meter
YSI 30 Salinity, Conductivity, and Temperature Meter
Magnetic stir plate

5.0 PROCEDURE

5.1 pH

1. Calibrate the Orion-model A325 pH Meter using standard pH buffers 4, 7, and 10.
2. Rinse probe with milli-Q water to remove any prior contaminant.
3. Remove the small blue rubber stopper from the probe
4. Submerge the tip of the probe in the sample and gentle stir the sample with the probe.
5. When the pH reading has stabilized, record pH in S.U. to a tenth of a S.U.
6. Rinse probe with milli-Q water between measurements and return to holder when finished.

5.2 Dissolved Oxygen (DO)/Temperature

1. Calibrate the YSI 55 Dissolved Oxygen Meter.
2. Rinse probe with milli-Q water to remove any prior contaminant.
3. Completely submerge the tip of the probe in the sample.
4. When the DO reading has stabilized, record DO in mg/L. Also record the temperature to a tenth of a degree (i.e. 20.1°C).
5. Rinse probe with milli-Q water between measurements and return to holder when finished.

5.3 Conductivity

1. Turn on the YSI 30 Salinity, Conductivity, and Temperature Meter.
2. Rinse probe with milli-Q water to remove any prior contaminant.
3. Submerge the probe in the sample and gently stir the sample with the probe.
4. When the conductivity reading has stabilized the conductivity will record in (mS/cm and temperature in degrees Celsius.

5. Rinse probe with milli-Q water and return to holder.
6. When finished turn off the meter

5.4 Alkalinity
1. Using a graduated cylinder, measure 50 mL of sample water and pour it into a 100-mL beaker with a magnetic stir-bar.
2. Place sample beaker on magnetic stir-plate. Turn on stir-plate to begin mixing sample.
3. Calibrate Orion-model 420A pH meter. Place probe in the appropriate stand, with the tip completely submerged in the sample water. (Make sure the stir-bar does not hit the pH probe).
4. Record the initial level of titrant (0.02 N H_2SO_4) in the burette (fill burette as necessary).
5. Slowly drip titrant into the sample, allowing time for the pH meter to stabilize.
6. Titrate to pH 4.5.
7. Record the volume (mL) of titrant used to reach the pH endpoint (pH=4.5).
8. Calculate: Total Alkalinity (mg/L as $CaCO_3$) = col. Titrant (mL)x 20
9. Turn off stir-plate and discard sample.

5.5 Hardness
1. Using a graduated cylinder, measure 50 mL of sample water and pour it into a 100-mL beaker with a magnetic stir-bar. (Dilutions can be made to conserve EDTA titrant, be sure to calculate dilutions into the final equation.)
2. Add 2-5 mL of buffer solution (to give the sample a pH of 10.0-10.1).
3. Add 2-4 drops of Eriochrome Black T Indicator. Sample should turn pink.
4. Place sample beaker on magnetic stir-plate. Turn on plate to mix sample.
5. Record the level of titrant (EDTA) in the burette (fill burette as necessary).
6. Slowly drip titrant into the sample, allowing time for the color change to stabilize.
7. Titrate until pink turns to a blue-green color.
8. Record the volume of titrant (mL) used to reach the color change.
9. Calculate: Hardness (mg/L $CaCO_3$) = volume titrant (mL) x 20.
10. Turn off stir-plate and discard sample.

6.0 QUALITY CONTROL CHECKS AND ACCEPTANCE CRITERIA
All procedures are subject to review by the Quality Assurance Unit.

METHOD FOR MEASURING OXIDATION-REDUCTION POTENTIAL

Sarah E. Sundberg, Derek Eggert, J. Chris Arrington, John H. Rodgers Jr.

1.0 OBJECTIVE

Oxidation and reduction (redox) reactions mediate the behavior of many chemical constituents in wastewaters. The reactivities and mobilities of important elements in biological systems, as well as those of a number of other metallic elements, depend strongly on redox conditions. Like pH, Eh (redox) represents an intensity factor; it does not characterize the capacity of the system for oxidation or reduction. Measurements are made by potentiometric determination of electron activity (or intensity) with an inert indicator electrode and a suitable reference electrode. Electrodes made of platinum are most commonly used for Eh measurements. This protocol describes the method used to measure redox in the hydrosoil of a constructed wetland treatment system.

