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Comparative aquatic toxicity of a commercial naphthenic acid and processes for mitigating risks

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COMPARATIVE AQUATIC TOXICITY OF A COMMERCIAL NAPHTHENIC ACID AND PROCESSES FOR MITIGATING RISKS

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ABSTRACT

Comparative toxicity data can inform predictions of relative risk, and can be used to implement strategies for altering exposures to mitigate risk. Naphthenic acids (NAs) are a complex group of carboxylic acids that naturally occur in petroleum sources and energy-derived process waters (e.g. refinery effluents and oil sands process affected waters). These compounds are relatively persistent in water and can be a source of toxicity to aquatic organisms. In the first experiment of this thesis, responses of sentinel aquatic organisms to 7-d exposures of commercial (Fluka) NAs were measured (in terms of acute toxicity) to discern relative sensitivities. In terms of sensitivities, fish>invertebrates>plant for exposures to Fluka NAs. Once toxicity was determined, two potential processes for altering exposures were investigated. In the second experiment, photocatalytic degradation of Fluka NAs was measured using fixed-film titanium dioxide (TiO_2) irradiated with sunlight for 8 hours. Confirmation of changes in NA concentrations by photocatalytic degradation was accomplished analytically and with toxicity tests using sentinel vertebrate and invertebrate species. The half-life for Fluka NAs achieved by photocatalytic degradation was approximately 1 hour, with toxicity eliminated to both test species (*Pimephales promelas* and *Daphnia magna*) by the 5^{th} hour of the sunlight exposure. In the third experiment, environmental conditions that can influence aerobic degradation for altering exposures of NAs were evaluated. Effects of nitrogen and phosphorus concentrations, dissolved oxygen concentrations, pH, and temperature on rates and extents of aerobic degradation of Fluka NAs were measured. Environmental conditions that positively influenced aerobic degradation rates of Fluka

NAs included nutrients (C:N 10:1-500:1, C:P 100:1-5000:1), DO (4.76-8.43 mg/L), pH (6-8), and temperature (5-25ºC). At an initial Fluka NA concentration of 61 mg/L (±8), a removal rate of 11.7 mg/L day^{-1} was achieved (half-life approximately 2.5 days) in treatments with C:N and C:P molar ratios of 10:1 and 100:1, respectively (with other macro- and micronutrients supplied), DO >8 mg/L, pH ≈8, and temperatures >23ºC. Commercial NAs differ structurally from energy-derived NAs (e.g oil sands process affected waters), but environmental conditions systematically evaluated in this study are also expected to affect rates and extents of aerobic degradation of compositionally complex NAs.

Ultimately, experiments conducted in this thesis can serve as a model approach for evaluating comparative toxicity of NAs, in terms of relative sensitivities of a taxonomic range of sentinel species, and using that information to implement effective strategies for mitigating risks in aquatic systems.

DEDICATION

This thesis is dedicated to my parents, who taught me responsibility and discipline, and have always been my biggest supporters; to my boyfriend Howie, who uprooted his own life to support my professional goals and has been there every step of the way; and to mine and Howie's families who have always supported us, even though the distance has been difficult.

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CHAPTER ONE

INTRODUCTION

Naphthenic acids (NAs) are a complex group of organic acids associated with crude oils (Seifert and Teeter, 1969; Tomcyzk et al., 2001) and energy-derived waters such as refinery effluents and oil sands process affected waters (Dorn, 1992; Allen, 2008). NAs consist of thousands of compounds, and are described by the formula: ($C_nH_{2n+Z}O_2$), where n is the number of carbons and Z is either zero or a negative even integer representing the hydrogen deficiency of the molecule due to rings or double bonds (Holowenko et al., 2002; Clemente and Fedorak, 2005). NAs pose potential risks in aquatic systems due to their relative persistence in water (Scott et al., 2005; Han et al., 2009; Headley et al., 2010) and observed toxicity in energy-derived waters such as oil sands process affected waters, with acute and chronic toxicity reported for fish, macro- and microinvertebrates, aquatic macrophytes, and microorganisms (Nero et al., 2006; Frank et al., 2008; Armstrong et al., 2009; Kavanagh et al., 2012; Leclair et al., 2013; Swigert et al., 2015). To mitigate potential risks due to NAs, exposures must be altered sufficient to eliminate toxicity to aquatic organisms. Before evaluating potential processes for altering exposures, relative sensitivities of sentinel aquatic organisms to NA exposures must be measured (Figure 1.1). To predict ecological risks of NAs, studies of responses of sentinel species to unconfounded or defined exposures of NAs are needed (e.g. Swigert et al., 2015).

Comparative toxicity studies provide data regarding relative sensitivities of a taxonomic range of sentinel species to exposures, which can be used for prediction and

mitigation of ecological risks. Responses measured in exposed species should encompass the potential modes of action (Armstrong et al., 2009; Swigert et al., 2015). *Pimephales promelas* Rafinesque (fathead minnow) is a sensitive sentinel fish species which inhabits freshwater systems throughout North America (USEPA, 2002). *Ceriodaphnia dubia* Richard (microcrustacean), *Hyalella azteca* Saussure (amphipod), and *Chironomus dilutus* Fabricus (midge) are invertebrates that inhabit surface waters, sediment-water interfaces, and sediments, respectively, and differ in terms of structure and physiology (USEPA, 2002; APHA et al., 2007). *Typha latifolia* Linnaeus (common cattail) has been used as a sentinel species to evaluate potential phytotoxicity to rooted aquatic macrophytes (Muller et al., 2001; Moore et al., 1999). After relative sensitivities of sentinel aquatic organisms to exposures of NAs have been measured, extents to which NA exposures must be altered in order to mitigate risk can be determined.

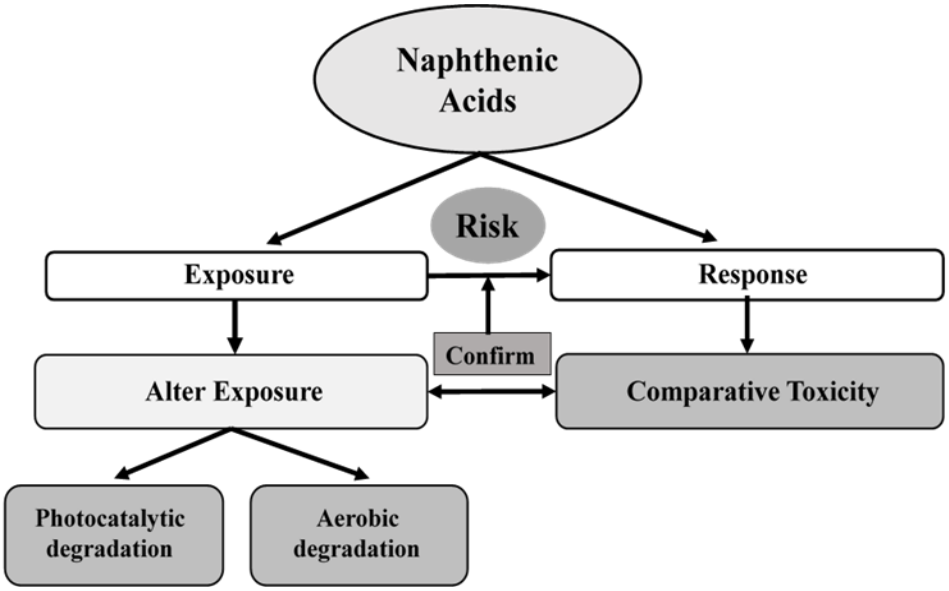


Figure 1.1 Conceptual model for context of experiments

Potential processes for altering NA exposures include a physical/chemical process, photocatalytic degradation (Headley et al., 2009; Mishra et al., 2010) and a biological process, aerobic degradation (Clemente et al., 2004; Scott et al., 2005; Han et al., 2008; Headley et al., 2010).

Naphthenic acids are susceptible to photolysis (USEPA, 2012; Headley et al., 2009), and the rate of this transformation process can be enhanced using catalysts (i.e. titanium dioxide [TiO_2]). Photocatalytic degradation of NA mixtures has been accomplished using TiO_2 in aqueous suspensions (i.e. "slurries") with both artificial ultraviolet (UV) irradiation (e.g. UV_{254} lamps) and natural sunlight (McMartin et al., 2004; Headley et al., 2009; Mishra et al., 2010). These prior bench-scale studies have demonstrated that rates of NA degradation using photocatalysis are of practical significance, with half-lives achieved in hours (Headley et al., 2009; Mishra et al., 2010). However, there are photocatalytic design features that may limit full-scale application. Post-treatment recovery of aqueous suspensions of TiO_2 particles may be challenging (Kinsinger et al., 2015) and artificial UV is energy intensive (Parsons, 2004; Metcalf and Eddy, 2004). Immobilizing TiO_2 on a fixed-film eliminates the need to amend and recover catalysts, potentially offering greater flexibility in treatment design. In addition, utilizing natural sunlight may provide sufficient energy to accomplish photocatalytic degradation of NAs while decreasing operational costs of treatment. Decreased toxicity to sensitive species can confirm alteration of exposures of NAs achieved by fixed-film photocatalytic degradation. Sentinel species such as fathead minnow (*Pimephales promelas* Rafinesque) and microinvertebrate (*Daphnia magna* Straus) are relatively

sensitive to NAs compared to non-vascular plants and bacteria (Swigert et al., 2015). Elimination of toxicity to these sensitive species in post-photocatalytic degradation samples can confirm alteration of NA exposures and mitigation of risks (Figure 1.1).

A potential biological transformation process for altering NA exposures is aerobic degradation. Studies of aerobic degradation of NAs have primarily focused on effects of structural compositions of NAs on rates and extents of removal (Clemente et al., 2004; Scott et al., 2005; Han et al., 2008; Headley et al., 2010). Environmental conditions that may influence aerobic degradation of NAs include nitrogen and phosphorus concentrations, dissolved oxygen concentration, pH, and temperature. Information gained from evaluating effects of environmental conditions on aerobic degradation of NAs could be used to further management strategies implemented to achieve efficacious treatment of NAs in energy-derived waters.

To accomplish evaluations of comparative toxicity and strategies for altering NA exposures, commercial NAs provide several advantages including stable and repeatable exposures, and availability and transferability (permitting inter- and intra-laboratory comparisons of toxicity data). Fluka commercial NAs were selected for these studies due to ready availability, cost-effectiveness, and thorough characterization of compositions in literature (Rudzinski et al., 2002; Barrow et al., 2004; Scott et al., 2005; Armstrong et al., 2007; Headley et al., 2010). In addition, Fluka NAs have been previously used to evaluate photolysis and photocatalysis (McMartin et al., 2004; Headley et al., 2009; Mishra et al., 2010) and aerobic degradation (Scott et al., 2005; Headley et al., 2010) of NAs, which allowed for comparisons of results among studies.

Objectives

Chapter 2

"Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid"

The overall objective of this study was to compare responses of freshwater aquatic organisms (in terms of acute toxicity) to Fluka commercial NA water accommodated fractions (WAFs). To achieve this overall objective, specific objectives were to 1) measure responses of a vertebrate: fathead minnow (*Pimephales promelas*), invertebrates: microcrustacean (*Ceriodaphnia dubia*), amphipod (*Hyalella azteca*), and midge (*Chironomus dilutus*) in terms of mortality and a macrophyte: common cattail (*Typha latifolia*) in terms of early seedling root and shoot growth to 7-d exposures of Fluka commercial NA WAFs, 2) compare the estimated 7-d lowest observed effect concentrations (LOECs), median lethal effect concentrations (LC50s) for animal species, and median effect concentration (EC50) for *T. latifolia*, 3) compare toxicity of Fluka NAs measured in this study with aquatic toxicity data for Fluka and other commercial NA sources in peer-reviewed scientific literature, and 4) compare with published aquatic toxicity data for NAs from energy-derived waters in peer-reviewed scientific literature.

Chapter 3

"Photocatalysis of a Commercial Naphthenic Acid in Water using Fixed-film TiO_2"

The overall objective of this study was to measure rates and extents of photolysis and photocatalytic degradation of a commercially available (Fluka) NA using bench-scale fixed-film TiO_2 and confirm changes in NA concentrations using sensitive vertebrate (fish = *Pimephales promelas*) and invertebrate (*Daphnia magna*) species. To achieve this overall objective, specific objectives were to: 1) measure the rates and extents of removal of commercial (Fluka) NAs when exposed to an 8 hour duration of natural sunlight ("photolysis") and natural sunlight in the presence of fixed-film TiO_2 ("photocatalysis"), and 2) measure changes in toxicity after photolysis and photocatalysis treatments (in terms of mortality) with sentinel fish (*P. promelas*) and microinvertebrate (*D. magna*) species in 96-hr static tests.

Chapter 4

"Effects of environmental conditions on aerobic degradation of a commercial naphthenic acid"

The overall objective of this experiment was to determine the relative influence of nitrogen (as ammonia) and phosphorus (as phosphate) concentrations, dissolved oxygen concentrations, temperatures, and pH on aerobic degradation of Fluka NAs in bench-scale laboratory reactors. To achieve this overall objective, specific objectives were to 1) measure and compare changes in Fluka NA concentrations with time for a range of C:N

(10:1-500:1) and C:P ratios (100:1-5000:1), 2) measure and compare changes in Fluka NA concentrations with time for a range of dissolved oxygen concentrations (<1-9 mg/L), 3) measure and compare changes in Fluka NA concentrations with time for a range of pH (6-8), 4) measure and compare changes in Fluka NA concentrations with time for a range of temperatures (5-25°C), 5) measure and compare relative abundance and diversity of microbial populations in treatments and 6) rank environmental conditions in terms of influence on extents of removal of Fluka NAs with time.

Organization of Thesis

This thesis is arranged in subsequent chapters intended for publication in peer-reviewed journals. Therefore, chapters two through four were written and formatted for a specific journal, and some introductory information, and materials and methods were repeated. Chapter Two is targeted for submission to *Ecotoxicology and Environmental Safety*, Chapter Three is targeted for submission to *Chemosphere*, and Chapter Four is targeted for submission to *Chemosphere*.

References

Allen, E.W. (2008). Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. *Journal of Environmental Engineering Science*, *7*, 123-138.

American Public Health Association (APHA). (2007). *Standard methods for the examination of water and wastewater*, 21st Ed. American Public Health Association, Port City Press: Baltimore, MD.

Armstrong, S. A., Headley, J. V., Peru, K. M., & Germida, J. J. (2007). Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. *Journal of Environmental Science and Health, Part A*, *43*, 36-42.

Armstrong, S. A., Headley, J. V., Peru, K. M., & Germida, J. J. (2009). Differences in phytotoxicity and dissipation between ionized and nonionized oil sands naphthenic acids in wetland plants. *Environmental Toxicology and Chemistry*, *28*, 2167-2174.

Barrow, M. P., Headley, J. V., Peru, K. M., & Derrick, P. J. (2004). Fourier transform ion cyclotron resonance mass spectrometry of principal components in oil sands naphthenic acids. *Journal of Chromatography A*, *1058*, 51-59.

Clemente, J. S., MacKinnon, M. D., and Fedorak, P. M. (2004). Aerobic biodegradation of two commercial naphthenic acids preparations. *Environmental Science & Technology*, *38*, 1009-1016.

Clemente, J. S., & Fedorak, P. M. (2005). A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. *Chemosphere*, *60*, 585-600.

Dorn, P. B. (1992). Case histories—the petroleum industry. Toxicity Reduction: Evaluation and Control. In: Ford, DL (Ed.), *Water Quality Management Library*, *3*, 183-223.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R. (2008). Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. *Environmental Science & Technology*, *43*, 266-271.

Han, X., Scott, A. C., Fedorak, P. M., Bataineh, M., & Martin, J. W. (2008). Influence of molecular structure on the biodegradability of naphthenic acids. *Environmental Science & Technology, 42*, 1290-1295.

Han, X., MacKinnon, M. D., & Martin, J. W. (2009). Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. *Chemosphere, 76*, 63-70.

Headley, J., Du, J., Peru, K., McMartin, D. (2009). Electrospray ionization mass spectrometry of the photodegradation of naphthenic acids mixtures irradiated with titanium dioxide, *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 44,* 591-597.

Headley, J. V., Peru, K. M., Adenugba, A. A., Du, J. L., & Mcmartin, D. W. (2010). Dissipation of naphthenic acids mixtures by lake biofilms. *Journal of Environmental Science and Health Part A, 45*, 1027-1036.

Holowenko, F.M., MacKinnon, M.D., and Fedorak, P.M. (2002). Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. *Water Resources, 36*, 2843-2855.

Kavanagh, R. J., Frank, R. A., Burnison, B. K., Young, R. F., Fedorak, P. M., Solomon, K. R., & Van Der Kraak, G. (2012). Fathead minnow (Pimephales promelas) reproduction is impaired when exposed to a naphthenic acid extract. *Aquatic Toxicology, 116*, 34-42.

Kinsinger, N., Honda, R., Keene, V., & Walker, S. L. (2015). Titanium Dioxide Nanoparticle Removal in Primary Prefiltration Stages of Water Treatment: Role of Coating, Natural Organic Matter, Source Water, and Solution Chemistry. *Environmental Engineering Science, 32,* 292-300.

Leclair, L. A., MacDonald, G. Z., Phalen, L. J., Köllner, B., Hogan, N. S., & van den Heuvel, M. R. (2013). The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (Oncorhynchus mykiss). *Aquatic Toxicology, 42*, 185-194.

McMartin, D. W., Headley, J. V., Friesen, D. A., Peru, K. M., & Gillies, J. A. (2004). Photolysis of naphthenic acids in natural surface water. *Journal of Environmental Science and Health, Part A 39*, 1361-1383.

Metcalf & Eddy., Tchobanoglous, G., Burton, F. L. 1., & Stensel, H. D., (2004). *Wastewater engineering: Treatment and reuse* (4th ed.). McGraw-Hill. Boston, MA.

Mishra, S., Meda, V., Dalai, A., McMartin, D., Headley, J., and Peru, K. (2010). Photocatalysis of Naphthenic Acids in Water. *Journal of Water Resource and Protection, 2,* 644-650.

Moore, M.T., Huggett, D.B., Huddleston III, G.M., Rodgers Jr., J.H., Cooper, C.M., (1999). Herbicide effects on Typha latifolia (Linneaus) germination and root and shoot development. *Chemosphere, 38,* 3637-3647.

Muller, S.L., Huggett, D.B., Rodgers Jr., J.H., (2001). Effects of copper sulfate on *Typha latifolia* seed germination and early growth in aqueous and sediment exposures. *Archives of Environmental Contamination and Toxicology, 40,* 192-197.

Nero, V., Farwell, A., Lee, L. E. J., Van Meer, T., MacKinnon, M. D., and Dixon, D. G. (2006). The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. *Ecotoxicology and Environmental Safety, 65,* 252-264.\

Parsons, S.(ed.). (2004). *Advanced oxidation processes for water and wastewater treatment.* IWA Publishing. London, UK.

Rudzinski, W. E., Oehlers, L., Zhang, Y., & Najera, B. (2002). Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. *Energy & Fuels, 16,* 1178-1185.

Scott, A.C., Mackinnon MD, and Fedorak, P.M. (2005). Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. *Environmental Science and Technology, 39,* 8388-8394.

Seifert, W. K., Teeter, R. M. (1969). Preparative thin-layer chromatography and high-resolution mass spectrometry of crude oil carboxylic acids. *Analytical Chemistry, 41,* 786-795.

Swigert, J. P., Lee, C., Wong, D. C., White, R., Scarlett, A. G., West, C. E., and Rowland, S. J. (2015). Aquatic hazard assessment of a commercial sample of naphthenic acids. *Chemosphere, 124,* 1-9.

Tomczyk, N. A., Winans, R. E., Shinn, J. H., and Robinson, R. C. (2001). On the nature and origin of acidic species in petroleum. 1. Detailed acid type distribution in a California crude oil. *Energy & Fuels, 15,* 1498-1504.

United States Environmental Protection Agency (USEPA). (2002). Methods for measuring acute toxicity of effluents and receiving waters to freshwater and marine organisms. Washington DC. EPA-821-R-02-12.

United States Environmental Protection Agency (USEPA). (2012). Screening-level hazard characterization: Reclaimed substances (Naphthenic acids category). Washington DC. EPA-821-R-02-12.

CHAPTER TWO

COMPARATIVE RESPONSES OF FRESHWATER ORGANISMS TO EXPOSURES OF A COMMERCIAL NAPHTHENIC ACID

Abstract

Comparative toxicity studies using unconfounded exposures can prioritize the selection of sensitive sentinel test species and can refine methods for evaluating ecological risks of complex constituents like naphthenic acids (NAs), a group of problematic organic acids associated with crude oils and energy-derived waters. Unconfounded exposures of NAs can be prepared with relatively stable and repeatable commercially available sources in water accommodated fractions (WAFs). The overall objective of this study was to compare responses of freshwater aquatic organisms (in terms of acute toxicity) to Fluka commercial NA WAFs. To achieve this overall objective, specific objectives were to 1) measure responses of a vertebrate: fathead minnow (*Pimephales promelas*), invertebrates: microcrustacean (*Ceriodaphnia dubia*), amphipod (*Hyalella azteca*), and midge (*Chironomus dilutus*) in terms of mortality and a macrophyte: common cattail (*Typha latifolia*) in terms of early seedling root and shoot growth to 7-d exposures of Fluka commercial NA WAFs, 2) compare the estimated 7-d lowest observed effect concentrations (LOECs), median lethal effect concentrations (LC50s) for animal species, and median effect concentration (EC50) for *T. latifolia*, 3) compare toxicity of Fluka NAs measured in this study with aquatic toxicity data for Fluka and other commercial NA sources in peer-reviewed scientific literature, and 4) compare results with published aquatic toxicity data for NAs from energy-derived waters in peer-reviewed scientific literature. Exposures were confirmed using high performance liquid

chromatography. Responses (7-d LC50s/EC50) ranged from 1.9 mg/L for *P. promelas* to 56.2 mg/L for *T. latifolia*. Following *P. promelas* in order of decreasing sensitivity were *C. dubia* (7-d LC50=2.8 mg/L), *H. azteca* (7-d LC50= 4.1 mg/L), *C. dilutus* (7-d LC50= 6.5 mg/L), and *T. latifolia* (7-d EC50= 56.2 mg/L), indicating that in terms of sensitivities, fish>invertebrates>plant for Fluka NAs in this study. Factors that affect exposures and measurements of exposures differ among commercial and energy-derived NAs and severely limit comparisons. However, fish species evaluated were relatively sensitive to both commercial and energy-derived NAs from different sources and are likely appropriate sentinel species for risk evaluations in aquatic systems.

Introduction

Comparative toxicity studies using unconfounded exposures are needed for evaluating ecological risks of complex constituents, such as naphthenic acids (NAs), which are a group of problematic organic acids associated with crude oils (Seifert and Teeter, 1969; Tomcyzk et al., 2001) and energy-derived waters (Dorn, 1992; Allen, 2008). NAs are described by the formula: ($C_nH_{2n+Z}O_2$), where n is the number of carbons and Z is either zero or a negative even integer representing the hydrogen deficiency of the molecule due to rings or double bonds (Holowenko et al., 2002; Clemente and Fedorak, 2005). NAs can be sources of toxicity in energy derived waters such as refinery effluents and oil sands process affected waters (Dorn, 1992; Schramm, 2000), with acute and chronic toxicity observed for fish, macro- and microinvertebrates, aquatic macrophytes, and microorganisms (Nero et al., 2006; Frank et al., 2008; Armstrong et al., 2009; Kavanagh et al., 2012; Leclair et al., 2013; Swigert et al., 2015). Unconfounded exposures of NAs can be prepared with relatively stable and repeatable commercial sources in standardized toxicity testing to prioritize test species and refine methods to acquire relative sensitivity information for NAs.

Availability and transferability of commercial NAs permits inter- and intra-laboratory comparative studies providing data for species sensitivities. To conduct a comparative toxicity study using sentinel aquatic species with a commercial NA, reliable and repeatable exposures must be prepared. Fluka NAs were selected for this study due to ready availability, cost-effectiveness, and thoroughly characterized composition in the literature (Rudzinski et al., 2002; Barrow et al., 2004; Scott et al., 2005; Armstrong et al.,

2007; Headley et al., 2010). Commercial NAs are complex mixtures containing compounds with a wide range of aqueous solubilities, and methods have been developed for preparing homogenous exposures to accommodate this range in composition. Tests to determine the aquatic toxicity of poorly water-soluble complex mixtures are conducted using the water accommodated fraction (WAF) of the mixture (Girling et al., 1994), which in this case is the portion of NAs that remains in solution after low-energy mixing for 12 hours.

