The effectiveness of photocatalysis on reducing the toxicity of oil sands process-affected water to *Lemna minor*

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Oil sands process-affected water (OSPW) is a by-product produced by the surface-mining of oil sands in Alberta, Canada. OSPW has been found to be acutely and chronically toxic to many forms of wildlife, and due to the industry's zero-discharge policy, it is stored on-site in tailings ponds. There is a need to establish a method to treat OSPW for environmental release as none currently exist. OSPW is a complex saline solution composed of high concentrations of clays, dissolved organic compounds, trace heavy metals, inorganic compounds, and trace amounts of solvents and bitumen. Naphthenic acids are a group of structurally diverse compounds found in the dissolved organics fraction and are among the most toxic organic pollutants present in OSPW. Photocatalysis using TiO_2 is a promising method for reducing the toxicity of such compounds via organic mineralization, and it has been proposed that after a primary photocatalytic treatment step, the treated OSPW can be discharged into wetlands for bioremediation and phytoremediation to treat the remaining pollutants. This research is focused on demonstrating the effectiveness of photocatalysis in reducing naphthenic acid toxicity by exposing the treated OSPW to the freshwater macrophyte *Lemna minor*. Photocatalytic treatment of OSPW reduces the toxicity of dissolved organics such as naphthenic acids to *L. minor*, but not that of trace heavy metals. This report shows that photocatalysis is effective at degrading organic compounds present in OSPW, and it also provides a better understanding of the effects of different OSPW components on *L. minor*.

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Dedication

This master's thesis is dedicated to my parents. Thank you for providing me with continuous encouragement and unfailing support throughout my seven years of study at the University of Waterloo.

Table of Contents

List of Figures

List of Tables

List of Equations

List of Abbreviations

% GI – Percent of growth inhibition

AEO – Acid-extractable organics

AOP – Advanced oxidation processes

APHA – American Public Health Association

ATP – Adenosine triphosphate

αOX – α-oxidation

BTEX – Benzene, toluene, ethylbenzene, xylene

βOX – β-oxidation

C_{db} – Control dried biomass

C_{fc} – Control frond count

CF – Coagulation/flocculation

CI – Confidence interval

COD – Chemical oxygen demand

CWTS – Constructed wetland treatment system

DCM – Dichloromethane

DNA – Deoxyribonucleic acid

DOM – Dissolved organic matter

EDTA – Ethylenediaminetetraacetic acid

EPA – Environmental Protection Agency

FPC(s) – Floating photocatalyst(s)

IC_{50} – Half maximal inhibitory concentration

MW – Molecular weight

n-BPBA – (4'-*n*-butylphenyl)-4-butanoic acid

NA(s) – Naphthenic acid(s)

NP(s) – Nanoparticle(s)

OSPW – Oil sands process-affected water

OXPHOS – Oxidative phosphorylation

PolyDADMAC – polydiallyldimethylammonium chloride

ROS – Reactive oxygen species

SD – Standard deviation

t-BPBA – (4'-t-butylphenyl)-4-butanoic acid

T_{db} – Test sample dried biomass

T_{fc} – Test sample frond count

TOC – Total organic carbon

UV – Ultraviolet

UV-Vis – Ultraviolet-visible spectroscopy

Chapter 1
General Introduction

1.1 Overview

Oil sands process-affected water (OSPW) is a by-product generated from the Clark hot-water bitumen extraction process, and due to the industry's zero-discharge policy, these tailings are stored on-site in tailings ponds[1–5]. There is currently no standardized treatment method rendering OSPW safe enough to be released into the environment; however, oil sands mining companies are required to remediate OSPW and incorporate the treated OSPW into the environment somehow upon completion of mining operations[1,2,5,6].