2.0 HEALTH AND SAFETY

Proper lab attire, including scrubs, lab coat, gloves and safety glasses must be worn at all times.

3.0 PERSONNEL/TRAINING/RESPONSIBILITES

Any graduate research assistant familiar with the equipment and laboratory techniques and trained in this and referenced SOPs may perform this procedure.

4.0 REQUIRED MATERIALS

4.1 Supplies

Potassium ferrocyanide $K_4Fe(CN)_6 \cdot 3H_2O$
Potassium ferricyanide, $K_3Fe(CN)_6$
Potassium chloride, KCl

4.2 Equipment
pH or millivolt meter
Reference electrode
Oxidation-reduction indicator electrode
Beakers
Magnetic Stirrer

5.0 PROCEDURE
Prepare ZoBell's standard redox solution by adding 1.4080 g potassium ferrocyanide, 1.0975 g potassium ferricyanide and, 7.4555 g potassium chloride to 1000 mL of Milli-Q water at 25°C. These measurements must be as accurate as possible to result in a reliable solution. When stored in dark plastic bottles in a refrigerator, this solution is stable for several months.
Follow the manufacturer's instructions for using the pH/millivolt meter and in preparing electrodes for use. Immerse the reference electrode connected to the millivolt meter and

the redox indicator electrode (platinum tip end) in the gently stirred, standard solution in a beaker. Connect the millivolt meter to the end of the indicator electrode opposite the platinum tip. Allow several minutes for electrode equilibrium then record the reading to the nearest millivolt. If the reading is within ±10 mV from the theoretical redox standard value at 25°C (+183 mV), record the reading. The indicator electrode is ready for placement in the hydrosoil. If the reading is not within +10 mV, the indicator electrode must be remade.

Place the indicator electrode's platinum tip into the sediment making certain it is not near the plant roots. Secure the electrode with cable ties. Allow the electrode to equilibrate for 24 hours prior to taking any readings. When measuring the redox potential of the hydrosoil place the reference electrode in the same water column as the probe. Connect the millivolt reader to the end of the indicator electrode opposite the platinum tip. Record the redox potential in mV. Repeat a second time by placing the reference electrode in another location. Successive reading s that vary less than +10 mV over 10 minutes are adequate for most purposes. Adjust the reading according to field corrections and electrode calibration corrections.

Example: The field measurement of a hydrosoil was -206 mV. When the electrode was initially calibrated in the lab, the redox reading was +193mV, which is +10mV different from the theoretical redox standard value of +183 mV. The field redox measurement must be corrected for this difference by subtracting 10 mV from -206 mV. This gives a redox measurement of -216 mV. The standard correction factor for field redox measurements for the millivolt reader is +240 mV. Therefore, this correction factor is added to the redox measurement of -2216 mV to yield a final redox measurement of +24 mV.

$$Eh_{system} = Eh_{observed} - Eh_{reference\ observed} + Eh_{field\ correction}$$
$$Eh_{system} = -206mV + 183mV - 193mV + 240mV$$

6.0 QUALITY CONTROL CHECKS AND ACCEPTANCE CRITERIA

All procedures are subject to review by the Quality Assurance Unit.

7.0 REFERENCES

Faulkner, S.P., W.H. Patrick, Jr., R.P. Gambrell, 1989. Field techniques for measuring wetland soil parameters. Soil Sci. Soc. Am. J. 53, 883-890.

ZoBell, C. E., 1946. Studies on redox potential of marine sediments. Bulletin of the American Association of Petroleum Geologists 30, 477-513.

METHOD FOR DERIVATIZING NAPHTHENIC ACIDS FOR HPLC ANALYSIS
Sam Muller
1.0 OBJECTIVE
The objective of this standard operating procedure is to clearly outline the methods for derivatizing naphthenic acids in solution for HPLC analysis.
2.0 HEALTH AND SAFETY
Proper personal protective equipment will be worn for the entirety of this procedure. Reagents for derivatization should be prepared and handled within a fume hood.
PERSONNEL/TRAINING/RESPONSIBILITIES
Any graduate research assistant familiar with the equipment and laboratory techniques and trained in this SOP may perform this procedure.
4.0 REQUIRED AND RECOMMENDED MATERIALS
4.1 SUPPLIES
1 M NaOH
3 M HCl
0.4 M HCl
Ethanol, HPLC grade
Methanol, HPLC grade
Nano-pure water
Pyridine
KOH salt, reagent grade
2-NPH
1-EDC-HCl
50 mL opaque vials with screw caps
2-mL amber glass HPLC vials with caps and septa