To capture the potential range of responses or effects, this comparative toxicity study of commercial NAs (Fluka) required a range of sentinel species spanning kingdoms. Responses measured in exposed species should encompass the potential modes of action for NAs (Armstrong et al., 2009; Siwik et al., 2010; Swigert et al., 2015). *Pimephales promelas* Rafinesque (fathead minnow) inhabits freshwater bodies throughout North America and was used in this study as a sensitive sentinel fish species (USEPA, 2002). *Ceriodaphnia dubia* Richard (microcrustacean), *Hyalella azteca* Saussure (amphipod), and *Chironomus dilutus* Fabricus (midge) are invertebrates that inhabit surface waters, sediment-water interfaces, and sediments, respectively, and were used due to anticipated differences in sensitivities related to structure and physiology (USEPA, 2002; APHA, 2007). *Typha latifolia* Linnaeus (common cattail) was used as a sentinel species to evaluate potential phytotoxicity to rooted aquatic macrophytes (Moore et al., 1999; Muller et al., 2001). Given the stability of commercial NAs, seven day exposure durations can be maintained and provide time for responses of exposed species to be manifested. To accomplish these defined exposures, modified formulated

moderately hard water (USEPA, 2002) was used for culture and to prepare WAFs (exposures) for this study.

Comparisons of measured toxicity data for Fluka NAs in the present study with published toxicity data for other commercial NAs provides information for relative toxicity of different commercial NAs as well as relative sensitivities of other species evaluated. Further, comparisons of toxicity data among commercial NAs and energy-derived NAs can provide context for the relative toxicity of these different sources.

The overall objective of this study was to compare responses of freshwater aquatic organisms (in terms of acute toxicity) to Fluka commercial NA water accommodated fractions (WAFs). To achieve this overall objective, specific objectives were to 1) measure responses of a vertebrate: fathead minnow (*Pimephales promelas*), invertebrates: microcrustacean (*Ceriodaphnia dubia*), amphipod (*Hyalella azteca*), and midge (*Chironomus dilutus*) in terms of mortality and a macrophyte: common cattail (*Typha latifolia*) in terms of early seedling root and shoot growth to 7-d exposures of Fluka commercial NA WAFs, 2) compare the estimated 7-d lowest observed effect concentrations (LOECs), median lethal effect concentrations (LC50s) for animal species, and median effect concentration (EC50) for *T. latifolia*, 3) compare toxicity of Fluka NAs measured in this study with aquatic toxicity data for Fluka and other commercial NA sources in peer-reviewed scientific literature, and 4) compare with published aquatic toxicity data for NAs from energy-derived waters in peer-reviewed scientific literature.

Materials and Methods

Preparation of Fluka naphthenic acid exposures

Fluka commercial NAs were obtained from Sigma-Aldrich® (St. Louis, MO; Table 2.1). Stock solutions were prepared by mixing 100 mg of NAs in 1L (100 mg/L) of reconstituted formulated moderately hard water (pH 7.7 ± 0.5 SU, alkalinity 65 ± 8 mg/L as $CaCO_3$, hardness 88 ± 10 mg/L as $CaCO_3$, conductivity 350 ± 20 µs/cm) prepared using reverse osmosis filtered water and reagent grade chemicals based on recommended culture methods (USEPA, 2002). The formulated water contained 5 mg/L $CaCO_3$, 102 mg/L $NaHCO_3$, 48 mg/L $MgSO_4$-$7H_2O$, 33 mg/L $CaSO_4$-$2H_2O$, 65 mg/L $CaCl_2$-$2H_2O$, 2 mg/L KCl, 0.8 mg/L KNO_3, 0.02 mg/L K_2PO_4, and 0.002 mg/L of each Cu, Se, and Zn (from aqueous standards). All reagents were obtained from Fisher Scientific® (Pittsburgh, PA). A modified water accommodated fraction (WAF) method was used to prepare NA stock solutions, where solutions were mixed with magnetic stir bars for 12 hours at a speed sufficient to create a vortex which extended 30-50% of the solution depth (OECD, 2000). Stock solutions were adjusted to pH 10±0.1 S.U. prior to mixing to increase solubility of NAs. After stirring, undissolved fractions were decanted and the remaining dissolved fraction in solution was used for testing. Experimental concentrations were prepared by serial dilution of the stock solution with moderately hard water. pH of exposures was adjusted to 8.3±0.1 with 0.1M HCl (Fisher Scientific, Pittsburgh, PA), in all toxicity tests in order to ensure homogenous exposures while remaining within environmental tolerances of organisms. Initial NA concentrations were measured in all treatments using high performance liquid chromatography (HPLC;

Dionex, UltiMate-3000; Sunnyvale, CA) according to a derivatization method described in Yen et al. (2004). The HPLC analytical column was an Agilent LiChrospher 100 RP-18 (5 µm particle size, 125mm x 4 mm) with a guard column packed with 2 µm RP-18 solid phase material. Column temperature was maintained at 40° C with a sample injection volume of 60 µL mobilized with HPLC grade methanol (Fisher Scientific) at a flow rate of 1.5 mL/min. Calibration standards were prepared with Fluka NAs using WAFs as described for stock solutions. The detection limit for this method is approximately 5 mg/L. In order to accurately measure exposures below the detection limit, stock solutions containing concentrations 10x (or 100x) greater than the nominal concentration were prepared, samples were collected for quantification of NAs, and solutions were then diluted 10x (or 100x) with moderately hard water for test exposures.

Toxicity Testing Procedures

Freshwater organisms (*P. promelas, C.dubia*, *H. azteca*, and *C. dilutus*) were cultured at Clemson University's Aquatic Animal Research Laboratory (AARL) according to methods of the United States Environmental Protection Agency (USEPA, 2002), under protocols in compliance with Clemson University's Institutional Animal Care and Use Committee (IACUC). Toxicity tests for *P. promelas* were conducted by exposing 30 organisms (< 24h old) per concentration (10 organisms per replicate for 3 replicates) in 250ml borosilicate beakers. During exposures, fish were fed once daily with *Artemia sp.* Toxicity tests for *C. dubia* were conducted by exposing 20 organisms (< 24h old) per concentration (1 organism per replicate for 20 replicates) in 15ml borosilicate vials. During exposures, *C. dubia* were fed once daily with 200µL of a 1:1

mixture of *Pseudokirchneriella subcapitata* and YCT (yeast, cerophyll, trout chow). Toxicity tests for *H. azteca* were conducted by exposing 30 organisms (≈2 weeks old) per concentration (10 organisms per replicate for 3 replicates) in 250ml borosilicate beakers. Amphipods were fed at test initiation with 2-3 maple leaf disks. Toxicity tests for *C. dilutus* were conducted by exposing 30 organisms (2^{nd}-3^{rd} instar larvae) per concentration (10 organisms per replicate for 3 replicates) in 250 ml borosilicate beakers. Aqueous solutions were gently aerated, and midges were fed at test initiation with a 1:10 mixture of fish flake food and reverse osmosis filtered water. Food was replaced as it was consumed from the overlying water. All untreated control exposures were moderately hard water only. Toxicity tests were conducted with a 16hr light/8hr dark photoperiod at 24 ± 1°C. After 7 days, live organisms for each exposure concentration were counted.

For phytotoxicity testing, mature *T. latifolia* inflorescences were collected in August and September, 2014 from a rural wetland site at Clemson University, Clemson, SC (34°40'7.12"N, 82°50'53.98"W). Inflorescences were stored in plastic bags and incubated at 20 ± 1°C until testing. Seeds were separated from bristle hairs by placing in a blender filled with NANOpure® water and blending for 30s. Seeds that sank to the bottom after blending were considered viable and used for testing (Muller et al., 2001; Moore et al., 1999). Viable seeds were then added to a small volume (about 1ml) of moderately hard water and incubated for 2 days to induce germination. Toxicity experiments for *T. latifolia* were initiated by adding 10 germinated *T. latifolia* seedlings (2 days old) to each replicate 50mL beaker (three replicates/concentration) under fluorescent lighting (1,500-3,000 Lux) with a 16hr light/8hr dark photoperiod at 24 ±

1°C. Exposure concentrations were pipetted into treatment chambers and volumes were maintained as necessary. Control (untreated) exposures were moderately hard water. After 7 days, seedlings were removed from exposures and preserved in 70% ethanol until analysis. Root and shoot lengths (mm) of seedlings were measured using a Leica® M80 Stereoscope and software (Leica Microsystems®).

Reference toxicity tests were conducted for all test species using copper sulfate ($CuSO_4 \cdot 5H_20$; Fisher Scientific) to measure the health of organisms used in tests (USEPA, 2002; USEPA, 1991). Acid soluble copper concentrations (exposures) were confirmed using flame atomic absorption spectroscopy and graphite atomic absorption spectroscopy (Agilent PSD 120 atomic absorption spectrometer; APHA, 2007).

Water characteristics of exposures were measured at test initiation and completion, with the exception of *T. latifolia* exposures, which were measured at test initiation. Dissolved oxygen, pH, and conductivity of exposure waters were measured using a YSI® Model 52 dissolved oxygen meter, Orion® Model 250A pH and meter Triode® electrode, and Orion® Model 142 conductivity meter, respectively. Hardness and alkalinity of samples were measured according to *Standard Methods for Examination of Water and Wastewater* (APHA, 2007).

Statistical Analyses

No observable effect concentrations (NOECs) and lowest observable effect concentrations (LOECs) of commercial NAs and copper as copper sulfate for *T. latifolia* root and shoot growth and mortality in animals were determined by statistically significant differences relative to untreated controls using one way analysis of variance

(ANOVA) and Dunnett's multiple range test ($\alpha = 0.05$; JMP Pro V.11). Median lethal effect concentrations (LC50s) were estimated using the Probit model (Bliss, 1935). The median effect concentration (EC50) for *T. latifolia* was estimated using non-linear regression, with a sigmoid logistic fit function. Inflection points calculated are synonymous with EC_{50} values.

Comparisons with commercial and energy-derived NA sources

To compare toxicity data from this study with toxicity data from other commercial NAs and toxicity data for NAs from energy derived waters in peer-reviewed literature, data were organized and summarized by species. Information included source of NAs, analytical methods and extraction techniques (where appropriate), duration of tests, species and age of organisms used, endpoint measured, estimated toxicity values, and pH measured in exposures (since pH influences solubility of NAs, and therefore exposures).

Results and Discussion

Confirmation of Fluka NA exposures

Measured exposure concentrations of Fluka NAs were within ±0.6 mg/L of nominal concentrations for animal species, which provided sufficient precision to discern differences in responses due to exposures in a narrow range of concentrations (Table 2.2). For *T. latifolia*, measured exposures of Fluka NAs were within ±25% of nominal concentrations (Table 2.2). In this study, the derivatization method with analysis using HPLC (Yen et al., 2004) confirmed exposures that elicited responses of organisms as a function of increasing NA concentrations.

Responses of organisms to Fluka naphthenic acid exposures

In response to Fluka NA exposures, the 7-d LC50 for *P. promelas* was 1.9 mg/L (Figure 2.1; Table 2.4), and the 7-d NOEC and LOEC were 0.4 and 1.2 mg/L, respectively ($\alpha = 0.05$; $p = 0.9429$ and 0.0003). Next in order of decreasing sensitivity, the 7-d LC50 for *C. dubia* was 2.8 mg/L (Figure 2.1; Table 2.4), and the 7-d NOEC and LOEC were 1.5 and 2.2 mg/L, respectively ($\alpha = 0.05$; $p = 1.000$ and 0.0009). For the benthic amphipod, *H. azteca*, the 7-d LC50 was 4.1 mg/L (Figure 2.1; Table 2.4), and the 7-d NOEC and LOEC were 1.5 and 2.6 mg/L ($\alpha = 0.05$; $p = 0.9015$ and 0.0221). For the midge, *C. dilutus*, the 7-d LC50 was 6.5 mg/L (Figure 2.1; Table 2.4), and the 7-d NOEC and LOEC were 3.3 and 4.6 mg/L ($\alpha = 0.05$; $p = 0.09877$ and 0.0334, respectively). The least sensitive species evaluated was the common cattail, *T. latifolia.* Responses of *T. latifolia* shoots (in terms of growth) were not sensitive enough to discern adverse effects from Fluka NA exposures, therefore, root growth was used to derive median effect

concentrations and the NOEC and LOEC. In terms of root growth, the 7-d EC50 for *T. latifolia* was 56.2 mg/L (Figure 2.2; Table 2.4), and the NOEC and LOEC were 25.2 and 49.8 mg/L, respectively ($\alpha = 0.05$; $p = 0.2318$ and 0.0003). Based on these results, *P. promelas* was the most sensitive species to Fluka NA exposures, followed by *C. dubia*, *H. azteca*, *C. dilutus*, and *T. latifolia*.

Explanatory parameters

Dissolved oxygen, conductivity, and hardness were within ranges for tolerances of organisms at test initiations and completions (Table 2.5). At test completions, pH (7.64-8.39) and alkalinity (56-96 mg/L as $CaCO_3$; Table 2.5), in exposures were sufficient to maintain solubility of NAs. In a study to measure the influence of salts on toxicity of NAs, Kavanagh et al. (2012) measured approximately 50% decreased toxicity to *P. promelas* larvae when 700 mg/L sodium bicarbonate ($NaHCO_3$) was added to exposures of NAs extracted from oil sands process affected waters (relative to exposures without additions of $NaHCO_3$). Kavanagh et al. (2012) suggested that bicarbonate may interfere with NA movement through cell membranes via competition for adsorption sites. Bicarbonate concentrations in exposures in the present study (100 mg/L) were likely not sufficient to interfere with NA uptake by organisms.

Reference toxicant exposures and responses

Measured acid soluble copper concentrations for reference toxicity tests were within ±20% of nominal concentrations with the exception of one exposure for *C. dilutus* (+24% of nominal concentration 0.2 mg/L; Table 2.3). In response to exposures of

copper as copper sulfate, the 7-d LC50 for *P. promelas* was 0.169 mg/L (Table 2.4), and the 7-d NOEC and LOEC were 0.049 and 0.095 mg/L, respectively ($\alpha = 0.05$; $p = 0.7143$ and 0.0032). *C. dubia* was more sensitive than *P. promelas*, with a 7-d LC50 of 0.037 mg/L (Table 2.4), and 7-d NOEC and LOEC of 0.027 and 0.035 mg/L, respectively ($\alpha = 0.05$; $p = 0.8546$ and <0.0001). The benthic amphipod, *H. azteca*, was the least sensitive species evaluated to aqueous copper exposures, with a 7-d LC50 of 0.503 mg/L (Table 2.4), and 7-d NOEC and LOEC of 0.231 and 0.351 mg/L, respectively ($\alpha = 0.05$; $p = 0.7856$ and 0.0462). The 7-d LC50 for *C. dilutus* was 0.215 mg/L (Table 2.4), with a NOEC and LOEC of 0.102 and 0.248 mg/L, respectively ($\alpha = 0.05$; $p = 0.4083$ and 0.0075). In terms of root growth, the EC50 for *T. latifolia* was 0.089 mg/L (Table 2.4). The NOEC and LOEC for *T. latifolia* were 0.012 and 0.068 mg/L, respectively ($\alpha = 0.05$; $p = 1.000$ and 0.0326). Copper sulfate reference toxicity data in the present study were consistent with reported inter- and intra-laboratory toxicity data (Suedel et al., 1996; Deaver and Rodgers, 1996; Mastin and Rodgers, 2000; Muller et al., 2001; Murray-Gulde et al., 2002). Measurements of exposures and responses in reference toxicity tests are an important quality assurance tool to confirm health of organisms used in testing (Jop et al., 1986). Dissolved oxygen, pH, conductivity, alkalinity, and hardness were consistent with explanatory parameters in Fluka NA toxicity tests (Table 2.5).

Comparisons with other commercial NAs

Exposures of commercial NAs in published toxicity studies varied in terms of source, duration, and analytical techniques used to confirm exposures. Swigert et al. (2015) used Merichem NAs in 48-h and 96-h exposures confirmed using FTIR

spectroscopy. Nero et al. (2006) used Acros Organics NAs in 96-h exposures confirmed using FTIR spectroscopy. Melvin and Trudeau (2012) used Fluka NAs in 72-h exposures, however, methods for exposure confirmation were not specified. Armstrong et al. (2007) used Fluka NAs in 30-d exposures confirmed with triple quadrupole mass spectrometry-electrospray ionization (ESI-MS). Peters et al. (2007) used Pfaltz-Bauer NAs in 9-d exposures confirmed using FTIR. Direct comparisons among toxicity data for commercial NAs should be made with caution, as sources were different, exposure durations ranged from 48-h to 30-d, and analytical techniques used to confirm exposures included unknown, FTIR, and ESI-MS (Table 6).

In response to Fluka NA exposures, Melvin and Trudeau (2012) estimated 72-h LC50s of 4.1 mg/L and 2.95 mg/L for Gosner stage 5 Northern leopard frog (*Lithobates pipiens*) and Nieuwkoop and Faber Stage 4 Western clawed frog (*Silurana tropicalis*), respectively. Analytical techniques for Fluka NA exposures and pH were not specified by Melvin and Trudeau (2012). Armstrong et al. (2007) reported decreases in water uptake by mature *T. latifolia* exposed to 60 mg/L Fluka NAs at pH 7.8 over 30 days (decrease from 200 mL on day 0 to 150 mL on day 30) relative to untreated controls (increase from 200 mL on day 0 to ≈500 mL on day 30). Armstrong et al. (2007) confirmed exposures using triple quadrupole mass spectrometry-electrospray ionization (MS-ESI). With a limited array of species evaluated for toxicity of Fluka NAs, the ability to compare responses is constrained.

In response to Merichem NA exposures, Swigert et al. (2015) measured effects on *P. promelas*, *D. magna*, and *P. subcapitata*. Swigert et al. (2015) estimated a 96-h LC50

of 5.6 mg/L for juvenile *P. promelas* at pH 8.0-8.4, a 48-h EC50 of 20 mg/L for <24-h *D. magna* at pH 7.5-8.6, and a 96-h EC50 of 30 mg/L for *P. subcapitata* at pH 6.8-8.9. Relative sensitivities between fish, invertebrates, and algae estimated by Swigert et al. (2015) were consistent with relative sensitivities of fish, invertebrates, and the macrophyte estimated in the present study. Hagen et al. (2012) measured acute and chronic immunotoxic effects to goldfish (*Carassius auratus*). In 7-d exposures, Hagen et al. (2012) reported increased pro-inflammatory cytokine gene expressions in gills, livers, and spleens at 10 and 20 mg/L Merichem NAs (nominal concentrations reported and pH not reported). In 7-d exposures, there were no differences in macrophage production in kidneys in 1, 5, and 10 mg/L exposures, but macrophage production increased in 20 mg/L exposures. In 8 week exposures, there were no differences in mortality in 1, 5, and 10 mg/L in fish exposed to a blood parasite, however, there was increased mortality in 20 mg/L exposures (Hagen et al., 2012). In 12 week exposures at 10 mg/L, several pro-inflammatory cytokines in kidneys and spleens were down-regulated (Hagen et al., 2012). The information provided by Hagen et al. (2012) in this biomarker study could benefit from further contextual information such as survival, growth, and reproduction. Relative to other sources of NAs, there is a wider variety of species evaluated for Merichem NAs, with sufficient information to develop a species' sensitivity distribution.

Information regarding responses to exposures is limited for three commercial NA sources (Acros, Eastman Chemicals, and Pfaltz Bauer). In response to Acros organics NA exposures, Nero et al. (2006) estimated a 96-h LC100 of 3.6 mg/L for juvenile yellow perch (*Perca flavescens*; confirmed using FTIR) at pH 8.38. Peters et al. (2007)

estimated threshold effects concentrations (calculated as the mean of the NOEC and LOEC) in 9-d exposures for larval yellow perch (0.88 mg/L) and larval Japanese medaka (*Oryzias latipes*; 1.44 mg/L) for Pfaltz-Bauer NAs confirmed using FTIR at pH 6.78-8.34. These studies provide information regarding the relative sensitivity of yellow perch and Japanese medaka to commercial NA exposures, although direct comparisons cannot be made due to different exposure sources and durations.

In response to exposures of Eastman Chemical NAs, Dorn (1992) estimated a 96-h LC50 of ≈5.0 mg/L (reported as nominal concentration) for juvenile three spine stickleback to exposures spiked in a non-toxic refinery effluent at pH 8.0. Since there are apparently no other toxicity data for Eastman Chemical NAs or three spine stickleback to exposures of commercial NAs, these findings cannot be used for toxicity comparisons in this context. However, the study conducted by Dorn (1992) illustrates a useful approach for confirming toxicity due to the NA fraction in refinery effluent.

Interestingly, for a variety of fish species, responses were measured within a relatively narrow range of commercial NA concentrations (0.88 mg/L-5.6 mg/L), although exposures differed in terms of durations and sources of NAs. It is apparent that fish are relatively sensitive species and are in some cases, more sensitive than invertebrates (see Table 2.6). Due to relatively high potencies of commercial NAs, differences in sensitivities of fish species are not discernable within this range of effect concentrations. If age or life history of fish influence measured toxicity, then specificity in terms of the age of test species is important for these comparisons.

Toxicity studies for benthic invertebrates exposed to commercial NAs are lacking. Since it is reasonable that benthic species would be exposed as other aquatic species, and results from the present study demonstrate their relative sensitivities, additional data are needed for accurate predictions of risk. In the peer-reviewed literature, plants and algae are less sensitive than fish and invertebrates to exposures of commercial NAs (Armstrong et al. 2007; Swigert et al., 2015; Table 2.6).

Chronic toxicity studies are limited in the literature for commercial NAs. The focus on acute effects in studies of commercial NAs is likely due to short exposure durations (<10 days) of relatively lower molecular weight (<<500 Da) NA compounds (Scott et al., 2005; Han et al., 2008). Nonetheless, lack of chronic toxicity studies for commercial NAs represents a significant data gap. Acute/chronic ratios permit predictions of concentrations expected to elicit chronic toxicity from available acute toxicity data when chronic toxicity data are unavailable (USEPA, 1991). Without chronic toxicity data for commercial NAs, these ratios cannot be estimated.

Comparisons with energy-derived NAs

NAs have been identified as a source of toxicity in oil sands process-affected waters (OSPWs; Mackinnon and Boerger, 1986; Schramm, 2000; Clemente and Fedorak, 2005) and have been studied more intensely than NAs in other energy-derived sources. NAs extracted from petroleum distillates for commercial production are not sourced from bituminous oil sands (Brient et al., 1995), and therefore original petroleum sources for commercial and OSPW-derived NAs are different. Caustic hot water processes used to separate bitumen from sand deposits permit solubilization of NAs in process waters

(Clemente and Fedorak, 2005; Allen, 2008). Biodegradation of NAs over time in process waters decreases concentrations of relatively labile, lower molecular weight NAs, altering profiles to larger proportions of more recalcitrant, higher molecular weight compounds, referred to as weathering (Holowenko et al., 2002). Similar caustic extraction processes are used to retrieve NAs from petroleum distillates for commercial production (Brient et al. 1995), however, refined commercial NAs are stored in stable conditions. For these reasons, exposures of commercial and OSPW-derived NAs are fundamentally different, and toxicity data are not comparable.

The majority of published toxicity data for NAs from OSPWs report chronic effects, which is appropriate due to the relative abundance of recalcitrant weathered NAs in OSPWs (compared to commercial mixtures) expected to elicit longer durations of exposures. In terms of chronic effects, Kavanagh et al. (2012) measured decreased reproductive toxicity in terms of fecundity, number of male tubercles, and number of spawns in 9-month old fathead minnows at 10 mg/L NAs extracted from OSPW in 21-d exposures at pH 8.6 (Table 2.7). Peters et al. (2007) measured decreased growth in larval yellow perch and Japanese medaka at 1.92 and 6.18 mg/L NAs, respectively in 9-d exposures to whole OSPWs at pH 6.78-8.34 (NAs not extracted; Table 2.7). Leclair et al. (2013) measured decreased blood platelets in spleens of adult rainbow trout at 8 mg/L NAs extracted from OSPW at pH 8.26 (Table 2.7). These data indicate that among a range of NA exposures from OSPWs, fish were relatively sensitive, with adverse chronic effects observed at 1.92-16 mg/L NAs. Macrophytes were relatively less sensitive, with adverse chronic effects observed around 50-60 mg/L NAs (decreased fresh weight gain in

mature *Phragmites australis*; Armstrong et al., 2009; Armstrong et al., 2010). For invertebrates, Anderson et al. (2012) conducted a study to measure responses of *C. dilutus* to exposures of OSPW in which NAs were quantified, and found that NA concentrations in OSPW strongly correlated with survival, pupation, and emergence.