4.2 EQUIPMENT
Water bath
Thermometer
Magnetic stir plate
Magnetic stir bar
pH meter with needle tip
100 mL volumetric flask
30 mL beakers
30 mL medicine cups
100-1000 μL volumetric pipette and tips
5-10 mL volumetric pipette and tips
Syringe and 0.22 μm syringe filter
5.0 PROCEDURE
5.1 Reagent Preparation
Prepare a 95% ethanol in water (v/v) solution in a 1-L volumetric flask. For the 2-NPH reagent, mix 15 mL of the 95% ethanol solution, 5 mL 0.4 M HCl, and 60 mg of 2-NPH in a beaker. Stir fifteen minutes on a stir plate and store in an opaque vial with screw top at 4° C in refrigerator. Prepare a 3% pyridine solution by adding 3 mL pyridine to a 100

mL volumetric flask and filling with 95% v/v ethanol in Nano-pure water. Add 10 mL 95% ethanol solution, 10 mL 3% pyridine solution, and 480 mg EDC-HCl to a beaker and place on stir plate for fifteen minutes. Store the solution in an opaque vial with screw top at 4° C. Prepare a 1 M KOH solution by dissolving 5.61 g KOH salt in 100 mL 80% v/v HPLC grade methanol solution in Nano-pure water. Dilute to 140 mM by adding 14 mL of the prepared 1 M KOH solution to a 100 mL volumetric flask. Fill to 100 mL using 80% (v/v) methanol in Nano-pure water.

5.2 Sample Preparation

Pour 5 mL of sample into a medicine cup. Add 1 M NaOH until pH reaches 12. Filter with a syringe and 0.22 µm syringe filter. Adjust pH of filtered sample to between 8 and 10 with 3 M HCl solution, measuring with needle tip pH meter.

5.3 Derivitization

Turn on water bath and set temperature to 60°C. Pipet 600 µL of pH-adjusted, filtered sample into a clean HPLC vial. Add 240 µL of 2-NPH reagent and 240 µL of 1-EDC-HCl solution to each vial. Tightly cap the vial and place samples in water bath for 20 minutes. Remove samples from water bath. Add 120 µL of 140 mM KOH in 80% (v/v) methanol/water to each vial. Recap vials and place in water bath for 15 minutes. Measure pH to ensure it does not exceed 7.5. pH should read between 5.5 and 6.5. Cool samples and take to HPLC.

6.0 REFERENCE

Yen TW, Marsh WP, MacKinnon MD, Fedorak PM. 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. J. of Chromatogaphy 1033: 83-90.

APPENDIX B

Chapter 2 Measured Explanatory Parameters

Initial conditions

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L CaCO$_3$)	Dissolved oxygen (mg/L)	ORP (mV)
BFP 1[a]	20.1	8.32	764	72	8.46	385
BFP 2[a]	20.	8.35	804	78	8.62	401
BFP 3[a]	19.5	8.37	760	84	8.51	391
Corn syrup 1	19.6	8.39	744	80	8.56	389
Corn syrup 2	19.8	8.34	816	76	8.71	404
Corn syrup 3	19.6	8.36	820	84	8.69	392
Hay 1	19.5	8.35	760	88	8.38	384
Hay 2	19.8	8.31	800	82	8.75	391
Hay 3	19.7	8.37	740	78	8.62	402
Control 1	19.6	8.36	760	74	9.01	399
Control 2	19.4	8.35	740	90	8.56	395
Control 3	19.1	8.38	760	84	8.74	381