NAs are a complex group of carboxylic acids in a range of structural compositions obtained from various sources. Due to fundamental differences in exposures, published aquatic toxicity data among commercial and energy-derived (i.e. OSPW) NAs are not comparable. Factors that influence exposures (source, structure, duration, and pH) and measurements of exposures (extraction and analytical techniques) must be thoroughly considered when interpreting toxicity data. Regardless of differences in exposures for commercial and energy-derived NAs, fish were relatively sensitive species and are likely appropriate sentinel species for risk evaluations.

Conclusions

This comparative study of the acute toxicity of Fluka NAs to sentinel aquatic species serves to provide context for toxicity of commercial NA sources relative to energy derived sources of NAs. In general, the responses (7-d LC50s/EC50) ranged from 1.9 mg/L for *Pimephales promelas* to 56.2 mg/L for *Typha latifolia*. Following *P. promelas* in order of decreasing sensitivity were *Ceriodaphnia dubia* (7-d LC50=2.8 mg/L), *Hyalella azteca* (7-d LC50= 4.1 mg/L), *Chironomus dilutus* (7-d LC50= 6.5 mg/L), and *Typha latifolia* (7-d EC50= 56.2 mg/L), indicating that in terms of sensitivities, fish>invertebrates>plant for Fluka NAs. Since exposures differed in terms of source, duration, and analytical technique to confirm exposures, published toxicity data for commercial NAs were not directly comparable. However, effect concentrations ranged between 0.88 mg/L and 5.6 mg/L NAs for five fish species (fathead minnow, yellow perch, three spine stickleback, Japanese medaka, and goldfish). Based on data for other species, the fish evaluated were more sensitive to commercial NA exposures than invertebrates and vascular and non-vascular plants. Factors that affect exposures (i.e. source, structure, duration, and pH) and measurements of exposures (i.e. extraction and analytical techniques) differ among commercial and energy-derived NAs and severely limit comparisons. However, fish species evaluated (fathead minnow, yellow perch, Japanese medaka, and rainbow trout) were also more sensitive than a bacterium and several macrophytes to energy-derived NAs from different sources in chronic tests and are likely appropriate sentinel species for risk evaluations in aquatic systems.

References

Allen, E. W., 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science 7, 123-138.

American Petroleum Institute (API), 2012. Naphthenic acids category analysis and hazard characterization. Petroleum HPV Testing Group Technical Report # 1100997.

American Public Health Association (APHA), 2007. *Standard methods for the examination of water and wastewater*, 21st Ed. American Public Health Association, Port City Press: Baltimore, MD, pp. 1368.

Anderson, J., Wiseman, S. B., Moustafa, A., El-Din, M. G., Liber, K., & Giesy, J. P., 2012. Effects of exposure to oil sands process-affected water from experimental reclamation ponds on Chironomus dilutus. Water Research 46, 1662-1672.

Armstrong, S.A., Headley, J.V., Peru, K.M., & Germida, J.J., 2007. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering 43, 36-42.

Armstrong, S.A., Headley, J.V., Peru, K.M., & Germida, J.J., 2009. Differences in phytotoxicity and dissipation between ionized and nonionized oil sands naphthenic acids in wetland plants. Environmental Toxicology and Chemistry 28, 2167-2174.

Armstrong, S. A., Headley, J. V., Peru, K. M., Mikula, R. J., & Germida, J. J., 2010. Phytotoxicity and naphthenic acid dissipation from oil sands fine tailings treatments planted with the emergent macrophyte Phragmites australis. Journal of Environmental Science and Health Part A 45, 1008-1016.

Barrow, M. P., Headley, J. V., Peru, K. M., & Derrick, P. J., 2004. Fourier transform ion cyclotron resonance mass spectrometry of principal components in oil sands naphthenic acids. Journal of Chromatography A 1058, 51-59.

Bliss, C. I., 1935. The calculation of the dosage-mortality curve. Annals of Applied Biology 22, 134-167.

Brient, J. A., Wessner, P. J., & Doyle, M. N., 1995. Naphthenic acids. *In Kirk-Othmer*

encyclopedia of chemical technology, 5th ed. by Kirk-Othmer (ed.). John Wiley & Sons, Inc. Hoboken, NJ, USA.

Clemente, J.S. & Fedorak, P.M., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Deaver E., & Rodgers, J.H., 1996. Measuring bioavailable copper using anodic stripping voltammetry. Environmental Toxicology and Chemistry 15, 1925-1930.

Dorn, P.B., 1992. Case histories—the petroleum industry. Toxicity Reduction: Evaluation and Control. In: Ford, DL (Ed.), Water Quality Management Library 3, 183-223.

Farwell, A., Nero, V., Croft, M., Bal, P., & Dixon, D. G., 2006. Modified Japanese medaka embryo-larval bioassay for rapid determination of developmental abnormalities. Archives of Environmental Contamination and Toxicology 51, 600-607.

Frank, R. A., Kavanagh, R., Burnison, B. K., Arsenault, G., Headley, J. V., Peru, K. M., Van Der Kraak, G., & Solomon, K. R., 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 72, 1309-1314.

Girling, A. E., Whale, G. F., & Adema, D. M. M., 1994. A guideline supplement for determining the aquatic toxicity of poorly water-soluble complex mixtures using water-accommodated fractions. Chemosphere 29, 2645-2649.

Hagen, M. O., Garcia-Garcia, E., Oladiran, A., Karpman, M., Mitchell, S., El-Din, M. G., Martin, J.W., & Belosevic, M., 2012. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquatic Toxicology 109, 143-149.

Han, X., Scott, A. C., Fedorak, P. M., Bataineh, M., & Martin, J. W., 2008. Influence of molecular structure on the biodegradability of naphthenic acids. Environmental Science & Technology 42, 1290-1295.

Headley, J. V., Peru, K. M., Adenugba, A. A., Du, J. L., & Mcmartin, D. W., 2010. Dissipation of naphthenic acids mixtures by lake biofilms. Journal of Environmental Science and Health Part A 45, 1027-1036.

Holowenko, F.M., MacKinnon, M.D., & Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research 36, 2843-2855.

Jop, K.M., Rodgers, J.H., Dorn, P.B., & Dickson, K.L., 1986. Use of hexavalent chromium as a reference toxicant in aquatic toxicity tests. Aquatic Toxicology and Environmental Fate, ASTM STP 921, 390-403.

Kavanagh, R. J., Frank, R. A., Burnison, B. K., Young, R. F., Fedorak, P. M., Solomon, K. R., & Van Der Kraak, G., 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116, 34-42.

Leclair, L. A., MacDonald, G. Z., Phalen, L. J., Köllner, B., Hogan, N. S., & van den Heuvel, M. R., 2013. The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 142, 185-194.

MacKinnon, M. D., & Boerger, H., 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Quality Research Journal of Canada 21, 496-512.

Mastin, B. J., & Rodgers Jr, J. H., 2000. Toxicity and bioavailability of copper herbicides (Clearigate, Cutrine-Plus, and copper sulfate) to freshwater animals. Archives of Environmental Contamination and Toxicology 39, 445-451.

Melvin, S. D., & Trudeau, V. L., 2012. Growth, development and incidence of deformities in amphibian larvae exposed as embryos to naphthenic acid concentrations detected in the Canadian oil sands region. Environmental Pollution 167, 178-183.

Moore, M.T., Huggett, D.B., Huddleston III, G.M., Rodgers Jr., J.H., Cooper, C.M., 1999. Herbicide effects on *Typha latifolia* (Linneaus) germination and root and shoot development. Chemosphere 38, 3637-3647.

Muller, S.L., Huggett, D.B., Rodgers Jr., J.H., 2001. Effects of copper sulfate on *Typha latifolia* seed germination and early growth in aqueous and sediment exposures. Archives of Environmental Contamination and Toxicology 40, 192-197.

Murray-Gulde, C. L., Heatley, J. E., Schwartzman, A. L., & Rodgers Jr, J. H., 2002. Algicidal effectiveness of Clearigate, Cutrine-Plus, and copper sulfate and margins of safety associated with their use. Archives of Environmental Contamination and Toxicology 43, 19-27.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., & Dixon, D.G., 2006. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicology and Environmental Safety 65, 252-264.

OECD (Organization for Economic Cooperation and Development), 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Series on Testing and Assessment No. 23, ENV/JM/MONO (2000), vol. 6. OECD, Paris, France.

Peters, L. E., MacKinnon, M., Van Meer, T., van den Heuvel, M. R., & Dixon, D. G., 2007. Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) embryonic development. Chemosphere 67, 2177-2183.

Rudzinski, W. E., Oehlers, L., Zhang, Y., & Najera, B., 2002. Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. Energy & Fuels 16, 1178-1185.

Schramm, L. L., 2000. *Surfactants: fundamentals and applications in the petroleum industry*. Cambridge University Press.

Scott, A.C., Mackinnon, M.D., & Fedorak, P.M., 2005. Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environmental Science and Technology 39, 8388-8394.

Seifert, W.K. & Teeter, R.M., 1969. Preparative thin-layer chromatography and high-resolution mass spectrometry of crude oil carboxylic acids. Analytical Chemistry 41, 786-795.

Sigma-Aldrich, 2015. Material Safety Data Sheet for Naphthenic Acids.

Siwik, P. L., Van Meer, T., MacKinnon, M. D., & Paszkowski, C. A., 2000. Growth of fathead minnows in oil sand-processed wastewater in laboratory and field. Environmental Toxicology and Chemistry 19, 1837-1845.

Suedel, B.C., Deaver, E., & Rodgers Jr., J.H., 1996. Experimental factors that may affect toxicity of aqueous and sediment-bound copper to freshwater organisms. Archives of Environmental Contamination and Toxicology 30, 40-46.

Swigert, J.P., Lee, C., Wong, D.C., White, R., Scarlett, A.G., West, C.E., Rowland, S.J., 2015. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere 124, 1-9.

Tomczyk, N.A., Winans, R.E., Shinn, J.H., & Robinson, R.C., 2001. On the nature and origin of acidic species in petroleum. 1. Detailed acid type distribution in a California crude oil. Energy & Fuels 15, 1498-1504.

United States Environmental Protection Agency (USEPA)., 1991. Technical support document for water quality-based toxics control. Washington DC. EPA/505/2-90-001.

United States Environmental Protection Agency (USEPA)., 2002. Methods for measuring acute toxicity of effluents and receiving waters to freshwater and marine organisms. Washington DC. EPA-821-R-02-12.

United States Environmental Protection Agency (USEPA), 2012. Screening-level hazard characterization: Reclaimed substances (Naphthenic acids category). Washington DC. EPA-821-R-02-12.

Yen, T.W., Marsh, W.P., MacKinnon, M.D., & Fedorak, P.M., 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. Journal of Chromatography A 1033, 83-90.

Table 2.1: Physical and chemical characteristics of Fluka commercial naphthenic acids (Sigma-Aldrich)[1]

Parameter	General Characteristic	Cite
Identification	1338-24-5 (CAS No)	Sigma-Aldrich, 2015
Color	Pale yellow	Sigma-Aldrich, 2015
Physical State	Viscous liquid	Sigma-Aldrich, 2015
Molecular Weight[2]	210-250 amu	Brient et al., 1995
Water Solubility[1]	88.1 mg/L at pH 7.5	API, 2012
Vapor pressure	1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C	USEPA, 2012
Partition coefficient octanol/ water[3]	˜4 at pH 1	Schramm, 2000
(Log Kow)[3]	˜2.4 at pH 7	Schramm, 2000
	< 0.1 at pH 10	Schramm, 2000
Density	0.92 g/mL at 20°C	Sigma-Aldrich, 2015
Flash Point	101°C	Sigma-Aldrich, 2015
Initial Boiling Point	106.4-333.6°C	Sigma-Aldrich, 2015
Viscosity	22 mm2/s	Sigma-Aldrich, 2015
pKa	5 to 6	Brient et al., 1995

[1]Alkylated cyclopentane carboxylic acids (mixture)
[2]Average molecular weight for refined naphthenic acids
[3]Weathered naphthenic acid mixture

Table 2.2: Nominal and average measured NA concentrations in exposures (n=3). (-) indicates measured peak areas were below blank peak areas.

Species	Nominal Concentration (mg/L)	Measured Concentration (mg/L)
P. promelas	0	(-)
	0.5	0.4
	1	1.2
	2	2.3
	3	2.8
	4	3.8
C. dubia	0	0.8
	1	1.5
	2	2.2
	3	3.2
	4	3.9
	5	5.1
	7	7.2
H. azteca	0	(-)
	1	1.5
	2	2.6
	3	3.1
	4	4.1
	5	5.3
	7	6.7
C. dilutus	0	(-)
	2	1.8
	5	4.6
	8	8.6
	10	9.7
	13	12.4
T. latifolia	0	2.6
	20	25.2
	40	49.8
	60	61.6
	80	67.1
	100	100.2

Table 2.3: Nominal and average measured copper concentrations for reference toxicity tests (n=3)

Species	Nominal Concentration (mg/L)	Measured Concentration (mg/L)
P. promelas	0	0.007
	0.05	0.050
	0.1	0.095
	0.2	0.190
	0.3	0.299
	0.4	0.365
	0.5	0.476
C. dubia	0	0.002
	0.005	0.004
	0.01	0.009
	0.02	0.018
	0.03	0.035
	0.05	0.046
	0.07	0.067
H. azteca	0	0.045
	0.2	0.231
	0.35	0.351
	0.5	0.466
	0.6	0.578
	0.9	0.879
C. dilutus	0	0.029
	0.1	0.102
	0.2	0.248
	0.3	0.307
	0.5	0.503
	0.6	0.656
T. latifolia	0	0.006
	0.015	0.012
	0.075	0.068
	0.1	0.099
	0.150	0.144
	0.200	0.200

Table 2.4: 7d NOECs, LOECs, and LC50s/EC50 for *P. promelas, C. dubia, H. azteca*, and *C. dilutus*, and in terms of root growth for *T. latifolia* for Fluka NAs and copper sulfate. C.I.= confidence interval.

Species	Fluka NA concentration (mg/L)*	Copper Concentration (mg/L as $CuSO_4$)**
P. promelas		
NOEC	0.4	0.050
LOEC	1.2	0.095
LC50 (95% C.I.)	1.9 (0.8-3.2)	0.169 (0.089-0.284)
C. dubia		
NOEC	1.5	0.027
LOEC	2.2	0.035
LC50 (95% C.I.)	2.8 (1.9-3.8)	0.037 (0.027-0.054)
H. azteca		
NOEC	1.5	0.231
LOEC	2.6	0.351
LC50 (95% C.I.)	4.1 (2.8-6.1)	0.503 (0.319-1.039)
C. dilutus		
NOEC	3.3	0.102
LOEC	4.6	0.248
LC50 (95% C.I.)	6.5 (2.3-10.9)	0.215 (0.023-0.462)
T. latifolia		
NOEC	25.2	0.012
LOEC	49.8	0.067
EC50 (95% C.I)	(52.4-60.1)	0.089 (0.081-0.098)

*Measured according to derivatization methods of Yen et al. (2004)
**Measured as acid soluble copper

Table 2.5: Water characteristics (ranges) at test initiation and completion for toxicity tests.

Organism	Time	Exposure	pH (S.U)	D.O. (mg/L)	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
P. promelas	Initial	NAs	8.30-8.39	7.55-8.26	321.5-379.4	68-74	99-128
		$CuSO_4$	8.08-8.32	7.01-8.32	318.2-328.3	54-66	96-122
	Final	NAs	8.03-8.31	6.87-8.70	437.8-551.0	92-96	94-136
		$CuSO_4$	8.14-8.30	6.84-8.34	405.0-459.0	70-86	102-126
C. dubia	Initial	NAs	8.29-8.31	6.76-7.82	345.8-417.8	62-80	96-112
		$CuSO_4$	8.37-8.41	7.73-8.62	318.0-320.8	68-82	100-130
	Final	NAs	8.18-8.33	7.32-8.02	366.3-428.5	64-80	100-122
		$CuSO_4$	8.15-8.30	7.89-8.64	315.2-340.4	58-78	96-112
H. azteca	Initial	NAs	8.27-8.38	7.11-8.54	337.6-388.0	66-78	92-102
		$CuSO_4$	8.20-8.38	8.33-8.53	350.0-370.0	68-88	98-110
	Final	NAs	8.21-8.39	6.99-7.56	353.0-402.6	60-80	110-114
		$CuSO_4$	8.19-8.36	7.53-8.11	390.0-410.0	68-74	100-110
C. dilutus	Initial	NAs	8.20-8.39	9.15-9.21	320.4-350.5	62-72	70-92
		$CuSO_4$	7.83-7.96	9.39-9.52	380.3-388.0	58-72	88-96
	Final	NAs	7.64-7.88	8.90-9.25	370.9-383.0	56-68	80-88
		$CuSO_4$	7.57-7.78	9.48-9.65	420.6-430.9	62-84	96-108
T. latifolia	Initial	NAs	8.30-8.38	7.88-8.13	359.8-388.0	72-80	102-114
		$CuSO_4$	8.08-8.35	7.99-8.75	398.0-433.6	64-70	98-116

Table 2.6: Reported toxicity values and test conditions for exposures of commercial NAs. NR= not reported.

NA Source	Analytical Method	Test Species	Test Species Age/Density	Exposure Duration	Toxicity Values	Endpoint	pH	Reference
Fluka	HPLC	*Pimephales promelas*	< 24h	7-d	LC50: 1.9 mg/L	Mortality	8.03-8.39	Current Study
Merichem	FTIR	*Pimephales promelas*	Juvenile	96-h	LC50: 5.6 mg/L	Mortality	8.0-8.40	Swigert et al. 2015
Pfaltz-Bauer	FTIR	*Perca flavescens* Yellow perch	Larvae	9-d	a. 1.67 mg/L[a] b. 0.88 mg/L[a]	a. % deformed b. length	7.1-7.19	Peters et al. 2007
Acros Organics	FTIR	*Perca flavescens*	Juvenile	96-h	LC100: 3.6 mg/L	Mortality	8.38	Nero et al. 2006
Eastman Chemicals	n/a; nominal	*Gasterosteus aculeatus* Three-spine stickleback	Juvenile	96-h	LC50: ~ 5 mg/L	Mortality	8.0	Dorn, 1992
Pfaltz-Bauer	FTIR	*Oryzias latipes* Japanese medaka	Larvae	9-d	a. 1.51 mg/L b. 1.44 mg/L	a. % deformed b. length	7.1-7.19	Peters et al. 2007
Merichem	n/a; nominal	*Carassius auratus* Goldfish	NR	7-d	10 mg/L	Increased cytokine gene expressions in gills, liver, and spleen	NR	Hagen et al. 2012
Merichem	n/a; nominal	*Carassius auratus*	NR	7-d	20 mg/L	Increased kidney macrophage production	NR	Hagen et al. 2012
Merichem	n/a; nominal	*Carassius auratus*	NR	a. 8 wks b. 12 wks	a. 20 mg/L b. 10 mg/L	a. Increased mortality due to infection b. Down-regulation of pro-inflammatory cytokines in kidney and spleen	NR	Hagen et al. 2012

Table 2.6 continued

NA Source	Analytical Method	Test Species	Test Species Age/Density	Exposure Duration	Toxicity Values	Endpoint	pH	Reference
Fluka	HPLC	*Ceriodaphnia dubia*	< 24h	7-d	LC50: 2.8 mg/L	Mortality	8.18-8.33	Current Study
Merichem	FTIR	*Daphnia magna*	< 24h	48-h	EC50: 20 mg/L	Immobilization	7.5-8.6	Swigert et al. 2015
Fluka	HPLC	*Hyalella azteca*	≈ 2 wks	7-d	LC50: 4.1 mg/L	Mortality	8.21-8.39	Current Study
Fluka	HPLC	*Chironomus dilutus*	2nd instar larvae	7-d	LC50: 6.5 mg/L	Mortality	7.64-8.39	Current Study
Fluka	NR	*Lithobates pipiens* Northern Leopard frog	Gosner Stage 5	72-h	LC50: 4.1 mg/L	Mortality	NR	Melvin and Trudeau, 2012
Fluka	NR	*Silurana tropicalis* Western clawed frog	Nieuwkoop & Faber Stage 4	72-h	LC50: 2.95 mg/L	Mortality	NR	Melvin and Trudeau, 2012
Merichem	FTIR	*Pseudokirchneriella subcapitata*	10^4cells/ mL	96-h	EC50: 30 mg/L	% growth rate inhibition	6.8-8.9	Swigert et al. 2015
Fluka	HPLC	*Typha latifolia*	2d	7-d	EC50: 56.2 mg/L	Seedling root growth	8.30-8.38	Current Study
Fluka	Triple quadrupole MS-ESI	*Typha latifolia*	Mature	10-d	60 mg/L	Water uptake	7.8	Armstrong et al. 2007
Merichem	n/a; nominal	*Vibrio fischeri*	≈$1x10^6$ cells/mL	15 min.	EC50: 46 mg/L	% inhibition of luminescence	NR	Swigert et al. 2015

[a]Toxicity values defined as "threshold concentrations" calculated as the mean of estimated NOECs and LOECs

Table 2.7: Reported toxicity values and test conditions for NAs in energy-derived waters. OSPW= oil sands process affected waters. NR= not reported.

Source	Analytical Method (Extraction Technique)	Test Species	Test Species Age	Exposure Duration	Toxicity Values	Endpoint	pH	Reference
OSPW	ESI-MS (Acid extraction)	*Pimephales promelas*	Embryos	9-d	LC50: 32.8 mg/L	Mortality	8.6±0.2	Kavanagh et al. 2012
OSPW	ESI-MS (Acid extraction)	*Pimephales promelas*	5-d larvae	96-h	LC50: 51.8 mg/L	Mortality	8.6±0.2	Kavanagh et al. 2012
OSPW	ESI-MS (Acid extraction)	*Pimephales promelas*	9-mo.	21-d	10 mg/L	Fecundity, male tubercles, # of spawns	8.6±0.2	Kavanagh et al. 2012
OSPW	FTIR[b]	*Perca flavescens* Yellow perch	Embryo/ Larvae	9-d	1.92 mg/L[c]	Length	6.78-8.34	Peters et al. 2007
OSPW	FTIR (Acid extraction)	*Perca flavescens*	Juvenile	96-h	LC100: 6.8 mg/L	Mortality	8.0-8.3	Nero et al. 2006
OSPW	FTIR[b]	*Oryzias latipes* Japanese medaka	Embryo/ Larvae	9-d	6.18 mg/L[c]	Length	6.78-8.34	Peters et al. 2007
OSPW	FTIR (Acid extraction)	*Oryzias latipes*	Larvae	18-d	LOEC: 16 mg/L	% heart deformities and cranial-skeletal deformities	NR	Farwell et al. 2006
OSPW	LC-HRMS (C18 extraction)	*Oncorhynchus mykiss* Rainbow trout	Adult	7-d	8 mg/L	Thrombocytes counts in spleen	8.26±0.05	Leclair et al. 2013
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Phragmites australis*	Mature	30-d	52.1 mg/L	Fresh weight gain	7.8	Armstrong et al. 2010

Table 2.7 (continued)

Source	Analytical Method	Test Species	Test Species Age/Density	Exposure Duration	Toxicity Values	Endpoint	pH	Reference
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Phragmites australis*	Mature	30-d	52.1 mg/L	Fresh weight gain	7.8	Armstrong et al. 2010
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Phragmites australis*	Mature	30-d	60 mg/L	Fresh weight gain	7.8	Armstrong et al. 2009
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Phragmites australis*	Mature	30-d	30 mg/L	Mortality	5.0	Armstrong et al. 2009
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Typha latifolia*	Mature	30-d	60 mg/L	Mortality	5.0	Armstrong et al. 2009
OSPW	Triple quadrupole MS-ESI (Liquid-liquid)	*Scirpus acutus*	Mature	30-d	30 mg/L	Fresh weight gain	5.0	Armstrong et al. 2009
OSPW	ESI-MS (Acid extraction)[a]	*Vibrio fischeri*	n/a	15 min.	EC50: 41.9 mg/L	Inhibitory effect	7.5±0.1	Frank et al. 2008b
OSPW	ESI-MS (Acid extraction)[a]	*Vibrio fischeri*	n/a	15 min.	EC50: 54.7 mg/L	Inhibitory effect	7.5±0.1	Frank et al. 2008b
OSPW	ESI-MS (Acid extraction)[a]	*Vibrio fischeri*	n/a	15 min.	EC50: 64.9 mg/L	Inhibitory effect	7.5±0.1	Frank et al. 2008b

[a]NA stocks were quantified using ESI-MS. Exposures were serial dilutions of stocks.
[b]NAs were not extracted from OSPW, but were quantified in the mixture using FTIR.
[c]Toxicity values defined as "threshold concentrations" calculated as the mean of the estimated NOECs and LOECs.