[a]Biofermentation product

Day 4

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L CaCO$_3$)	Dissolved oxygen (mg/L)	ORP (mV)
BFP 1 [a]	19.7	8.91	632	74	8.56	401
BFP 2 [a]	19.4	8.87	616	82	8.78	314
BFP 3 [a]	19.5	8.84	672	78	8.25	454
Corn syrup 1	19.5	8.32	684	82	8.19	368
Corn syrup 2	19.7	8.41	760	88	8.78	352
Corn syrup 3	19.6	8.40	732	84	8.10	476
Hay 1	19.4	8.35	804	78	9.05	352
Hay 2	19.4	8.41	800	74	8.75	398
Hay 3	19.3	8.28	784	82	8.42	390
Control 1	19.6	8.40	788	92	8.63	411
Control 2	19.5	8.47	808	84	8.52	417
Control 3	19.4	8.43	680	84	9.22	379

[a]Biofermentation product

Day 8

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L $CaCO_3$)	Dissolved oxygen (mg/L)	ORP (mV)
BFP 1[a]	19.3	8.42	648	78	8.75	319
BFP 2[a]	19.5	8.46	636	72	8.31	438
BFP 3[a]	19.5	8.41	680	72	8.35	374
Corn syrup 1	19.6	8.51	692	80	8.28	357
Corn syrup 2	19.8	8.48	760	84	8.71	409
Corn syrup 3	19.6	8.39	740	76	8.50	370
Hay 1	19.5	8.41	804	82	8.91	384
Hay 2	19.8	8.48	760	88	8.39	359
Hay 3	19.7	8.45	788	76	8.75	461
Control 1	19.6	8.37	764	72	9.17	414
Control 2	19.4	8.41	796	96	8.76	395
Control 3	19.1	8.38	672	84	8.98	375

[a]Biofermentation product

Day 12

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L CaCO$_3$)	Dissolved oxygen (mg/L)	ORP (mV)
BFP 1[a]	21.1	8.55	728	80	8.39	381
BFP 2[a]	21.0	8.61	764	80	8.93	409
BFP 3[a]	20.8	8.49	820	76	8.70	375
Corn syrup 1	20.9	8.68	712	84	8.26	358
Corn syrup 2	21.4	8.54	644	80	8.53	401
Corn syrup 3	21.1	8.70	656	76	8.45	392
Hay 1	21.5	8.52	592	88	9.02	345
Hay 2	21.5	8.56	536	80	8.77	397
Hay 3	21.4	8.49	644	88	8.39	486
Control 1	22.0	8.39	660	88	8.41	399
Control 2	21.7	8.44	644	84	8.67	395
Control 3	21.7	8.35	708	88	8.82	381

[a]Biofermentation product

Day 16

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L CaCO3)	Dissolved oxygen (mg/L)	ORP (mV)
BFP 1[a]	20.4	8.86	800	84	8.78	434
BFP 2[a]	20.6	9.05	780	88	8.53	322
BFP 3[a]	20.4	9.11	780	92	8.37	397
Corn syrup 1	20.6	8.89	780	84	8.66	345
Corn syrup 2	20.9	9.05	744	80	8.72	501
Corn syrup 3	20.8	9.04	800	92	8.45	441
Hay 1	20.1	8.87	784	96	8.69	480
Hay 2	20.8	8.75	704	104	8.55	459
Hay 3	20.4	8.68	720	88	8.82	420
Control 1	21.0	8.48	716	92	8.76	415
Control 2	20.5	8.45	656	84	9.22	372
Control 3	20.6	8.38	680	88	9.01	399

[a]Biofermentation product

Day 20

	Temperature (°C)	pH (SU)	Alkalinity (mg/L CaCO3)	Hardness (mg/L CaCO$_3$)	Dissolved oxygen (mg/L)	Redox (mV)
BFP 1[a]	23.2	9.05	812	84	8.48	405
BFP 2[a]	22.9	9.19	820	88	9.05	512
BFP 3[a]	23.1	9.14	780	92	8.78	488
Corn syrup 1	22.8	8.98	792	84	8.25	456
Corn syrup 2	22.6	9.12	760	80	8.67	422
Corn syrup 3	23.1	9.04	808	92	8.54	389
Hay 1	22.6	9.05	784	96	9.21	378
Hay 2	22.6	8.87	728	104	8.82	397
Hay 3	22.8	8.93	704	88	8.56	480
Control 1	22.5	8.45	720	92	8.92	378
Control 2	22.2	8.41	644	84	8.65	418
Control 3	22.4	8.38	680	88	8.50	415