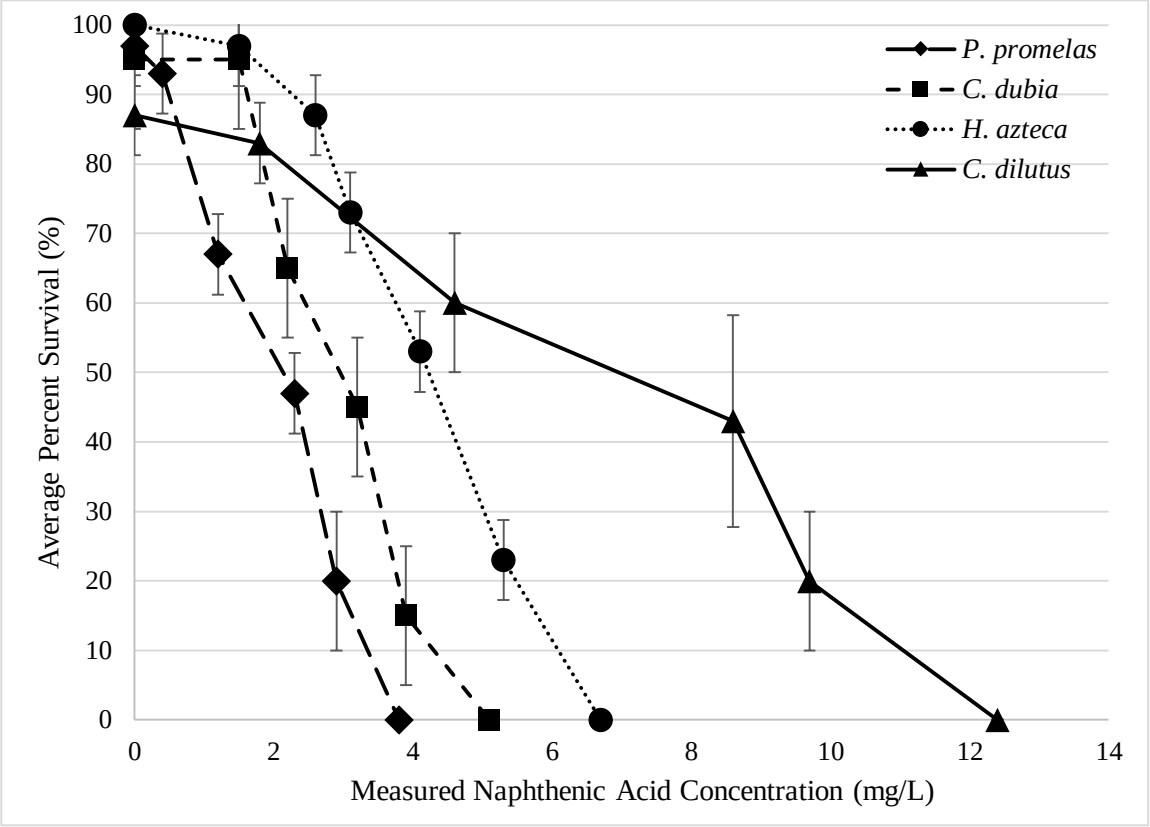


Figure 2.1: Responses of *P. promelas*, *C. dubia*, *H. azteca*, and *C. dilutus* in terms of survival to 7-d exposures of commercial NAs (*n*=3). Error bars indicate standard deviations.

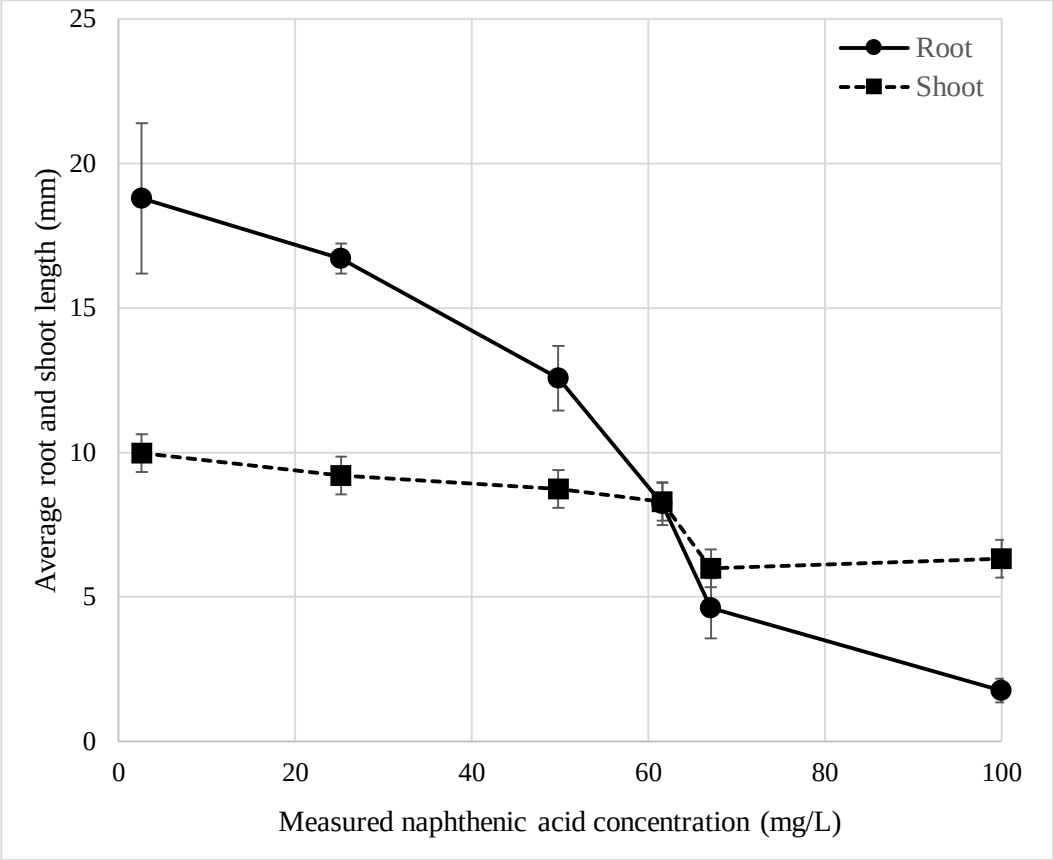


Figure 2.2: Responses of *T. latifolia* in terms of seedling root and shoot growth to 7-d exposures of commercial NAs (*n*=3). Error bars indicate standard deviations.

CHAPTER THREE

PHOTOCATALYSIS OF A COMMERCIAL NAPHTHENIC ACID IN WATER USING FIXED-FILM TiO_2

Abstract

Photolysis or photocatalysis may provide a process for mitigating ecological risks of naphthenic acids (NAs) contained in energy-derived waters such as refinery effluents and process waters. If effective, fixed-film TiO_2 photocatalysis of NAs could decrease operational expenses as well as capital costs for water treatment. Studies of photocatalytic NA degradation using slurries of titanium dioxide (TiO_2) have illustrated the potential utility of this approach. Alterations of exposures and mitigation of risks can be confirmed by coupling analytical quantification of NAs with responses of sentinel aquatic organisms. The overall objective of this study was to measure rates and extents of photolysis and photocatalytic degradation of Fluka commercial NAs using bench-scale fixed-film TiO_2 and confirm changes in NA concentrations using sensitive vertebrate (fish = *Pimephales promelas*) and invertebrate (*Daphnia magna*) species. To achieve this overall objective, specific objectives were to: 1) measure rates and extents of degradation of commercial (Fluka) NAs throughout an 8 hour duration of natural sunlight ("photolysis") and natural sunlight in the presence of fixed-film TiO_2 ("photocatalysis"), and 2) measure changes in toxicity after photolysis and photocatalysis treatments in terms of mortality with sentinel fish (*P. promelas*) and microinvertebrate (*D. magna*) species in 96-hr static tests. Bench-scale chambers were used to measure photocatalysis of NAs irradiated with sunlight in contact with a thin-film of TiO_2 and high performance liquid

chromatography was used to quantify NAs before and after treatment. After 4-hr in photocatalysis treatments, >92% decline was observed (initial concentration of 63 mg NA/L) with an average removal rate 15.5 mg/L hr^{-1} and half-live of 2 hours. There was no measurable NA toxicity for fish (*P. promelas*) or microinvertebrates (*D. magna*) after 5-hr. In sunlight only exposures (photolysis; no TiO_2), NA concentrations decreased by 42% following a 4-hr exposure (initial concentration of 65 mg NA/L). However, complete mortality was observed for sentinel species exposed to samples from 8-hr photolysis treatments and dark controls. Photocatalytic degradation using fixed-film TiO_2 irradiated with sunlight achieved efficacious rates (average removal rate 15.5 mg/L hr^{-1}) and extents (>92% decline in 4-hr) of removal of Fluka NAs and eliminated acute toxicity to sentinel aquatic organisms, indicating the potential for application of this technology for mitigating ecological risks associated with NAs. Coupled with existing treatment processes (i.e. aerobic biodegradation), photocatalysis can augment rates and extents of NA removal from impaired waters.

Introduction

Naphthenic acids (NAs) are a complex group of organic acids associated with crude oils (Seifert and Teeter, 1969; Tomcyzk et al., 2001) and energy derived process waters (Dorn, 1992; Allen, 2008). NAs are generally described by the formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and Z is either zero or a negative even integer representing the hydrogen deficiency of the molecule due to rings or double bonds (Holowenko et al., 2002; Clemente and Fedorak, 2005). NAs are sources of toxicity in energy derived waters such as refinery effluents and oil sands process affected waters (Dorn, 1992; Schramm, 2000), with adverse effects observed for fish, macro- and microinvertebrates, aquatic macrophytes, and microorganisms (Nero et al., 2006; Frank et al., 2008; Armstrong et al., 2008; Kavanagh et al., 2012; Leclair et al., 2013; Swigert et al., 2015). In addition, NAs are relatively persistent in water, with aerobic biodegradation half-lives for NAs in oil sands process affected waters ranging from months to years (Scott et al., 2005; Han et al., 2009; Headley et al., 2010). To mitigate risks due to NAs, exposures must be sufficiently altered to eliminate toxicity to aquatic organisms. To effectively alter exposures of NAs, potential transformation pathways with reasonable rates and extents of removal should be thoroughly evaluated.

Naphthenic acids are susceptible to photolysis (USEPA, 2012; Headley et al., 2009), and the rate of this transformation process can be enhanced using catalysts (e.g. titanium dioxide [TiO_2]). TiO_2 is widely used as a photocatalyst due to long term photo-stability, relative effectiveness, and stability in acidic and oxidative conditions (Bagheri et al., 2014). Degussa® and Aeroxide® P25 are commercial forms of TiO_2 previously

used in photocatalytic studies due to their relatively large surface area (~50 m^2/g) and high ratio (4:1) of anatase to rutile (Wold, 1993). Photocatalytic degradation of NA mixtures has been accomplished using TiO_2 in aqueous suspensions (i.e. "slurries") with both artificial ultraviolet (UV) irradiation (e.g. UV_{254} lamps) and natural sunlight (McMartin et al., 2004; Headley et al., 2009; Mishra et al., 2010). Bench-scale studies have demonstrated that rates of NA degradation using photocatalysis are of practical significance (in terms of scalability), with half-lives achieved in hours (Headley et al., 2009; Mishra et al., 2010). However, some photocatalytic design features may limit full-scale application. Post-treatment recovery of aqueous suspensions of TiO_2 particles may be challenging (Kinsinger et al., 2015) and artificial UV is energy intensive and impractical (Parsons, 2004; Metcalf and Eddy, 2004). Immobilizing TiO_2 on a fixed-film eliminates the need to amend and recover catalyst offering greater flexibility in treatment design. In addition, natural sunlight could provide sufficient energy to accomplish photocatalytic degradation of NAs while decreasing operational costs of treatment.

To evaluate the feasibility of fixed-film photocatalysis irradiated by natural sunlight for achieving degradation of NAs, commercially available NAs were used for this study. To assess performance of photolysis and photocatalysis, rates and extents of degradation of Fluka NAs have been measured (McMartin et al., 2004; Headley et al., 2009; Mishra et al., 2010), providing an opportunity to compare results from this study. In addition, Fluka NAs have been well studied as a simplistic analogue to understand more compositionally complex mixtures of NAs present in energy-derived waters (Headley and McMartin 2004; Barrow et al., 2004; Scott et al., 2005; Rudzinski et al.,

2002; Lo et al., 2006; Armstrong et al., 2008; Headley et al., 2010). Photolysis and photocatalytic oxidation can decrease concentrations and complexity of parent NA compounds (USEPA, 2012; McMartin et al., 2004; Headley et al., 2009); however, the question of post-treatment toxicity remains.

Decreased toxicity to sensitive species can confirm alteration of exposures of Fluka NAs achieved by fixed-film photocatalytic degradation. Sentinel species such as fathead minnow (*Pimephales promelas* Rafinesque) and microinvertebrate (*Daphnia magna* Straus) are relatively sensitive to NAs (Swigert et al., 2015; Kinley et al. in review). Commercial NA 96-hr LC_{50} for *P. promelas* is 5.6 mg NA/L and 48-hr EC_{50} (immobilization) for *D. magna* is 20 mg NA/L (Swigert et al., 2015), demonstrating mortality is informative to assess changes in NA concentrations. Elimination of toxicity to these sensitive species in post-photocatalytic degradation samples can confirm mitigation of risks, supporting observations of NA degradation measured analytically.

The overall objective of this study was to measure rates and extents of photolysis and photocatalytic degradation of a commercially available (Fluka) NA using bench-scale fixed-film TiO_2 and confirm changes in NA concentrations using sensitive vertebrate (fish = *Pimephales promelas*) and invertebrate (*Daphnia magna*) species. To achieve this overall objective, specific objectives were to: 1) measure the rates and extents of removal of commercial (Fluka) NAs throughout an 8 hour duration of natural sunlight ("photolysis") and natural sunlight in the presence of fixed-film TiO_2 ("photocatalysis"), and 2) measure changes in toxicity after photolysis and photocatalysis treatments (in

terms of mortality) with sentinel fish (*P. promelas*) and microinvertebrate (*D. magna*) species in 96-hr static tests.

Materials and Methods

Experimental Design

The experimental design (Figure 3.1) included three treatments: 1) Fluka NAs irradiated by natural sunlight in the presence of TiO_2 film ("photocatalysis"), 2) Fluka NAs irradiated by natural sunlight without TiO_2 film ("photolysis"), and 3) Fluka NAs in the presence of TiO_2 film with no sunlight ("dark control"). Treatments were conducted outdoors near Clemson, SC USA (34°40'6.14"N, 82°50'52.02"W) in November with clear, sunny conditions. All treatments were conducted in 28x43 cm Sterilite® high-density polyethylene (HDPE) containers. For photocatalytic and dark control treatments, a thin-film of silicone caulk (DAP®; 100% silicone rubber sealant) was applied to a thickness of <0.2 cm to 28x43 cm on the bottom of the containers (Figure 3.2). Immediately after silicone application, TiO_2 (Aeroxide™ P25; Fisher Scientific, Fairlawn NJ) particles were added to the surface of the film and air dried for 24-hrs. The primary particle size of the TiO_2 was approximately > 45 μm and the specific surface area was 35 - 65 m^2/g with a crystalline composition of 10-20% rutile and 80-90% anatase. Water depths of <1.0 cm were used for sufficient light penetration based on preliminary experimentation. Dark control containers were shaded with polypropylene covers. Aqueous samples were collected every hour through the 8-hr duration of the study in 60 mL amber glass vials for quantification of NAs and to conduct toxicity testing. During the 8-hr treatments, *in situ* dissolved oxygen, pH, and conductivity of NA amended water were measured every hour using a YSI® Model 52 dissolved oxygen meter, Orion® Model 250A pH meter and Triode® electrode, and Orion Model 142

conductivity meter, respectively (Table 3.1). Alkalinity and hardness of aqueous samples were determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 2007). Turbidity was measured using a Hach 2100AN Turbidimeter, (V2.2; APHA, 2007) and light intensity (LUX) was measured using a VWR® Traceable® dual-range light meter. Ultraviolet (UV) and visible irradiance were estimated using a methylene blue and peroxide actinometer (Alpert et al., 2010). A stock solution of 0.5 mg/L methylene blue with 15 µL of 30% hydrogen peroxide (Fisher Scientific) was used to estimate UV and visible light irradiance using sealed 1-cm quartz cuvettes at water depth intervals of 0, 0.5, and 1.0 cm. Methylene blue concentrations were estimated by measuring absorbance at 661nm using a SpectraMax®M2 spectrophotometer (Molecular Devices Corp. Sunnyvale, CA).

Fluka Naphthenic Acid Exposure Preparation and Analysis

Fluka NAs (Sigma-Aldrich; St. Louis, MO; Table 3.2) were used to prepare initial test concentrations and stock solutions in reconstituted moderately hard water (pH 8.2 ± 0.5 SU, alkalinity 65 ± 8 mg/L as $CaCO_3$, hardness 88 ± 10 mg/L as $CaCO_3$, conductivity 350 ± 20 µs/cm) which was prepared using reverse osmosis filtered water and reagent grade chemicals based on recommended culture methods (USEPA, 2002). The formulated water contained 5 mg/L $CaCO_3$, 102 mg/L $NaHCO_3$, 48 mg/L $MgSO_4$-$7H_2O$, 33 mg/L $CaSO_4$-$2H_2O$, 65 mg/L $CaCl_2$-$2H_2O$, 2 mg/L KCl, 0.8 mg/L KNO_3, 0.02 mg/L K_2PO_4, and 0.002 mg/L of each Cu, Se, and Zn (from aqueous standards). All reagents were obtained from Fisher Scientific® (Pittsburgh, PA). Fluka NAs were added to moderately hard water in a 20 L HDPE Nalgene® container (initial nominal

concentrations of 65 mg/L) and mixed to prepare a modified water accommodated fraction (WAF), where solutions were mixed with magnetic stir bars for 24 hours at a speed sufficient to create a vortex which extended 30-50% of the solution depth (OECD, 2000). Methods for NA derivatization and analysis were based on Yen et al. (2004) using high performance liquid chromatography (HPLC; Dionex, UltiMate-3000; Sunnyvale, CA). The HPLC analytical column was an Agilent LiChrospher 100 RP-18 (5 µm particle size, 125mm x 4 mm) with a guard column packed with 2 µm RP-18 solid phase material. Column temperature was maintained at 40° C with a sample injection volume of 60 µL mobilized with HPLC grade methanol (Fisher Scientific) at a flow rate of 60 µL per minute. The detection limit for this HPLC method is approximately 5 mg NA/L.

Removal Efficiency and Rate Calculations

Removal efficiencies (equation 1) were estimated using the following equation:

$$\text{Removal efficiency } (\%) = \frac{[C0]-[C]}{[C0]} \times 100 \qquad (1)$$

Where, measured initial concentrations of NAs are designated as $[C_0]$ (mg/L) and [C] (mg/L) is concentration of NAs at test completion. A linear relationship was observed between changes in NA concentration with time; therefore, removal rates (k=mg/L day^{-1}) were calculated using zero order kinetics, as the inverse slope of the line indicating change in concentration with change in time (hours). Correlation coefficients for the zero-order model are provided for each treatment. Half-lives were estimated using the following equation based on zero order kinetics:

$$T_{1/2} = [C_0] / 2k \qquad (2)$$

Where, $T_{1/2}$ is half-life (hours), $[C_0]$ (mg/L) is NA concentration at test initiation and k is degradation rate.

Toxicity Testing

Photolysis and photocatalysis treatments were assessed for their ability to alter toxicity of Fluka NAs using sensitive sentinel species *P. promelas* and *D. magna*. Larval (<24h old) fish (*P. promelas*) and *D. magna* (<24h old) were obtained from cultures at Clemson University Aquatic Animal Research Laboratory (AARL). Survival of *P. promelas* and *D. magna* were evaluated in 96-hr static/non-renewal toxicity tests conducted following a United States EPA (USEPA) freshwater toxicity testing protocol with (n=30) organisms per exposure (USEPA, 2002; Table 3.3). Water characteristics (dissolved oxygen, pH, conductivity, alkalinity, and hardness) of test waters were measured at test initiation and completion using methods described in Table 3.1.

Statistical Analysis

Data were tested for normal distribution and homogeneity of variance using Chi-square and Bartlett's tests, respectively. Normally distributed, homogeneous data were analyzed by one-way analysis of variance (ANOVA). Differences among treatments were conducted using 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).

Results and Discussion

Exposure conditions for photolysis and photocatalysis

Mean measured initial concentrations of NAs for aqueous samples were 63 (±9), 65 (±5), and 64 (±10) mg NA /L for photocatalysis, photolysis, and dark controls, respectively. Over the 8-hr treatment period in direct sunlight (excluding the dark control), measured light intensity ranged from 11,500 (at 8-hr) to 109,700 LUX (at 4-hr). There was no measureable incident light in dark controls. UV/Visible irradiance ranged from 12.6 W/m^2 at water surface to 6.27 W/m^2 at 1.0 cm water depth, respectively, indicating rapid light attenuation with water depth (attenuation coefficient [Kd] = 0.69 cm^{-1}). Ambient air temperatures during the 8-hr experiment ranged from 7.5 to 21.6°C and water temperatures in treatments and controls ranged from 13-19ºC. Water containing NAs had no detectable turbidity (<0.1 NTU). pH in treatments and controls ranged from 8.02-8.26. *In situ* water characteristics measured during the 8-hr treatment durations (e.g. temperature, pH, DO, conductivity) did not differ among treatments (Table 3.4).

Photocatalysis

After 4-hr in photocatalysis treatments, NA concentrations declined to below detection limit (method detection limit = 5 mg/L), resulting in >92% removal (Figure 3.2). Data were fit to a zero-order model with a correlation coefficient (R^2) of 0.9792, based on 5 data points from Time 0 (test initiation) to Time 5 (hour 4). The calculated NA removal rate (k) and half-life for photocatalysis were 15.5 mg/L hr^{-1} and 2.0-hr,

respectively (Table 3.5). Results from this study are similar to results for Fluka NAs using TiO_2 "slurries" in photocatalysis studies irradiated with sunlight and UV_{254} bulbs (Headley et al., 2009; Mishra et al., 2010; Table 3.6). For example, using sunlight, Headley et al. (2009) achieved 75% Fluka NA removal in 8-hr (initial concentration of 46 mg/L NA) using TiO_2 slurries with concentrations of 2 g TiO_2/L. Mishra et al. (2010) used aqueous suspensions of TiO_2 (at 0.3 g TiO_2/L concentrations) irradiated with UV_{254} (8W lamps) and achieved first order rate coefficients ranging from 0.05 to 0.34 hr^{-1} for Fluka NAs in South Saskatchewan River water and deionized water, respectively. Under the same laboratory conditions, Mishra et al. (2010) achieved relatively rapid degradation of NAs derived from oil sands process affected water (OSPW), with half-lives of 1.55 and 4.8-hr for deionized water and South Saskatchewan River water, respectively. Generally, results were similar among fixed-film and slurries of TiO_2, irradiance from sunlight and artificial UV_{254}, and Fluka and OSPW NAs (Headley et al., 2009; Mishra et al., 2010). Among these treatments, measured photocatalysis rates and extents of removal are of practical significance with NA half-lives achieved in hours.

Photolysis and Dark Control

In photolysis treatments, 51% removal of the initial NA concentration (65 mg/L ±5 SD) was achieved after 8-hr (final concentration 32 mg NA/L ±11 SD; Figure 3.2; Table 3.5). NA concentrations after 4-hr (α =0.05; $p<0.001$) were significantly different from test initiation, which corresponded with maximum measured light intensity during the experimental duration (109,700 LUX at 4-hr). Data were fit to a zero-order model with a correlation coefficient (R^2) of 0.9122, based on 9 data points from Time 0 (test

initiation) to Time 9 (hour 8). The calculated NA removal rate (k) and half-life for photolysis were 4 mg/L hr^{-1} and 8.1-hr, respectively (Table 3.5). Since one NA half-life was achieved with this treatment (rather than reaching the detection limit), this removal rate (4 mg/L hr^{-1}) applies to the first measured half-life only, and application of this estimated rate beyond the measured bounds in this study should be undertaken carefully. These photolysis results using Fluka NAs in sunlight are similar to modeled half-lives estimated for photo-degradation of commercial NAs (USEPA, 2012). Empirical models indicate photo-degradation half-lives for 1- to 4-ring structures with molecular weights of 254–325 amu (as compared to 210-250 amu of the NA used in this study) range from 3 to 6.8-hr (USEPA, 2012). Headley et al. (2009) estimated < 3% removal of Fluka NAs (initial concentration 46 mg/L) in 8-hr photolysis (no TiO_2) experiments irradiated with sunlight in Milli-Q water. A number of factors influence the photolysis of NAs, including structure of the molecule (Headley et al., 2009), and exposure conditions (i.e. sunlight intensity, water depth, turbidity; Kirk, 1994). In this study, conditions that may confound photolysis and photocatalysis such as water depth, pH, and turbidity were managed to minimize their effects.

Dark controls containing fixed-film TiO_2 were used to account for sorption and volatilization of NAs, which are other factors that may influence the measured concentration of NAs. Based on a one-way ANOVA, NA concentrations in dark controls did not differ from test initiation (64 mg/L ±10 SD) to completion at 8-hr (56 mg/L ±4 SD; $\alpha = 0.05$; $p = 0.8487$). In the absence of light, minimal decrease in NA concentration was anticipated due to the limited volatility (1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C) and

sorption (log Kd <0.5 [quartz sand]) of naphthenic acids at the experimental pH of 8.02-8.26 (Schramm, 2000; USEPA, 2012).

Toxicity testing to confirm changes in NA exposures

Toxicity tests using fish and microinvertebrates were used to confirm degradation of NAs within treatments. For all treatments at test initiation, complete mortality (0% survival) of both *D. magna* and *P. promelas* was observed in 96-hr exposures (NA concentrations 63-65 mg/L). In photocatalysis treatments, toxicity was completely eliminated (100% survival) for both *D. magna* and *P. promelas* after 5-hr (Table 3.7). Both photolysis and dark control treatments did not have any measurable change in toxicity after 8-hr durations (i.e. 100% mortality throughout treatments).

In this study, larval fish (*P. promelas*) were more sensitive to Fluka NA exposures than the microcrustacean *D. magna*, with 0% and 57% survival observed for *P. promelas* and *D. magna*, respectively, after 3-hr of photocatalytic treatment. Changes in percent mortalities with time observed for *D. magna* and *P. promelas* in this study provided an opportunity to compare with reported endpoint estimations (i.e. EC_{50s} and LC_{50s}) for commercial NAs (Swigert et al., 2015; Kinley et al. in review). In photocatalysis treatments, *Daphnia magna* mortality was 43% in Fluka NA concentrations of 15 mg/L NA (±5), similar to the reported 48-hr EC_{50} (immobilization) of 20 mg/L (17-23 mg/L C.I.) for *D. magna* exposed to Merichem NAs (Swigert et al., 2015). After photocatalysis treatment in this study, mortality of *P. promelas* declined from 100% to 40% after 4-hr and 0% mortality was observed after 5-hr with measured NA concentrations below the analytical detection limit of 5 mg/L NA. Swigert et al. (2015) measured a 96-hr LC_{50} for

juvenile *P. promelas* of 5.6 mg/L Merichem NAs. Comparatively, Kinley et al. (in review) observed a 7-day LC_{50} for larval *P. promelas* of 1.9 mg/L as Fluka NAs. In this study, coupling bioassay response data with analytical quantification of NAs provided a robust approach for discerning changes in NA exposures and mitigation of ecological risk.

Conclusions

Greater than 90% removal of Fluka NAs (initial concentration 63 mg/L) was achieved in 4-hr with photocatalysis in fixed-film (TiO_2) reactors in direct sunlight. Photocatalysis also eliminated acute toxicity to sentinel species, with mortality decreasing from 100% to 0% after 5-hr of photocatalytic treatment for fathead minnow (*Pimephales promelas*) and after 4-hr for the freshwater invertebrate (*Daphnia magna*). In this experiment, measuring responses of aquatic organisms concomitantly with analytical quantification of Fluka NAs over time confirmed alteration of exposures as well as mitigation of risk. Fixed-film TiO_2 application may provide an alternative solution for scaling the technology for larger applications. Photocatalytic degradation using fixed-film TiO_2 irradiated with sunlight achieved efficacious rates and extents of removal of Fluka NAs, indicating the potential for application of this technology for mitigating ecological risks associated with NAs.

References

Allen, E.W., 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering Science 7, 123-138.

Alpert, S.M., 2010. Modeling the UV/hydrogen peroxide advanced oxidation process using computational fluid dynamics. Water Research 44, 1797-1808.

American Petroleum Institute (API), 2012. Naphthenic acids category analysis and hazard characterization. Petroleum HPV Testing Group Technical Report # 1100997.

American Public Health Association (APHA), 2007. *Standard methods for the examination of water and wastewater*, 21st Ed. American Public Health Association, Port City Press: Baltimore, MD, pp. 1368.

Armstrong, S. A., Headley, J. V., Peru, K. M., Germida, J. J., 2007. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. Journal of Environmental Science and Health. Part A, Toxic/hazardous substances & environmental engineering 43, 36-42.

Bagheri, S., Muhd Julkapli, N., & Bee Abd Hamid, S., 2014. Titanium Dioxide as a Catalyst Support in Heterogeneous Catalysis. The Scientific World Journal.

Barrow, M. P., Headley, J. V., Peru, K. M., & Derrick, P. J., 2004. Fourier transform ion cyclotron resonance mass spectrometry of principal components in oil sands naphthenic acids. Journal of Chromatography A 1058, 51-59.

Brient, J. A., Wessner, P. J., Doyle, M. N., 1995. Naphthenic acids. *Kirk-Othmer encyclopedia of chemical technology*, 4th ed., 1017-1029.

Clemente, J. S., Fedorak, P. M., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Dorn, P. B., 1992. Case histories—the petroleum industry. Toxicity Reduction: Evaluation and Control. In: Ford, DL (Ed.), Water Quality Management Library 3, 183-223.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R., 2008. Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. Environmental Science & Technology 43, 266-271.

Han, X., MacKinnon, M.D., & Martin, J.W., 2009. Estimating the *in situ* biodegradation of naphthenic acids in oil sands process waters. Chemosphere 76, 63-70.

Headley, J. V., & McMartin, D. W., 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. Journal of Environmental Science and Health, Part A 39, 1989-2010.

Headley J, Du J, Peru K, McMartin D., 2009. Electrospray ionization mass spectrometry of the photodegradation of naphthenic acids mixtures irradiated with titanium dioxide. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering 44, 591-597.

Headley, J. V., Peru, K. M., Adenugba, A. A., Du, J. L., & Mcmartin, D. W., 2010. Dissipation of naphthenic acids mixtures by lake biofilms. Journal of Environmental Science and Health Part A 45, 1027-1036.

Holowenko, F.M., MacKinnon, M.D., & Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research 36, 2843-2855.

Kavanagh, R. J., Frank, R. A., Burnison, B. K., Young, R. F., Fedorak, P. M., Solomon, K. R., & Van Der Kraak, G., 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116, 34-42.

Kinley, C.M., McQueen, A.D., & Rodgers, Jr., J.H., (In review). Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid. Ecotoxicology and Environmental Safety.

Kinsinger, N., Honda, R., Keene, V., Walker, S. L., 2015. Titanium dioxide nanoparticle removal in primary prefiltration stages of water treatment: role of coating, natural organic matter, source water, and solution chemistry. Environmental Engineering Science 32, 1-9.

Kirk, J. T. 1994. Light and photosynthesis in aquatic ecosystems. Cambridge University Press. New York. NY.

Leclair, L. A., MacDonald, G. Z., Phalen, L. J., Köllner, B., Hogan, N. S., & van den Heuvel, M. R., 2013. The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 142, 185-194.

Lo, C. C., Brownlee, B. G., & Bunce, N. J., 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. Water Research 40, 655-664.

McMartin, D. W., Headley, J. V., Friesen, D. A., Peru, K. M., & Gillies, J. A., 2004. Photolysis of naphthenic acids in natural surface water. Journal of Environmental Science and Health, Part A 39, 1361-1383.

Metcalf & Eddy., Tchobanoglous, G., Burton, F. L. 1., & Stensel, H. D., 2004. *Wastewater engineering: Treatment and reuse* (4th ed.). McGraw-Hill. Boston, MA.

Mishra, S., Meda, V., Dalai, A., McMartin, D.W., Headley, J.V., Peru, K.M., 2010. Photocatalysis of naphthenic acids in water. Journal of Water Resource and Protection 2, 644-650.

Nero, V., Farwell, A., Lee, L. E. J., Van Meer, T., MacKinnon, M. D., Dixon, D. G., 2006. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicology and Environmental Safety 65, 252-264.

OECD (Organization for Economic Cooperation and Development), 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Series on Testing and Assessment No. 23, ENV/JM/MONO (2000), vol. 6. OECD, Paris, France.

Parsons, S.(ed.), 2004. *Advanced oxidation processes for water and wastewater treatment.* IWA Publishing. London, UK.

Rudzinski, W. E., Oehlers, L., Zhang, Y., & Najera, B., 2002. Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. Energy & Fuels 16, 1178-1185.

Schramm, L. L. (Ed.), 2000. *Surfactants: fundamentals and applications in the petroleum industry*. Cambridge University Press, Cambridge, UK.

Scott, A.C., Mackinnon, M.D., & Fedorak, P.M., 2005. Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environmental Science and Technology 39, 8388-8394.

Seifert, W. K., Teeter, R. M., 1969. Preparative thin-layer chromatography and high-resolution mass spectrometry of crude oil carboxylic acids. Analytical Chemistry 41, 786-795.

Sigma-Aldrich, 2015. MSDS, Naphthenic acid. St. Louis, Missouri.

Swigert, J. P., Lee, C., Wong, D. C., White, R., Scarlett, A. G., West, C. E., Rowland, S. J., 2015. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere 124, 1-9.

Tomczyk, N. A., Winans, R. E., Shinn, J. H., Robinson, R. C., 2001. On the nature and origin of acidic species in petroleum. 1. Detailed acid type distribution in a California crude oil. Energy & Fuels 15, 1498-1504.

United States Environmental Protection Agency (USEPA), 2002. Methods for measuring acute toxicity of effluents and receiving waters to freshwater and marine organisms. Washington DC. EPA-821-R-02-12.

United States Environmental Protection Agency (USEPA), 2012. Screening-level hazard characterization: Reclaimed substances (Naphthenic acids category). Washington DC. EPA-821-R-02-12.

Wold, A. 1993. Photocatalytic properties of Ti02. Chemistry of materials 5, 280-283.

Yen, T. W., Marsh, W. P., MacKinnon, M. D., Fedorak, P. M., 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. Journal of Chromatography 1033, 83-90.

Table 3.1: Methods for water characteristics, light, and NA concentrations.

Parameter	Method	Method Detection Limit
pH	Electrometric method 4500-H^+ B: Orion model 420A (APHA, 2007)	0.01 SU
Temperature	Laboratory method 2550 B: Orion Model 420A (APHA, 2007)	0.01 °C
Dissolved oxygen	Membrane electrode method 4500-O G: YSI Model 52 (APHA, 2007)	0.1 mg/L
Conductivity	Laboratory method 2510 B: YSI 30 (APHA, 2007)	0.1 μS/cm
Alkalinity	Titration method 2320 B (APHA, 2007)	2 mg/L as CaCO3
Hardness	EDTA Titrimetric Method 2340 C (APHA, 2007)	2 mg/L as CaCO3
Turbidity	Nephelometric Method 2130 B (APHA, 2007)	0.1 NTU
Light intensity	VWR® Traceable® Light Meter	0.1 LUX
UV attenuation	Methylene blue and peroxide actinometer (Alpert et al., 2010)	1 W/m^2
Naphthenic acid concentration	HPLC; Derivatization based on Yen et al. (2004)	5 mg/L

Table 3.2: Physical and chemical characteristics of Fluka naphthenic acids (Sigma-Aldrich)[a]

Parameter	General Characteristic	Cite
Identification	1338-24-5 (CAS No)	Sigma-Aldrich, 2015
Color	Pale yellow	Sigma-Aldrich, 2015
Physical State	Viscous liquid	Sigma-Aldrich, 2015
Molecular Weight[2]	210-250 amu	Brient et al., 1995
Water Solubility[1]	88.1 mg/L at pH 7.5	API, 2012
Vapor pressure	1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C	USEPA, 2012
Partition coefficient octanol/ water[3]	˜4 at pH 1	Schramm, 2000
(Log Kow)[3]	˜2.4 at pH 7	Schramm, 2000
	< 0.1 at pH 10	Schramm, 2000
Density	0.92 g/mL at 20°C	Sigma-Aldrich, 2015
Flash Point	101°C	Sigma-Aldrich, 2015
Initial Boiling Point	106.4-333.6°C	Sigma-Aldrich, 2015
Viscosity	22 mm2/s	Sigma-Aldrich, 2015
pKa	5 to 6	Brient et al., 1995

[1]Alkylated cyclopentane carboxylic acids (mixture)
[2]Average molecular weight for refined naphthenic acids
[3]Weathered naphthenic acid mixture

Table 3.3: Summary of toxicity test conditions for *P. promelas* and *D. magna* (USEPA, 2002).

Test Parameter	Description
Test type	static non-renewal
Endpoint measured[a]	mortality
Test Duration (hours)	96
Test temperature (°C)	25 ± 1
Test chamber	30 mL HDPE chamber
Test water	Formulated moderately hard water[b]
Number of organisms/ exposure	30
Number of organisms/ chamber[c]	10
Age of organisms	< 24 h[d]
Photoperiod	16 h light, 8 h dark

[a]Immobilization
[b]USEPA, 2002
[c]3 replicates
[d]Post-hatch for *P. promelas*; post-brood for *D. magna*

Table 3.4: Measurements of water characteristics during photocatalysis and photolysis treatments (and dark controls) and toxicity testing.

Treatment	Temp. (°C)	pH (S.U.)	DO (mg O_2/L)	Conductivity (µS/cm)	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
In Situ[a]						
Photocatalysis	13-19	8.02-8.22	8 ± 1	340-355	-	-
Photolysis	13-19	8.06-8.26	8 ± 1	345-355	-	-
Dark Control	13-19	8.08-8.23	8 ± 1	345-352	-	-
Toxicity Testing[b]						
Photocatalysis						
P. promelas	25 ± 1	8.08-8.26	8 ± 1	335-362	76-80	152-160
D. magna	25 ± 1	8.05-8.26	8 ± 1	340-366	70-78	155-164
Photolysis						
P. promelas	25 ± 1	8.05-8.22	8 ± 1	346-365	70-80	152-160
D. magna	25 ± 1	8.11-8.21	8 ± 1	344-353	76-84	155-160
Dark Control						
P. promelas	25 ± 1	8.12-8.28	8 ± 1	345-360	68-82	152-160
D. magna	25 ± 1	8.10-8.22	8 ± 1	344-364	70-85	155-160

[a]measured at 1 hour sampling intervals (n=8)
[b]measured at test completion (n=3)

Table 3.5: Removal rates and extents for photocatalysis, photolysis, and dark control treatments.

Parameter	Treatments		
	Photocatalysis	**Photolysis**	**Dark Control**
Initial [NA] mg/L (±SD)	63 (± 9)	65 (± 5)	64 (± 10)
Ending [NA] mg/L (±SD)	BDL[a]	32 (± 11)	56 (± 4)
Removal Efficiency, %	>92	51	NA[c]
Rate Equation[b]	Y = -15.502x + 64.727	Y = -0.0876 x + 4.1401	NA
R^2	0.9792	0.9122	NA
k average (mg/L $hour^{-1}$)[b]	15.5	4.0	NA
$T_{1/2}$ (hour)	2.0	8.1[d]	NA

NA = Not applicable

[a]BDL = Below Detection Limit, detection limit = 5 mg/L NA

[b]Zero Order Rate Equation; Linear plot [C] versus time (hours), slope = -k

[c]Ending [NA] not statistically different from initial [NA] in dark control (α =0.05; p= 0.8487)

[d]Estimation based on 1 observed half life

Note: Removal rate coefficient calculated from best fit using five data points (photocatalysis) and eight data points (photolysis)

Table 3.6: Comparative rates and extents of removal for photocatalytic degradation and photolysis of Fluka NAs.

Treatment (Condition)	Initial NA (mg/L) Mean (±SD)	Ending NA (mg/L), Duration Mean (±SD)	Removal Efficiency (%)	Removal Rate	Estimated Half Life Values (hours)	Citation
Photocatalysis (sunlight; TiO_2 film)	63 (± 9)	BDL, 8-hr	>92	15.5 mg/L hr^{-1}	2.0	Current study
Photocatalysis (sunlight; TiO_2 slurry[a])	46	9, 8-hr	~80	-	-	Headley et al., 2009
Photocatalysis (UV[b]; TiO_2 slurry[c])	40-100	NA; 5-hr	-	0.34 hr^{-1}	2.04	Mishra et al., 2010
Photocatalysis (UV[b]; TiO_2 slurry[c]; river water[d])	40-100	NA; 5-hr	-	0.05 hr^{-1}	13.86	Mishra et al., 2010
Photolysis (sunlight)	65 (± 5)	32 (± 11), 8-hr	51	4.0 mg/L hr^{-1}	8.1	Current study
Photolysis (sunlight)	46	45, 8-hr	~2	-	-	Headley et al., 2009
Photolysis (simulated solar radiation[e]; river water[f])	50	NA, 168-hr	-	-	8120 ± 100	McMartin et al., 2004
Photolysis (UV[b])	40-100	NA, 5-hr	-	0.22 hr^{-1}	3.15	Mishra et al., 2010
Photolysis (UV[b]; river water[d])	40-100	NA, 5-hr	-	0.04 hr^{-1}	17.33	Mishra et al., 2010
Photolysis (UV[g]; river water[f])	10	NA, 8-hr	-	-	1050 ± 20	McMartin et al., 2004
Photolysis (UV[g]; river water[f])	50	NA, 8-hr	-	-	1200 ± 40	McMartin et al., 2004
Photolysis (UV[h]; river water[f])	10	NA, 8-hr	-	-	50 ± 1	McMartin et al., 2004
Photolysis (UV[h]; river water[f])	50	NA, 8-hr	-	-	52 ± 1	McMartin et al., 2004

Dark Control (TiO_2 film)	46 (± 10)	45 (± 4), 8-hr	~2	-	-	Current study
Dark Control (TiO_2 slurry[a])	64	45, 8-hr	< 3	-	-	Headley et al., 2009

BDL = below detection limit (<5 mg/L)

NA = data not available

[a]TiO_2 (Degussa P25) slurry concentration = 2 g/L

[b]UV fluorescent tubes (8W)

[c]TiO_2 (Degussa P25) slurry concentration = 0.3 g/L

[d]NA mixtures prepared in South Saskatchewan River water (Saskatoon, SK)

Note: pH not reported in McMartin et al. (2004), Headley et al. (2009); Mishra et al. (2010)

[e]Simulated full spectrum solar radiation with incandescent and florescent lamps

[f]Fluka NA concentrations prepared in unaltered Athabasca River Water

[g]Philips medium pressure black light bulb; 300-400 nm (15W)

[h]Low pressure Phillips® UV_{254} tube

Note: Comparison studies quantified Fluka NAs using electrospray ionization mass spectrometry (ESI-MS)

Table 3.7: Summary of mean NA concentrations (mg/L) and 96-hr percent survival for microinvertebrates (*D. magna*) and fish (*P. promelas*) for photocatalysis, photolysis, and dark control treatments. NA concentrations with different letters are significantly different ($p<0.05$).

Treatment Duration (hours)	Treatments											
	Photocatalysis				Photolysis				Dark Control			
	NAs (±SD)		% Survival		NAs (±SD)		% Survival		NAs (±SD)		% Survival	
			D. Magna	*P. Promelas*			*D. Magna*	*P. Promelas*			*D. Magna*	*P. Promelas*
0	63 (±9)	A	0	0	65 (±5)	A	0	0	64 (±10)	A	0	0
1	54 (±12)	AB	0	0	57 (±15)	AB	0	0	60 (±12)	A	0	0
2	32 (±8)	BC	0	0	55 (±10)	AB	0	0	67 (±8)	A	0	0
3	15 (±5)	C	57	0	47 (±8)	ABC	0	0	65 (±5)	A	0	0
4	BDL		100	60	38 (±10)	BC	0	0	63 (±5)	A	0	0
5	BDL		100	100	44 (±5)	BC	0	0	58 (±5)	A	0	0
6	BDL		100	100	38 (±10)	BC	0	0	58 (±2)	A	0	0
7	BDL		100	100	33 (±12)	C	0	0	54 (±7)	A	0	0
8	BDL		100	100	32 (±11)	C	0	0	56 (±4)	A	0	0

BDL = below detection limit (<5 mg/L)

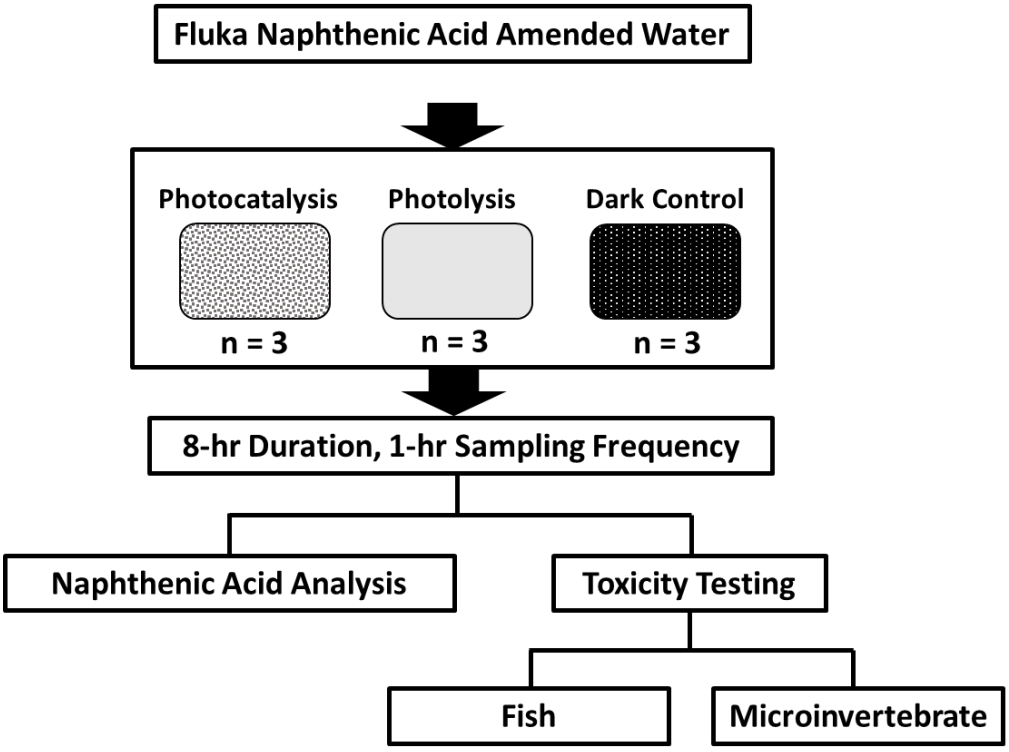


Figure 3.1: Experimental design schematic

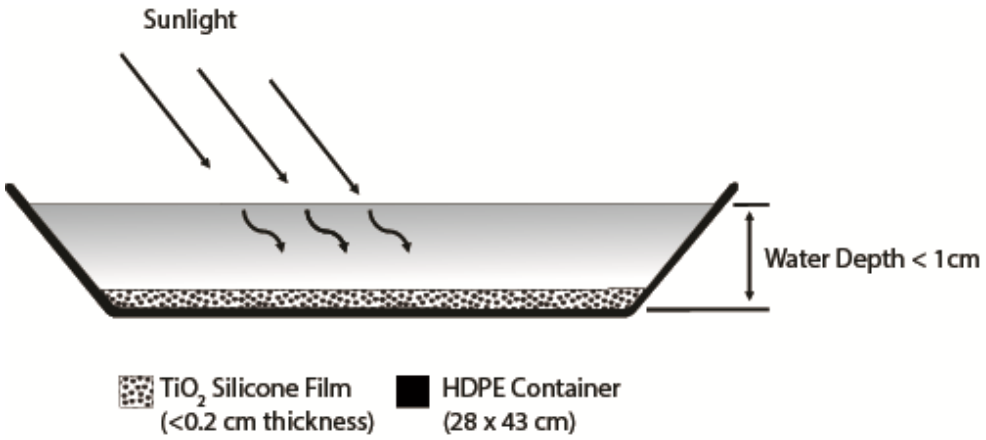


Figure 3.2: Schematic of photocatalytic reaction chamber

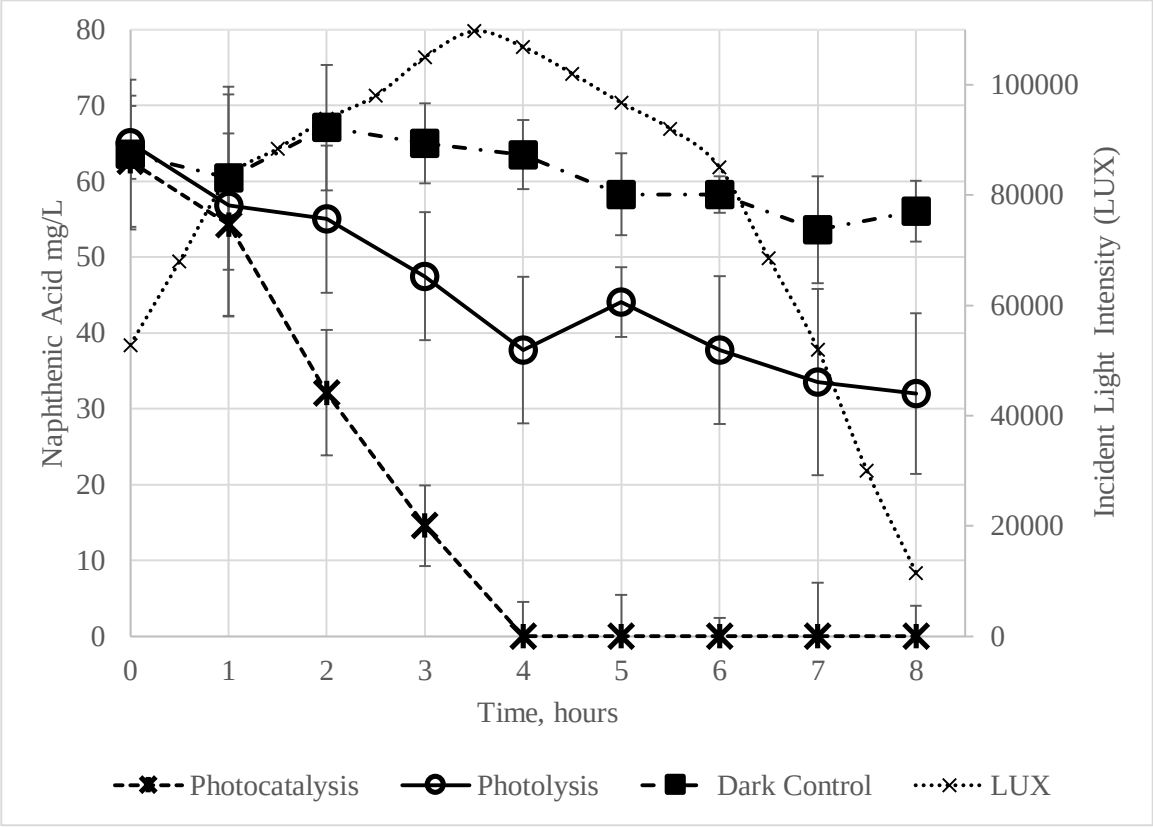


Figure 3.3: Concentrations of Fluka naphthenic acids (mg/L) with time in photocatalysis, photolysis, and dark control treatments.

CHAPTER FOUR

EFFECTS OF ENVIRONMENTAL CONDITIONS ON AEROBIC DEGRADATION OF A COMMERCIAL NAPHTHENIC ACID

Abstract

Environmental conditions influencing aerobic degradation of naphthenic acids (NAs) include nitrogen (N) and phosphorus (P) concentrations, dissolved oxygen (DO) concentrations, pH, and temperature. Since NAs are problematic constituents in energy derived waters (e.g. refinery effluents and oil sands process affected waters), aerobic degradation may provide a solution for mitigating risks to aquatic organisms. Commercial NAs provide repeatable exposures necessary to compare influences of environmental conditions on degradation. The overall objective of this study was to determine the relative influence of N (as ammonia) and P (as phosphate) concentrations, DO concentrations, pH, and temperatures on aerobic degradation of a commercial (Fluka) NA in bench-scale reactors. Changes of Fluka NA concentrations with time were measured and compared for environmentally relevant ranges of N and P concentrations, DO concentrations, pH, and temperatures. NAs were quantified using a derivatization method and high performance liquid chromatography. Environmental conditions that positively influenced aerobic degradation rates of Fluka NAs included nutrients (C:N 10:1-500:1, C:P 100:1-5000:1), DO (4.76-8.43 mg/L), pH (6-8), and temperature (5-25ºC). Approximately 50% removal of an initial Fluka NA concentration of 61±8 mg/L was achieved in less than 2 days after test initiation, reaching non-detect (5 mg/L) levels

by day 6 of the experiment in treatments with C:N and C:P molar ratios of 10:1 and 100:1, respectively (with other macro- and micronutrients supplied), DO >8 mg/L, pH ~8-9, and temperatures >23ºC. Based on results from this study, when macro- and micronutrients were available, increased DO, pH, and temperature (within environmentally relevant ranges) increased rates of aerobic degradation of Fluka NAs. Commercial NAs differ structurally from energy-derived NAs (e.g oil sands process affected waters), but environmental conditions in this study are also expected to affect rates and extents of aerobic degradation of compositionally complex NAs. This aerobic degradation study of Fluka NAs could serve as a model for systematically evaluating environmental factors that influence NA degradation.

Introduction

Aerobic degradation can alter structural compositions and decrease concentrations of naphthenic acids (NAs), a complex group of carboxylic acids associated with crude oils (Seifert and Teeter, 1969; Tomcyzk et al., 2001) and energy derived process waters (Dorn, 1992; Allen, 2008). NAs are described by the formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and Z is either zero or a negative even integer representing the hydrogen deficiency of the molecule due to rings or double bonds (Holowenko et al., 2002; Clemente and Fedorak, 2005). NAs are sources of toxicity in energy derived waters such as refinery effluents and oil sands process affected waters (Dorn, 1992; Schramm, 2000), with adverse effects observed for fish, macro- and microinvertebrates, aquatic macrophytes, and microorganisms (Nero et al., 2006; Frank et al., 2008; Armstrong et al., 2009; Kavanagh et al., 2012; Leclair et al., 2013; Swigert et al., 2015). To evaluate aerobic degradation of NAs for mitigating ecological risks in aquatic systems, commercially available NAs have storage stability and can be used to prepare repeatable exposures. Fluka commercial NAs have been used for assessing aerobic degradation of NAs (Scott et al., 2005; Headley et al., 2010) and have also been evaluated as a simplistic analogue to understand more compositionally complex mixtures of NAs present in energy-derived waters (Headley and McMartin 2004; Barrow et al., 2004; Scott et al., 2005; Rudzinski et al., 2002; Lo et al., 2006; Armstrong et al., 2007; Headley et al., 2010). By using a model NA (Fluka) with repeatable exposures, factors that influence degradation can be accurately measured.

Effects of NA structural compositions on rates and extents of aerobic degradation have been illustrated with NAs from various sources, with studies including comparisons between commercial NA sources (Clemente et al., 2004), comparisons between commercial and energy-derived NA sources (Scott et al., 2005; Han et al., 2008; Headley et al., 2010), and correlations of cyclicity of structures with half-lives of NAs (Han et al., 2008). Commercial NAs have been degraded with half-lives of less than 10 days (Clemente et al., 2004; Scott et al., 2005; Headley et al., 2010), while Scott et al. (2005) observed 25% removal of OSPW-derived NAs (initial concentration 37 mg/L) in 49 days. Similarly, Han et al. (2008) measured half-lives ranging from 1-8 days for Merichem commercial NAs and from 44-240 days for OSPW-derived NAs, illustrating that commercial NAs are relatively labile compared to energy-derived NAs. Environmental conditions that may influence aerobic degradation of NAs include nitrogen and phosphorus concentrations, dissolved oxygen concentration, pH, and temperature. Changes in rates and extents of removal by aerobic degradation can be due to effects on degradation pathways, microbial species (abundance), shifts in species (diversity), or bioavailability to species. Although commercial NAs differ structurally from energy-derived NAs, effects of environmental conditions on degradation can be discerned using a relatively labile model (Fluka) NA. Within the constraints of the compositional and structural differences among commercial and energy-derived NAs, environmental conditions affecting aerobic degradation for commercial NAs are also expected to affect degradation of energy-derived NAs.

Sufficient stoichiometric balances of nitrogen (N) and phosphorus (P) relative to supplied carbon (C) concentrations (from NAs) must be achieved to provide macronutrients for microbes capable of degrading complex organic molecules (e.g. NAs). Provision of essential aqueous molar ratios and concentrations is critical for establishing and maintaining biological activity for aerobic degradation (Herbert, 1976; Brock and Madigan, 1988). Stoichiometric balances of these nutrients necessary to sustain growth of microbial populations have been well studied. Redfield (1958) and Goldman et al. (1987) estimated requisite C:N:P ratios of 105:15:1 and 106:12:1, respectively. Based on nutrient requirements observed for microorganisms, C:N and C:P ratios of approximately 10:1 and 100:1, respectively, should be sufficient to establish and maintain microbial growth, given that all other necessary macro- and micronutrients are available. Bushnell-Haas aqueous culture medium (Bushnell and Haas, 1941) is advantageous for biodegradation studies because it does not supply an organic carbon source, so potential use of the constituent of interest (e.g. NAs) by microbes as carbon and energy sources can be evaluated. Bushnell-Haas medium has been used in NA aerobic degradation studies (Clemente et al., 2004; Scott et al., 2005; Del Rio et al., 2006; Han et al., 2008; Headley et al., 2010), allowing for comparisons of results between laboratory studies. Increasing C:N and C:P ratios in supplied nutrient medium can inform potential effects of N and P limitations on rates and extents of aerobic degradation, presumably due to limitations of growth of microbial populations. To minimize effects of limited N and P as a confounding variable, measures of degradation for a range of C:N and C:P ratios preceded evaluation of other treatment factors.

Once questions regarding C:N and C:P ratios are resolved, dissolved oxygen is the next critical parameter to evaluate, since a primary degradation pathway for aliphatic and alicyclic carboxylic acids by microorganisms is β-oxidation (Taylor and Trudgill, 1978; Trudgill et al., 1984), which utilizes molecular oxygen. Diatomic oxygen is sparingly soluble in water and rates of dissolved oxygen consumption by microorganisms (as a macronutrient and as an electron acceptor in degradation) can exceed rates of atmospheric oxygen diffusion into water (Brock and Madigan, 1988). Aerobic degradation of NAs results in biological oxygen demand (BOD) in water, and estimates of moles O_2/mole carbon necessary for NA degradation can provide data to promote efficacious removal.

Somewhat unique to NAs as a class of compounds, pH can precipitously influence their solubility and bioavailability. Solubility of NAs in water is influenced by pH, with log K_{ow} values ranging from about 1.8 at pH 6 to 0.8 at pH 8 for NAs found in oil sands process affected waters (Schramm, 2000). Susceptibility of organic constituents to biodegradation often correlates with water solubility and octanol-water partition coefficients (Alexander, 1999). Clearly, pH can be a confounding factor in measuring the rates and extents of removal of NAs. For evaluating degradation in bench-scale studies, maintaining pH at approximately 8 ensures solubility of NAs (Schramm, 2000) while remaining within environmental requirements and tolerances of microorganisms (USEPA, 1986). Decreasing pH from 8, within the range of requirements for microbes, provides information about potential effects on degradation, presumably due to decreased bioavailability of NAs.

Finally, ambient temperatures can affect aerobic degradation of NAs by altering microbial metabolic rates and physical state of the compounds (Atlas, 1981; Alexander, 1999). Increases in temperature (above freezing) can correlate with increases in enzymatic activity, improving hydrocarbon degradation rates (Leahy and Colwell, 1990; Atlas and Bartha, 1972; Gibbs et al., 1975). In addition, as temperature increases, viscosity of hydrocarbons decreases (Leahy and Colwell, 1990), improving transport and bioavailability to microorganisms (Atlas, 1981). Increased temperatures are anticipated to correlate with increased degradation rates and extents of NAs.

Measuring effects of environmental conditions on NA degradation (C:N and C:P ratios, DO, pH, and temperature) allows for ranking in terms of relative influence on degradation with time. Ranking the influence of these environmental conditions on degradation of model Fluka NAs provides context for managing these conditions to promote efficacious degradation of NAs associated with energy-derived waters.

The overall objective of this experiment was to determine the relative influence of nitrogen (as ammonia) and phosphorus (as phosphate) concentrations, dissolved oxygen concentrations, temperatures, and pH on aerobic degradation of Fluka NAs in bench-scale laboratory reactors. To achieve this overall objective, specific objectives were to 1) measure and compare changes in Fluka NA concentrations with time for a range of C:N (10:1-500:1) and C:P ratios (100:1-5000:1), 2) measure and compare changes in Fluka NA concentrations with time for a range of dissolved oxygen concentrations (<1-9 mg/L), 3) measure and compare changes in Fluka NA concentrations with time for a range of pH (6-8), 4) measure and compare changes in Fluka NA concentrations with

time for a range of temperatures (5-25°C), 5) measure and compare relative abundance and diversity of microbial populations in treatments and 6) rank environmental conditions in terms of influence on extents of removal of Fluka NAs with time.

Materials and Methods

Experimental Design and Preparation of Treatments

Degradation experiments were conducted in 300mL biological oxygen demand (BOD) bottles with bottle openings covered with parafilm to maintain sufficient volumes for sampling and to minimize head-space to decrease potential for evaporation. Bushnell-Haas culture medium (Bushnell and Haas, 1941) was used for all treatments to supply essential nutrients to establish microorganisms. The culture medium contained 0.2 g/L $MgSO_4$, 0.02 g/L $CaCl_2$, 0.02 g/L $FeCl_3$, and N and P modified to achieve targeted C:N and C:P ratios. Nitrogen was added as ammonium sulfate ($(NH_4)_2(SO_4)$; Fisher Scientific; Pittsburgh, PA) at concentrations of 21, 2, and 0.4 mg/L (corresponding to 4, 0.4, and 0.08 mg/L N) to achieve molar C:N ratios of 10:1, 100:1 and 500:1, respectively. Phosphorus was added as dipotassium phosphate (K_2HPO_4; Fisher Scientific) at concentrations of 5.5, 0.6, and 0.1 mg/L (corresponding to 0.9, 0.1, and 0.02 mg/L P) to achieve molar C:P ratios of 100:1, 1000:1, and 5000:1, respectively. Fluka NAs were obtained from Sigma-Aldrich® (St. Louis, MO; Table 4.1). The Fluka NA mixture is composed of alkylated cyclopentane carboxylic acids (Sigma-Aldrich, 2015). Nutrient ratios were calculated assuming that the NA mixture consists of 6 carbon NAs. NAs were weighed and added to Bushnell-Haas medium to obtain initial nominal concentrations of 60 mg/L for all experiments. A modified water accommodated fraction method was used to prepare initial NA concentrations, where solutions were mixed with magnetic stir bars for 12 hours at a speed sufficient to create a vortex which extended 30-50% of the solution depth (Girling et al., 1994; OECD, 2000). After stirring, dissolved

fractions were decanted and used for testing. pH was adjusted to 8±0.1 prior to initiation of experiments in all NA solutions, with the exception of treatments targeting pH 6±0.1 and 7±0.1. Initial NA concentrations were measured in all treatments using high performance liquid chromatography (HPLC; Dionex, UltiMate-3000; Sunnyvale, CA) according to a derivatization method (Yen et al., 2004). The HPLC analytical column was an Agilent LiChrospher 100 RP-18 (5 µm particle size, 125mm x 4 mm) with a guard column packed with 2 µm RP-18 solid phase material. Column temperature was maintained at 40° C with a sample injection volume of 60 µL mobilized with HPLC grade methanol (Fisher Scientific) at a flow rate of 60 µL. Calibration standards were prepared with Fluka NAs using WAFs as described for stock solutions. The detection limit for this method is approximately 5 mg NA/L.

General water characteristics were measured prior to initiation of experiments. pH, dissolved oxygen, and conductivity were measured using an Orion® Model 250A pH meter and Triode® electrode, YSI® Model 52 dissolved oxygen meter, and Orion® Model 142 conductivity meter, respectively. Hardness and alkalinity of samples were measured according to *Standard Methods for Examination of Water and Wastewater* (APHA et al., 2007). Untreated controls for all experiments were sterilized (autoclaved) 300mL BOD bottles containing NAs in NANOpure® water with no nutrient addition or aeration to account for sorption or photolysis occuring throughout experiments. All treatments were buffered with 1 g/L sodium bicarbonate ($NaHCO_3$; Fisher Scientific) to maintain targeted pH and solubility of NAs throughout experiments.

Degradation as a function of C:N and C:P ratios were measured first, followed by dissolved oxygen, pH, and temperature. Experimental conditions for all treatments are summarized in Table 4.2. Treatments were stored in darkness in a temperature controlled incubator and 600 µL was sampled daily and derivatized to determine NA concentrations. pH was adjusted if necessary in treatments using 1M NaOH and 1M HCl. To achieve targeted DO concentrations in treatments, compressed air was dispensed into BOD bottles through airline tubing not exceeding 100 bubbles/minute.

Estimates of changes in NA concentration with time in treatments

Since NAs are complex mixtures, in which structural compositions change with time during aerobic degradation (Holowenko et al., 2002; Scott et al., 2005; Han et al., 2009) and final structural compositions are expected to differ among treatments, degradation observed in this study was not modeled by chemical kinetics. However, based on commercial NA toxicity data (Swigert et al., 2015; Kinley et al., in review), NA concentrations would need to reach the detection limit of the Yen et al. (2004) HPLC method (<5 mg/L) to provide a line of evidence that aquatic toxicity could be eliminated by this process. Therefore, treatments were evaluated according to the time required to achieve non-detect concentrations.

Percent removal (Equation 1) was calculated for all treatments:

$$\text{Percent Removal (\%)} = \frac{C_0 - C}{C_0} \times 100 \quad (1)$$

Where, initial concentration of NAs is denoted [C_0] (mg/L) and [C] (mg/L) is concentration of NAs at test completion.

Microbial taxonomic diversity and abundance in treatments

Experiments were conducted in non-sterile conditions, allowing for bacteria from source materials (i.e. NAs) and resident bacteria (i.e. airborne) to colonize nutrient media in treatments. Measurements of diversity and relative abundance of microbial genera were prioritized by treatments with environmental conditions that showed changes in rates and extents of removal with changes in the treatment factor. Targeted DNA sequencing was used to identify bacteria present in samples via polymerase chain reaction (PCR) amplification of the v3/v4 region of the 16S ribosomal RNA gene (Klindworth et al., 2013). Bioinformatics pipelines consisting of internally developed scripts and selected QIIME scripts (Caporaso et al., 2010; Edgar, 2010) were used to process the reads. Taxonomic classification of the OTUs was performed using the Greengenes database version 13_8 (DeSantis et al., 2006; McDonald et al., 2012). Weighted UniFrac distance measure (Lozupone et al., 2011) was used for ordination analyses using the "phyloseq" package (McMurdie and Holmes, 2013) in R.

Results and Discussion

Fluka NA degradation for C:N and C:P ratios

For all C:N ratios evaluated in this study, 50% Fluka NA removal was achieved between 2.5 and 3.5 days after test initiation (Figure 4.1). Mean initial concentrations of NAs were 61, 62, and 56 mg/L for C:N ratios 10:1, 100:1, and 500:1, respectively. After 5 days, all NA concentrations were non-detect (detection limit 5 mg/L; Figure 4.1).

For all C:P ratios evaluated in this study, 50% Fluka NA removal was achieved between approximately 2.5 and 4 days after test initiation (Figure 4.2). Mean initial concentrations of NAs were 61, 60, and 55 mg/L for C:P ratios 100:1, 1000:1, and 5000:1, respectively. After 5 days, all concentrations were non-detect (Figure 4.2).

These two nutrients (N and P) in bioavailable forms (ammonia and phosphate) achieved comparable Fluka NA degradation at the lowest concentrations evaluated (80 and 20 µg/L, respectively), corresponding to a molar C:N:P ratio of 5000:10:1. Evaluating a range of N and P concentrations enabled minimum concentrations that achieved comparable degradation to be discerned, which has implications for amending N and P efficiently in field situations. Addition of nutrients clearly mattered, as initial and final measured concentrations of NAs in untreated controls (no added nutrients) were 64 mg/L and 62 mg/L (Table 4.4; Figures 4.1 and 4.2). Additions of N and P will only promote degradation of NAs if other macro- and micronutrients are available. Therefore, waters used for aerobic degradation should be evaluated to discern nutrients that may be limiting.

Fluka NA degradation for dissolved oxygen concentrations

Increased dissolved oxygen (DO) concentrations positively influenced degradation of Fluka NAs in this study. For the highest DO treatment evaluated (averaging 8.43 mg/L DO), 50% removal was achieved 2 days after test initiation from an initial average NA concentration of 61 mg/L, and non-detect concentrations were achieved by day 6 of the experiment (Figure 4.3). The two lower DO treatments evaluated (averaging 1.94 and 4.76 mg/L DO, respectively) achieved 50% removal between 4.5 and 5.5 days after test initiation (initial NA concentrations of 64 and 55 mg/L, respectively), however, did not reach the analytical detection limit within the 8 day experimental duration (Figure 4.3). Based on the criteria for achieving non-detect concentrations of NAs, DO treatments averaging 1.94 and 4.76 mg/L DO were not as effective for degradation of NAs in this study.

Daily sampling for NA concentration analyses created headspace in treatment chambers. Treatments targeting ≤1 mg/L and 4-5 mg/L DO were purged with nitrogen gas to achieve targeted concentrations, which was necessary on days 1 and 2 for 4.76 mg/L DO treatments, and each day for 1.94 mg/L DO treatments. It is possible that daily purging of N_2 into lowest targeted DO treatments volatilized NAs, and therefore, analytically measured degradation for this treatment (1.94 mg/L DO) may be an artifact of experimental design.

Sufficient DO is essential for mineralization of NAs to CO_2 and H_2O. To achieve mineralization of Fluka NAs in this study, approximately 1.25 mmol of O_2 per mmol C would be required (estimated using the molecular formula of Fluka NA [$C_6H_{10}O_2$]),

indicating diatomic oxygen needs to be maintained in excess of the NA to promote degradation. Estimating stoichiometric ratios of O_2/C necessary for NA mineralization *in situ* can inform strategies to promote sufficient DO for NA degradation. To efficiently manage DO in field situations, low-cost, low-energy solutions (e.g. thin film reactors) could be widely applicable.

Fluka NA degradation for pH

Treatments with the highest pH evaluated (measured pH range 7.90-9.19) achieved 50% removal 2 days after test initiation from an initial average NA concentration of 61 mg/L, and achieved non-detect NA concentrations by day 6 of the experiment (Figure 4.5). Treatments with near neutral pH (~7; measured range 6.93-7.67) achieved 50% removal approximately 3.5 days after test initiation (initial average NA concentration of 56 mg/L), and achieved non-detect NA concentrations after 7 days (Figure 4.4). Although treatments with the lowest pH evaluated (~6; measured pH range 5.93-6.42) achieved 50% NA removal 3.5 days after test initiation (initial NA concentration 69 mg/L), NA concentrations were approximately 30 mg/L for the remainder of the experiment, and therefore did not reach detection limits within the experimental duration of this study.

Changes in NA concentrations with time were not uniform in pH adjusted treatments. Biphasic degradation observed in the pH adjusted treatments may be due to the water soluble fractions of NAs being more bioavailable to NA degrading microbes. Prior to daily measurements of NA concentrations and water characteristics, a sheen was observed on the surface of treatments at pH~6 and ~7. Headley et al. (2002) observed

that decreasing pH from 7 (to 3 and 5) decreased solubility of cyclical NA compounds. Increasing pH from 7 to 9 did not change mass profiles, but did increase total ion abundance by 37%, indicating that total solubility of NAs is dependent on relatively higher pH values (>7; Headley et al., 2002).

At pH<6, NAs are primarily in non-ionized forms, and elicit greater toxicity than NAs at pH>6 (Armstrong et al., 2009). Microbial toxicity was not suspected in this study, since microbial diversity and relative abundance of species were similar in all pH treatments (Figure 4.6).

NA degradation increased (with time) with increasing pH in this study, with non-detect NA concentrations achieved 6 days after test initiation in treatments with the highest pH (~8-9) evaluated. In cases with lower pHs (~6 and ~7), alkalinity of water can be managed to provide stable pH and control the solubility of NAs.

Fluka NA degradation for temperature

Treatments with the highest temperature averaging 23˚C (range 23.0-24.3˚C) achieved 50% NA removal 2 days after test initiation (initial NA concentration 61 mg/L), and reached non-detect concentrations by day 6 of the experiment. Treatments with the two lower temperatures evaluated achieved slower degradation within the experimental duration in this study. These treatments (averaging 5.9 and 16.9ºC) achieved near non-detect NA concentrations by day 8 of the experiment (initial NA concentrations of 70 and 56 mg/L, respectively), 2 days after the highest temperature evaluated (Figure 4.5). NA degradation was biphasic in treatments stored at lower temperatures (5.9 and 16.9˚C) evaluated, with degradation slowing after days 1 and 2, respectively. Interestingly,

microbial species diversity and abundance differed in lower temperature treatments from all other treatments evaluated (DO and pH treatments) based on a hierarchial clustering analysis (Figure 4.6). Differences in microbial diversity and abundance observed in lower temperature treatments may have contributed to slower rates of NA degradation.

Temperature should be considered in aerobic degradation of NAs and managed when feasible. Warmer ambient temperatures in field situations could be utilized to promote NA degradation. Alternatively, waste heat may be an option to regulate temperatures in reactors in industrial situations.

The mean initial and final measured concentrations of NAs in untreated controls for dissolved oxygen, temperature, and pH treatments were 56 mg/L (±5) and 66 mg/L (±8; Table 4.4), indicating no measurable degradation in the absence of nutrients. Therefore, regardless of dissolved oxygen concentration, pH, or temperature, availability of macro- and micronutrients should be considered essential when promoting aerobic degradation.

Microbial diversity and relative abundance in treatments

Microbial diversity and relative abundance were measured in treatments evaluating dissolved oxygen concentrations, pH, and temperature. Species diversity and relative abundance were similar in the two lower temperature (5.9 and 16.9ºC) treatments but differed from all other treatments evaluated (Figure 4.6). These data serve as an additional line of evidence that effects of temperature on NA degradation may be due to effects on microbial populations. Prominent genera in lower temperature treatments were *Acinetobacter* and *Acidovorax*. Prominent genera in lower DO (average 1.94 and 4.76

mg/L) treatments were *Palimonas*, *Ralstonia*, and *Acidovorax*. Treatments containing highest DO (8.42 mg/L), pH (≈8), and temperature (23.4ºC) evaluated were most similar to lower pH treatments (≈6 and 7), with common prominent genera including *Acinetobacter, Acidovorax, Methyloversatilis,* and *Mycoplana.*

Comparisons with published commercial NA degradation studies

Based on results from this study and published studies of commercial NA degradation (Table 4.5), NA degradation rates and extents are affected by environmental conditions and NA structural compositions. Paslawski et al. (2009) evaluated aerobic degradation rates of a model NA (4 methyl-1-cyclohexane carboxylic acid) as a function of initial concentration, temperature, and pH. At pH of 7, temperature of 23°C, and initial concentration of 50 mg/L, the degradation rate was approximately 11.0 mg/L day^{-1} (Paslawski et al., 2009). Other studies used temperatures around 20°C and aerobic conditions (Clemente et al., 2004; Scott et al., 2005; Del Rio et al., 2006; Han et al., 2008; Headley et al., 2010). Unfortunately, measurements of pH and dissolved oxygen were not reported in many of the studies reviewed. Since pH and DO influenced rates and extents of Fluka NA removal in this study, these measurements would benefit interpretation of results.

Other commercial NA aerobic degradation studies have used NAs from various sources including Fluka, Kodak, and Merichem NAs as well as model carboxylic acids with formulas similar to NAs (Table 4.5). Clemente et al. (2004) measured biodegradation of Kodak and Merichem NAs at initial concentrations of 82 mg/L and 109 mg/L, respectively. In a modified Bushnell-Haas medium at room temperature (≈21°C)

under aerobic conditions, Kodak NA concentrations decreased from 82 mg/L to <10mg/L in 12 days and Merichem NA concentrations decreased from 109 mg/L to 8 mg/L in 10 days (Clemente et al., 2004; Table 4.5). Under aerobic conditions at 20°C using modified Bushnell-Haas medium to obtain initial nitrogen and phosphorus concentrations of 1 and 0.7 mM, respectively, Scott et al. (2005) measured 87% removal of Kodak NAs (81 mg/L to 11 mg/L) in 10 days (Table 4.5). Scott et al. (2005) measured biodegradation of three other commercial NAs (Fluka, Merichem and Kodak) under the same conditions and observed 69-93% removal in 10 days with initial NA concentrations ranging from 30-100 mg/L (Table 4.5).

Han et al. (2008) correlated half-lives of Merichem NAs with number of rings in NA structures. For example, 80% removal of acyclic structures (Z=0) was achieved in about 2 days, while NAs with 3 rings (Z=-6) were removed in about 10-15 days (Han et al., 2008). The number of rings in structures correlated more strongly with degradation rates than carbon numbers (Han et al., 2008). Half-lives for Merichem NAs ranged from 1-8 days, and increased with increasing cyclicity in structures (z=0<z=-2<z=-4<z=-6; Han et al., 2008).

Ranking of environmental conditions in terms of influence on degradation

In this study, treatment conditions were isolated (to the extent possible) to discern differences in aerobic degradation of initial Fluka NA concentrations with time for a range of C:N and C:P ratios, dissolved oxygen concentrations, pHs, and temperatures. The following treatment conditions were able to decrease Fluka NA concentrations to non-detect levels in less than 6 days: all C:N and C:P ratios (in the presence of other

macro- and micronutrients), DO concentrations averaging 8.43 mg/L, pH ~8-9, and temperatures averaging 23.4ºC (Table 4.6). By reaching this detection limit, a line of evidence was provided that toxicity could be eliminated based on commercial NA toxicity data (Swigert et al., 2015; Kinley et al. in review). Within the experimental duration (8 days), treatments that did not reach the analytical detection limit were considered ineffective in terms of environmental relevance. Treatments that did not achieve non-detect NA concentrations included: average DO concentrations of 1.94 and 4.76 mg/L, pH ~6, and temperatures averaging 5.9 and 16.9ºC (the two lower temperatures reached 6 and 7 mg NA/L by day 8).

This study demonstrated an approach for evaluating environmental conditions that influence aerobic degradation of NAs. Measurable changes in degradation were observed as a function of all of the environmental factors evaluated (N and P concentrations, DO, pH, and temp). Aerobic microbial degradation has been identified as a promising treatment pathway for energy-derived NAs (Dorn, 1992; Clemente et al., 2004; Allen, 2008; Whitby, 2010). Information gained from results in this study could be used to further management strategies implemented to achieve efficacious treatments of NAs in energy-derived waters.

Conclusions

The purpose of this study was to determine effects of environmental conditions on aerobic degradation of Fluka commercial NAs, with the criterion of achieving concentrations below the analytical detection limit (<5 mg/L), operationally defined by a derivatization method (Yen et al., 2004). Reaching analytically non-detect NA concentrations provided a line of evidence that aquatic toxicity could be eliminated (Swigert et al., 2015; Kinley et al., in review). Of the evaluated conditions, the lowest N (as ammonia) and P (as phosphate) concentrations (80 and 20 µg/L, respectively, corresponding to C:N and C:P ratios of 500:1 and 5000:1) were sufficient to achieve aerobic degradation of Fluka NAs to < 5 mg/L in 5 days. The highest nutrient ratios evaluated (C:N 10:1 and C:P 100:1) were then amended to the remaining treatments to discern influences of DO, pH, and temperature on rates of NA degradation. Treatments with the highest DO concentration (average 8.43 mg/L DO), highest pH (~8-9) and the highest temperature (23.4ºC) achieved degradation to below detection limits in 6 days. Treatments containing near neutral pH (~7) and lower temperatures (~6 and 16ºC) took 1-2 days longer to reach NA concentrations below detection. Fluka NA concentrations in treatments containing lower pH (~6) and lower DO (averaging 1.94 and 4.76 mg/L DO) did not reach the analytical detection limit within the 8 day experimental duration. Clearly, the environmental conditions evaluated in this study influenced rates of aerobic degradation of Fluka NAs. Although commercial NAs differ structurally from energy-derived NAs (e.g oil sands process affected waters, refinery waters), the conditions evaluated in this study should also affect rates and extents of removal of energy-derived

NAs, due to effects on the physical state of NAs or on microbial populations. This aerobic degradation study of Fluka NAs could serve as a model for systematically evaluating environmental factors that influence NA degradation.

References

Alexander, M. 1999. *Biodegradation and Bioremediation*, *2nd ed*. Academic Press, London, UK.

Allen, E.W. 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. *Journal of Environmental Engineering Science*, *7*, 123-138.

American Petroleum Institute (API). 2012. Naphthenic acids category analysis and hazard characterization. Petroleum HPV Testing Group Technical Report # 1100997.

American Public Health Association (APHA). 2007. *Standard methods for the examination of water and wastewater*, 21st Ed. American Public Health Association, Port City Press: Baltimore, MD.

Armstrong, S.A., Headley, J.V., Peru, K.M., & Germida, J.J. 2007. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering*, *43*, 36-42.

Armstrong, S. A., Headley, J. V., Peru, K. M., & Germida, J. J. 2009. Differences in phytotoxicity and dissipation between ionized and nonionized oil sands naphthenic acids in wetland plants. *Environmental Toxicology and Chemistry*, *28*, 2167-2174.

Atlas, R. M., & Bartha, R. 1972. Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorous. *Biotechnology and Bioengineering*, *14*, 309-318.

Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological Reviews*, *45*, 180.

Barrow, M. P., Headley, J. V., Peru, K. M., & Derrick, P. J. 2004. Fourier transform ion cyclotron resonance mass spectrometry of principal components in oil sands naphthenic acids. *Journal of Chromatography A*, *1058*, 51-59.

Brient, J. A., Wessner, P. J., & Doyle, M. N. 1995. Naphthenic acids. *Kirk-Othmer encyclopedia of chemical technology*.

Brock, T.D. & Madigan, M.T. 1988. *Biology of Microorganisms, 5th ed.* Prentice Hall Inc., Englewood Cliffs, NJ.

Bushnell, L. D., Haas, H. F. 1941. The utilization of certain hydrocarbons by microorganisms. *Journal of Bacteriology*, *41*, 653.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... & Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*, 335-336.

Clemente, J. S., MacKinnon, M. D., & Fedorak, P. M. 2004. Aerobic biodegradation of two commercial naphthenic acids preparations. *Environmental Science & Technology*, *38*, 1009-1016.

Clemente, J. S., Fedorak, P. M. 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. *Chemosphere*, *60*, 585-600.

Del Rio, L. F., Hadwin, A. K. M., Pinto, L. J., MacKinnon, M. D., & Moore, M. M. 2006. Degradation of naphthenic acids by sediment micro-organisms. *Journal of Applied Microbiology*, *101*, 1049-1061.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ... & Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*, 5069-5072.

Dorn, P. B., 1992. Case histories—the petroleum industry. Toxicity Reduction: Evaluation and Control. In: Ford, DL (Ed.), Water Quality Management Library 3, 183-223.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*, 2460-2461.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R. 2008. Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. *Environmental Science & Technology*, *43*, 266-271.

Gibbs, C. F., Pugh, K. B., & Andrews, A. R. 1975. Quantitative studies on marine biodegradation of oil. II. Effect of temperature. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *188*, 83-94.

Girling, A. E., Whale, G. F., & Adema, D. M. M. 1994. A guideline supplement for determining the aquatic toxicity of poorly water-soluble complex mixtures using water-accommodated fractions. *Chemosphere, 29,* 2645-2649.

Goldman, J.C., Caron, D.A., & Dennett, M.R. 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. *Limnology and Oceanography, 32,* 1239-1252.

Han, X., Scott, A. C., Fedorak, P. M., Bataineh, M., & Martin, J. W. 2008. Influence of molecular structure on the biodegradability of naphthenic acids. *Environmental Science & Technology, 42,* 1290-1295.

Headley, J. V., Peru, K. M., McMartin, D. W., & Winkler, M. 2002. Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry. *Journal of AOAC International, 85,* 182-187.

Headley, J. V., & McMartin, D. W. 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. *Journal of Environmental Science and Health, Part A, 39*(8), 1989-2010.

Headley, J. V., Peru, K. M., Adenugba, A. A., Du, J. L., & Mcmartin, D. W. 2010. Dissipation of naphthenic acids mixtures by lake biofilms. *Journal of Environmental Science and Health Part A, 45,* 1027-1036.

Herbert, D. 1976. Stoichiometric aspects of microbial growth. *Continuous Culture, 6,* 1-30.

Hill, Jr., C.G. & Root, T.W. 2014. *Introduction to Chemical Engineering Kinetics and Reactor Design*, 2nd ed. John Wiley & Sons, Inc. Hoboken, NJ, USA.

Holowenko F.M., MacKinnon, M.D., Fedorak, P.M. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. *Water Resources, 36,* 2843-2855.

Kavanagh, R. J., Frank, R. A., Burnison, B. K., Young, R. F., Fedorak, P. M., Solomon, K. R., & Van Der Kraak, G. 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. *Aquatic Toxicology, 116,* 34-42.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for

classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, gks808.

Leahy, J. G., & Colwell, R. R. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews*, *54*, 305-315.

Leclair, L. A., MacDonald, G. Z., Phalen, L. J., Köllner, B., Hogan, N. S., & van den Heuvel, M. R. 2013. The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, *142*, 185-194.

Lo, C. C., Brownlee, B. G., & Bunce, N. J. 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. *Water Research*, *40*, 655-664.

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. 2011. UniFrac: an effective distance metric for microbial community comparison. *The ISME Journal*, *5*, 169.

Massardier-Nageotte, V., Pestre, C., Cruard-Pradet, T., & Bayard, R. (2006). Aerobic and anaerobic biodegradability of polymer films and physico-chemical characterization. *Polymer Degradation and Stability*, *91*, 620-627.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., ... & Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*, *6*, 610-618.

McMurdie, P. J., & Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*, e61217.

Nero, V., Farwell, A., Lee, L. E. J., Van Meer, T., MacKinnon, M. D., Dixon, D. G., 2006. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. *Ecotoxicology and Environmental Safety*, *65*, 252-264.

Organization for Economic Cooperation and Development (OECD). 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Series on Testing and Assessment No. 23, ENV/JM/MONO (2000), vol. 6. OECD, Paris, France.

Paslawski, J. C., Headley, J. V., Hill, G. A., & Nemati, M. 2009. Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid. *Biodegradation*, *20*, 125-133.

Redfield, A.C. 1958. The biological control of chemical factors in the environment. *American Scientist*, *46*, 205-221.

Rudzinski, W. E., Oehlers, L., Zhang, Y., & Najera, B. 2002. Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. *Energy & Fuels*, *16*, 1178-1185.

Schramm, L. L. (Ed.), 2000. *Surfactants: fundamentals and applications in the petroleum industry*. Cambridge University Press, Cambridge, UK.

Scott, A. C., Mackinnon, M. D., & Fedorak, P. M. 2005. Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. *Environmental Science & Technology*, *39*, 8388-8394.

Seifert, W. K., Teeter, R. M. 1969. Preparative thin-layer chromatography and high-resolution mass spectrometry of crude oil carboxylic acids. *Analytical Chemistry*, *41*, 786-795.

Sigma-Aldrich. 2015. Material Safety Data Sheet for Naphthenic Acids.

Smith, J.M. 1981. *Chemical Engineering Kinetics*, 3rd ed. McGraw-Hill Book Company. New York, NY, USA.

Swigert, J. P., Lee, C., Wong, D. C., White, R., Scarlett, A. G., West, C. E., Rowland, S. J., 2015. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere *124*, 1-9.

Taylor, D. G., & Trudgill, P. W. 1978. Metabolism of cyclohexane carboxylic acid by Alcaligenes strain W1. *Journal of Bacteriology*, *134*, 401-411.

Tomczyk, N. A., Winans, R. E., Shinn, J. H., Robinson, R. C. 2001. On the nature and origin of acidic species in petroleum. 1. Detailed acid type distribution in a California crude oil. *Energy & Fuels*, *15*, 1498-1504.

Trudgill, P.W. 1984. Microbial degradation of the alicyclic ring. *In Microbial Degradation of Organic Compounds*; Gibson, D.T., Ed.; Marcel Dekker: New York, 131-180.

United States Environmental Protection Agency (USEPA). 1986. Quality criteria for water. Washington DC. EPA-440/5-86-001.

United States Environmental Protection Agency (USEPA). 2012. Screening-level hazard characterization: Reclaimed substances (Naphthenic acids category). Washington DC. EPA-821-R-02-12.

Whitby, C. 2010. Microbial naphthenic acid degradation. *Advances in Applied Microbiology 70*, 93-125.

Yen, T. W., Marsh, W. P., MacKinnon, M. D., Fedorak, P. M., 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. Journal of Chromatography A *1033*(1), 83-90.

Zhao, J. H., Wang, X. Q., Zeng, J., Yang, G., Shi, F. H., & Yan, Q. (2005). Biodegradation of poly (butylene succinate) in compost. *Journal of Applied Polymer Science*, *97*(6), 2273-2278.

Table 4.1: Physical and chemical properties influencing environmental fate and persistence of Fluka naphthenic acids (Sigma-Aldrich)[a]

Parameter	General Characteristic	Source
Identification	1338-24-5 (CAS No)	Sigma-Aldrich, 2015
Color	Pale yellow, dark amber	Sigma-Aldrich, 2015
Physical State	Viscous liquid	Sigma-Aldrich, 2015
Molecular weight[b]	210-250 amu	Brient et al., 1995
Water solubility	88.1 mg/L at pH 7.5	API, 2012
Vapor pressure	1.1×10^{-7} to 7.1×10^{-6} mm Hg at 25°C	USEPA, 2012
Partition coefficient octanol/ water (Log Kow)[c]; pH dependent	˜4 at pH 1	Schramm, 2000
	˜2.4 at pH 7	Schramm, 2000
	< 0.1 at pH 10	Schramm, 2000
Density	0.92 g/mL	Sigma-Aldrich, 2015
Viscosity	22 mm^2/s	Sigma-Aldrich, 2015
pKa	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 OSPW NAs

Table 4.2: Targeted test conditions for experimental treatments.

	Targeted Conditions			
Treatment Factor	**C:N and C:P**	**DO (mg/L)**	**Temp. (°C)**	**pH (S.U)**
C:N and C:P	C:N 10:1, 100:1, 500:1 C:P 100:1, 1000:1, 5000:1	8-9	25±1	8±0.1
DO	10:1 and 100:1	<1, 4-5, 8-9	25±1	8±0.1
Temperature	10:1 and 100:1	8-9	5±1, 15±1, 25±1	8±0.1
pH	10:1 and 100:1	8-9	25±1	6±0.1, 7±0.1, 8±0.1

Table 4.3: Measurements of water characteristics during treatments.

Treatment	Temperature (°C)[a] Mean (Range)	pH (S.U.)[a] Range	DO (mg O_2/L)[a] Mean (Range)	Conductivity (µS/cm)[b]	Alkalinity (mg CaCO3/L)[b]	Hardness (mg CaCO3/L)[b]
C:N						
10:1	24 (23.4-24.3)	7.90-9.15	8.38 (8.05-8.32)	1290	692	92
100:1	24 (23.4-24.3)	7.90-9.10	8.37 (8.17-8.49)	1233	592	102
500:1	24 (23.4-24.3)	7.90-9.17	8.44 (8.31-8.63)	1266	708	100
C:P						
100:1	24 (23.4-24.3)	7.90-9.15	8.38 (8.05-8.32)	1290	692	92
1000:1	24 (23.4-24.3)	7.94-9.18	8.35 (8.17-8.52)	1287	592	102
5000:1	24 (23.4-24.3)	7.93-9.01	8.41 (8.18-8.60)	1340	708	106
Control[c]	24 (23.4-24.3)	7.96-8.21	8.15 (7.06-8.32)	159.9	16	28
D.O.						
≤1 mg/L	23.4 (23.0-24.3)	7.90-8.33	1.94 (0.36-3.95)	1354	480	90
4-5 mg/L	23.4 (23.0-24.3)	7.91-8.88	4.76 (3.95-7.22)	1401	580	100
8-9 mg/L	23.4 (23.0-24.3)	7.90-9.19	8.43 (8.03-8.82)	1391	620	74
Temp.						
5°C	5.9 (5.0-8.0)	7.90-9.16	10.87 (8.32-11.67)	1375	608	78
15°C	16.9 (14.2-20.0)	7.90-9.08	9.52 (8.31-10.37)	1355	576	70
25°C	23.4 (23.0-24.3)	7.90-9.19	8.43 (8.03-8.82)	1391	620	74
pH						
6.0	23.4 (23.0-24.3)	5.93-6.42	8.69 (8.06-8.95)	1377	560	86
7.0	23.4 (23.0-24.3)	6.93-7.67	8.62 (8.03-8.82)	1339	508	84
8.0	23.4 (23.0-24.3)	7.90-9.19	8.43 (8.03-8.82)	1391	620	74
Control[d]	23.4 (23.0-24.3)	7.90-8.18	8.07 (7.09-8.53)	159.1	18	40

[a]measured daily (n=3)
[b]measured at test initiation from composite sample (n=1)
[c]control for C:N and C:P treatments
[d]control for all other treatments

Table 4.4: Measured initial and final Fluka NA concentrations and percent removal in treatments and untreated controls.

Treatment	Initial [NA] mg/L (±SD)	Ending [NA] mg/L (±SD)	Percent Removal (%)
C:N[4]			
10:1	61 (6)	BDL	91.8
100:1	62 (10)	BDL	91.9
500:1	56 (6)	BDL	91.1
C:P[4]			
100:1	61 (6)	BDL	91.8
1000:1	60 (6)	BDL	91.7
5000:1	55 (10)	BDL	90.9
Control[1]	64 (1)	62 (17.8)	NA
DO[5]			
1.94 mg/L	64 (3)	24	62.5
4.76 mg/L	55 (6)	16 (10)	70.9
8.43 mg/L	61 (8)	BDL	91.8
Temperature[5]			
5.9°C	70 (4)	6 (2)	91.3
16.9°C	56 (10)	7 (7)	87.5
23.4°C	61 (8)	BDL	91.8
pH[5]			
6.0	69 (1)	29 (7)	58.6
7.0	56 (4)	5 (3)	91.1
8.0	61 (8)	BDL	91.8
Control[2]	56 (5)	66 (8)	NA

NA= not applicable; BDL= below detection limit (5 mg/L)

[1]Control for C:N and C:P treatments

[4]5 day experiment duration

[5]8 day experiment duration

Table 4.5: Reported commercial and model NA biodegradation data and test conditions (if provided). NS=not stated.

Commercial/ Model NA	Nutrient Medium	Temp. (°C)	pH	DO (mg/L)	Initial, Ending NA concentrations (mg/L)	NA Removal (%), Incubation Time (d)	Reference
Fluka[e]	Bushnell-Haas	23.4	≈8	8-9	61, < 5	92, 8	Current Study
Fluka[e]	Bushnell-Haas	~ 20	NS	Aerobic[a]	NS[d]	81-87[b], 10	Scott et al. (2005)
Fluka[f]	Nutrients in lake water	NS	8.0[b]	5.46[b]	NS	NS, 24	Headley et al. (2010)
Kodak Acids[e]	Bushnell-Haas	~ 20	NS	Aerobic[a]	81, 11	87-88, 10	Scott et al. (2005)
Kodak Salts[e]	Bushnell-Haas	~ 21	NS	Aerobic[a]	82, 10	>90, 12	Clemente et al. (2004)
Kodak Salts[e]	Bushnell-Haas	~ 20	NS	Aerobic[a]	NS[d]	69-77, 10	Scott et al. (2005)
Kodak Salts[g]	Mineral salts medium	20	8.2	NS	NS	95, 28	Del Rio et al. (2006)
Merichem Acids[e]	Bushnell-Haas	~ 21	NS	Aerobic[a]	109, 8	93, 10	Clemente et al. (2004)
Merichem Acids[e]	Bushnell-Haas	~ 20	NS	Aerobic[a]	NS[d]	90-93, 10	Scott et al. (2005)
Merichem Acids[h]	Bushnell-Haas	~ 20	NS	Aerobic[a]	100, 2	98, 28	Han et al. (2008)
Trans-4MCHCA[c,i]	McKinney's Medium	23	7	NS	50, NS	NS	Paslawski et al. (2009)

[a] Measured DO concentration not reported

[b] Property of feedwater, conditions in reactor not measured

[c] Surrogate NA; a carboxylic acid fitting the description $C_nH_{n-Z}O_2$

Table 4.6. Ranked conditions in terms of time to achieve non-detect Fluka NA concentrations; MDL= method detection limit (5 mg/L); NA= not applicable.

*Condition	Initial NA mg/L (±SD)	Time to 50% removal (days)	Time to reach non-detect NA concentration (days)	Reach MDL?
C:N 10:1	61 (6)	2.5	5	Yes
C:N 100:1	62 (10)	3.2	5	Yes
C:N 500:1	56 (6)	3.2	5	Yes
C:P 100:1	61 (6)	2.5	5	Yes
C:P 1000:1	60 (6)	3.1	5	Yes
C:P 5000:1	55 (10)	4.2	5	Yes
DO 8.43 mg/L	61 (8)	2	6	Yes
pH 7.90-9.19	61 (8)	2	6	Yes
Temp 23.4ºC	61 (8)	2	6	Yes
pH 6.93-7.67	56 (4)	3.5	7	Yes
Temp 5.9ºC	70 (4)	5.5	NA	No
Temp 16.9ºC	56 (10)	2	NA	No
DO 4.76 mg/L	55 (6)	5.5	NA	No
DO 1.94 mg/L	64 (3)	4.3	NA	No
pH 5.93-6.42	69 (1)	3.5	NA	No

*All C:N and C:P ratios were stored at targeted pH 8±0.1, temperature 25±1ºC, and dissolved oxygen 8-9 mg/L. All DO, pH, and temperature treatments were evaluated separately (i.e. where one variable was altered, others were kept consistent) and prepared with Bushnell-Haas medium containing C:N ratios of 10:1 and C:P of 100:1.

Figure 4.1: Changes in NA concentrations over time for a range of C:N ratios (*n*=3). Error bars indicate standard deviations.

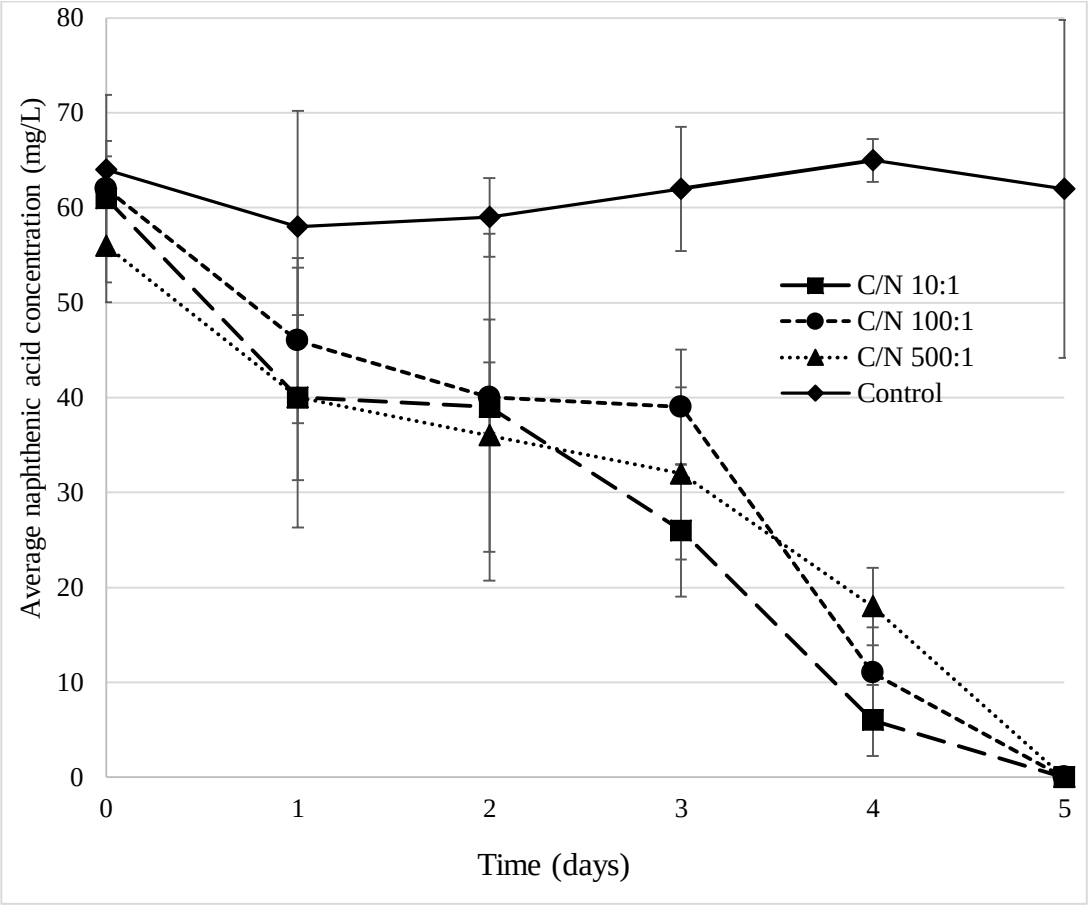


Figure 4.2: Changes in NA concentrations over time for a range of C:P ratios (*n*=3). Error bars indicate standard deviations.

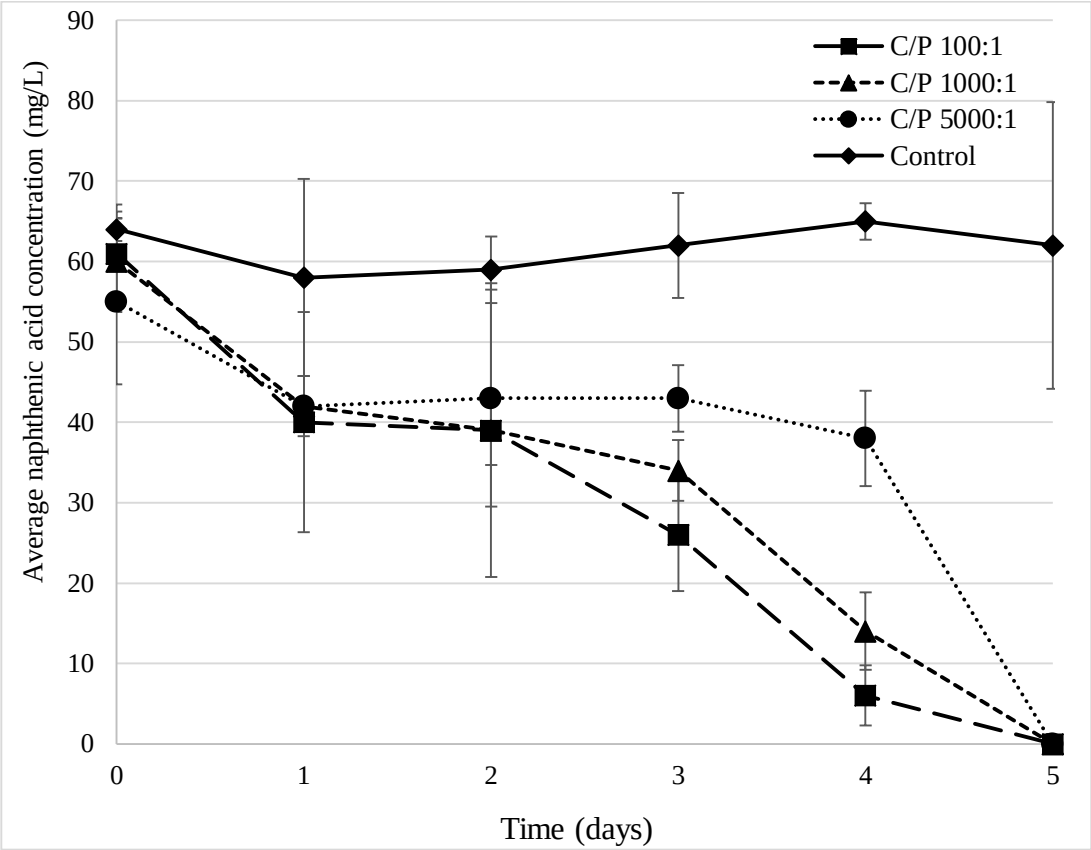


Figure 4.3: Changes in NA concentrations over time for a range of dissolved oxygen concentrations (*n*=3). Error bars indicate standard deviations.

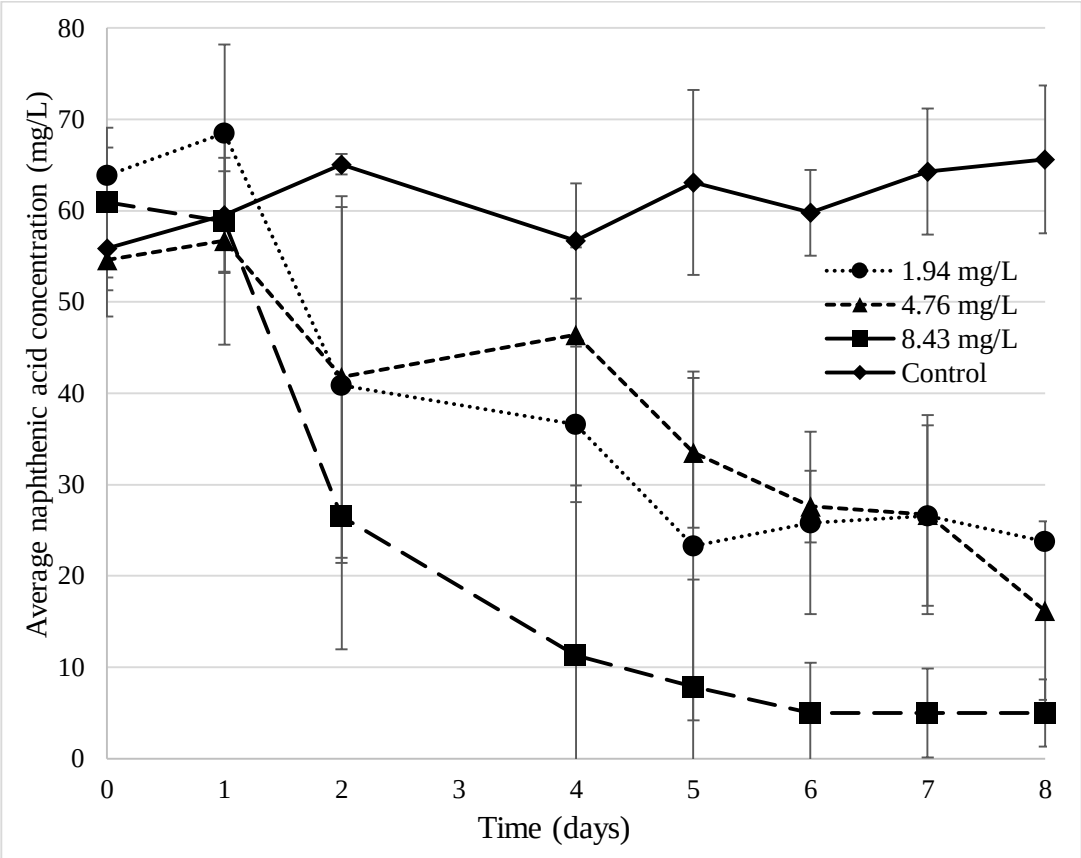


Figure 4.4: Changes in NA concentrations over time for a range of pH (*n*=3). Error bars indicate standard deviations.

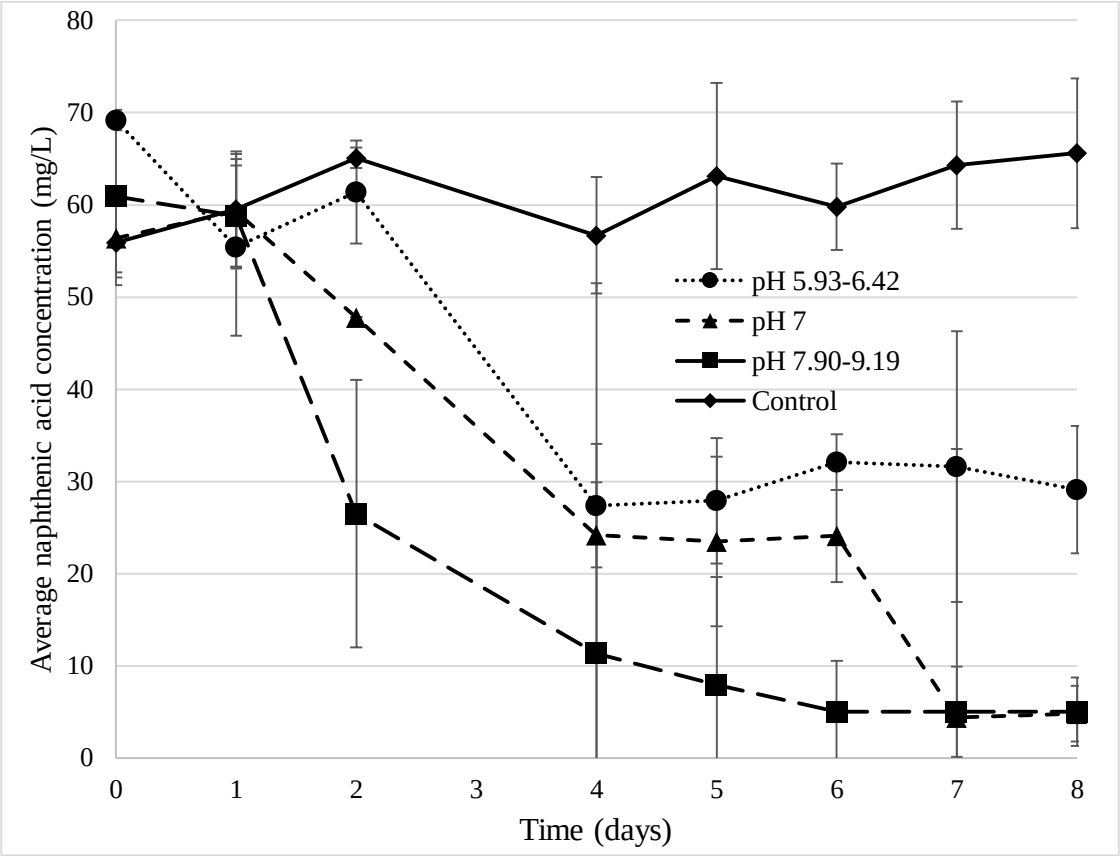


Figure 4.5: Changes in NA concentration over time for a range of temperatures (*n*=3). Error bars indicate standard deviations.

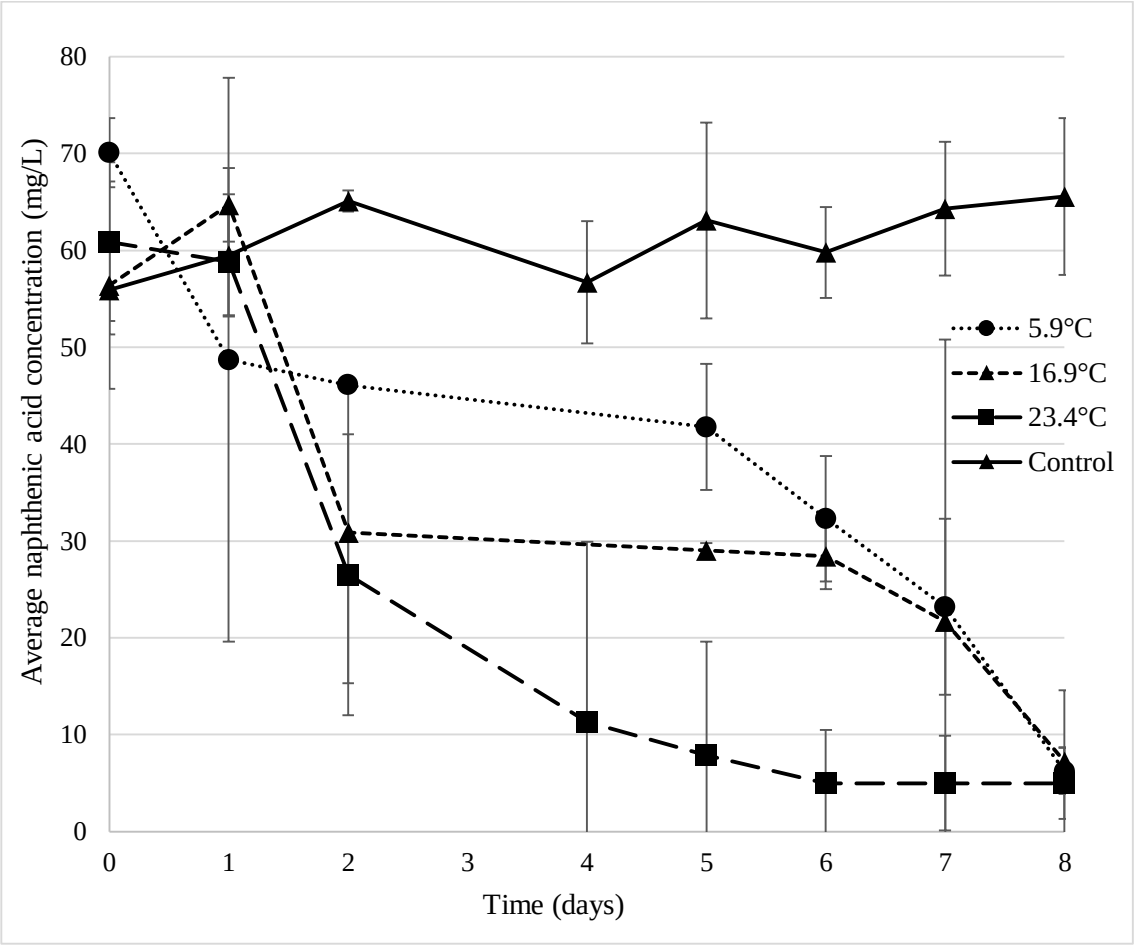
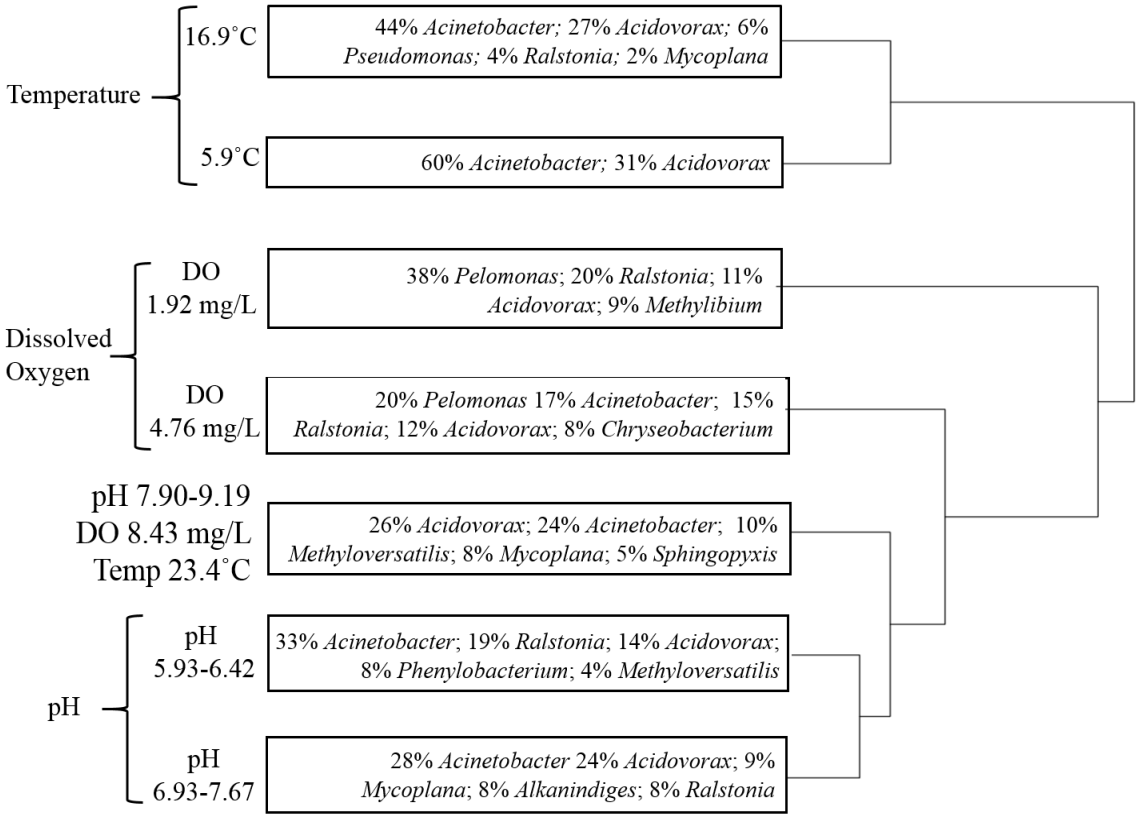


Figure 4.6: Hierarchial clustering analysis of microbial sequencing data showing prominent genera in each treatment evaluated.



CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Naphthenic acids (NAs) found in energy-derived waters such as refinery effluents and oil sands process affected waters are relatively persistent in water and are often sources of toxicity and ecological risk. To mitigate risk, exposures must be altered sufficient to eliminate toxicity. In this research, a commercial source of NAs (Fluka) was used to measure comparative toxicity and to evaluate two processes for altering NA exposures.

The manuscript "Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid" was a laboratory experiment utilizing stable and repeatable exposures of Fluka NAs to discern relative sensitivities of sentinel aquatic organisms. Sentinel aquatic organisms included a vertebrate: fathead minnow (*Pimephales promelas*), invertebrates: microcrustacean (*Ceriodaphnia dubia*), amphipod (*Hyalella azteca*), and midge (*Chironomus dilutus*) and a macrophyte: common cattail (*Typha latifolia*). Measured responses (7-d LC50s/EC50) ranged from 1.9 mg NA/L for *Pimephales promelas* to 56.2 mg NA/L for *Typha latifolia*. Following *P. promelas* in order of decreasing sensitivity were *Ceriodaphnia dubia* (7-d LC50=2.8 mg NA/L), *Hyalella azteca* (7-d LC50= 4.1 mg NA/L), *Chironomus dilutus* (7-d LC50= 6.5 mg NA/L), and *Typha latifolia* (7-d EC50= 56.2 mg NA/L), indicating that in terms of sensitivities, fish>invertebrates>plant for Fluka NAs. Factors that affect exposures (i.e. source, structure, duration, and pH) and measurements of exposures (i.e. extraction and analytical techniques) can fundamentally differ among commercial and energy-derived

NAs and severely limit comparisons. However, fish species evaluated in published studies were relatively sensitive to both commercial and energy-derived NAs from different sources and are likely appropriate sentinel species for risk evaluations in aquatic systems.

The manuscript "Photocatalytic Degradation of a Commercial Naphthenic Acid in water using Fixed-film TiO_2" was a bench-scale laboratory experiment designed to promote photocatalysis of Fluka NAs with fixed-film TiO_2 with sunlight irradiance. Changes in NA concentrations with time were confirmed with measures of toxicity to sentinel species fathead minnow (*P. promelas*) and a microcrustacean (*D. magna*). Greater than 90% removal of Fluka NAs (initial concentration 63 mg/L) was achieved in 4-hrs with photocatalysis in fixed-film (TiO_2) reactors in direct sunlight. Photocatalysis also eliminated acute toxicity to sentinel species, with mortality decreasing from 100% to 0% after 5-hrs of photocatalytic treatment for fathead minnow (*Pimephales promelas*) and after 4-hrs for the freshwater invertebrate (*Daphnia magna*). In this experiment, measuring responses of aquatic organisms concomitantly with analytical quantification of Fluka NAs over time confirmed alteration of exposures as well as mitigation of risk. Fixed-film TiO_2 application may provide an alternative solution for scaling the technology for larger applications. Photocatalytic degradation using fixed-film TiO_2 irradiated with sunlight achieved efficacious rates and extents of removal of Fluka NAs, indicating the potential for application of this technology for mitigating ecological risks associated with NAs.

The manuscript "Effects of environmental conditions on aerobic degradation of a commercial naphthenic acid" was a bench-scale laboratory experiment evaluating the relative influence of environmental conditions on rates and extents of aerobic degradation of Fluka NAs. Rates and extents of aerobic degradation of Fluka NAs were measured and compared in a laboratory culture medium for environmentally relevant ranges of N and P concentrations, DO concentrations, pH, and temperatures. Treatments were accomplished in a strategic order, where C:N and C:P ratios were measured first, to ensure nutrient limitation was not a confounding variable in other conditions (DO, pH, and temperature). These conditions were then ranked in terms of influence on Fluka NA half-lives. All conditions evaluated in this study affected rates and extents of aerobic degradation of Fluka NAs. Environmental conditions that positively influenced aerobic degradation rates of Fluka NAs were C:N and C:P molar ratios of 500:1 and 5000:1, respectively (with other macro- and micronutrients supplied), DO >8 mg/L, pH ≈8, and temperatures >23ºC. Under the above described conditions at an initial Fluka NA concentration of 61 mg/L (±8), a removal rate coefficient of 0.487 day^{-1} was achieved (half-life approximately 1.4 days). Clearly, environmental conditions influence aerobic degradation of NAs. This study demonstrated an approach for evaluating environmental conditions that may influence rates and extents of aerobic degradation of NAs. Commercial NAs differ structurally from energy-derived NAs (e.g oil sands process affected waters), but environmental conditions systematically evaluated in this study are also expected to affect rates and extents of aerobic degradation of compositionally complex NAs.

Ultimately, experiments conducted in this thesis can serve as a model approach for evaluating comparative toxicity of NAs, in terms of relative sensitivities of a taxonomic range of sentinel species, and using that information to implement effective strategies for mitigating risk in aquatic systems.