OSPW is a complex matrix of dissolved salts, clays, silt, residual solvents and bitumen, dissolved organic compounds, trace heavy metals, and inorganic compounds[1,4,7]. The primary toxic components in OSPW are naphthenic acids (NAs) and naphthenate salts[3,8,9]. Conventionally, NAs have the structure $C_nH_{2n+z}O_2$ where "n" indicates the number of carbon atoms and "z" represents zero or a negative even integer indicating the extent of cyclization[1,8–10]. Lower molecular weight (MW) NAs are more easily biodegraded by microbial populations than larger MW NAs, however complete microbial degradation under natural conditions does not seem to be occurring[3,11]. It has been shown in tailings ponds that there are residual NAs even 7-11 years after the last input of OSPW, and it is suggested that the persisting toxicity in older tailings is due to higher MW NAs that are more resistant to microbial degradation[11].

Of the different OSPW treatment methods considered, advanced oxidation processes (AOPs) are most effective at degrading NAs and reducing their toxicity[9]. Photocatalysis over TiO_2 is considered to be especially promising because TiO_2 is earth-abundant, chemically stable, inexpensive, and reusable over multiple treatments, while sunlight is a free and renewable resource[2,4,9]. This passive treatment method does not require the usage of water or electricity or the modification of existing infrastructure[4]. In addition, photocatalysis over TiO_2 can exploit the large surface areas of tailings ponds exposed to sunlight[4]. Passing OSPW through laboratory microcosms has shown that an aerobic wetland environment is capable of reducing total concentrations of NAs over time but is incapable of completely removing persistent NAs[11,12]. Since photocatalysis over TiO_2 is capable of fully mineralizing even the most recalcitrant NAs[9], it can be coupled with bioremediation and phytoremediation in constructed

wetlands for a hybrid OSPW treatment method as constructed wetlands are considered for treating problematic constituents such as metals and organics[12].

This thesis sought to determine whether photocatalysis is a viable method for reducing the concentration of organics present in OSPW prior to its incorporation into wetlands for further remediation. *Lemna minor* was used as the aquatic toxicity test organism and representative wetland plant. Firstly, the components within OSPW that were toxic to *L. minor* were identified, the amount of toxicity contributed by each component was determined, and possible remediation methods were examined. Afterwards, the effects of exposing the photocatalysts to *L. minor* were studied. The IC_{50} of NAs were then determined for *L. minor* frond number and dried biomass, and this was then used to determine the NA toxicity threshold for *L. minor*. The extent of photocatalysis mineralizing NAs in synthetic OSPW and thus reducing toxicity to *L. minor* was observed, and the effects of salts and heavy metals on NA toxicity were investigated. NAs were shown to be both acutely and chronically toxic to *L. minor* using various endpoints.

1.2 Research Objectives

The overall research objective of this research project was to demonstrate that photocatalysis is a viable method of degrading organic compounds in OSPW prior to its introduction into the environment for further bioremediation, and whether the photocatalyst is harmful to aquatic plants in wetlands.

The specific objectives of the study are as follows:

1. Investigate the toxicities of different OSPW components to *Lemna minor* and possible remediation methods
    - Determine the amount of toxicity contributed by organics, heavy metals, and oxidative compounds generated by photocatalysis to the overall toxicity
2. Observing the possible toxicity of the photocatalyst itself to *L. minor*
    - Investigate the effects of exposing the nanopowder photocatalyst P25 TiO_2 and the floating photocatalyst to *L. minor* using frond number and biomass as the endpoints
3. Determine the IC_{50} of synthetic NAs to *L. minor*
    - Determine the IC_{50} of synthetic naphthenic acids using frond number and dried biomass as the endpoints

- Determine the naphthenic acid concentration that is nontoxic to *L. minor* using frond number and dried biomass as the endpoints

4. Demonstrate the ability of photocatalysis in degrading naphthenic acids in synthetic OSPW
    - Determine the extent of organic mineralization throughout photocatalysis over TiO_2 using COD, TOC, and UV-Vis
    - Observe the effects of salts on NA toxicity to *L. minor*
    - Observe the reduction of NA toxicity to *L. minor* as the photocatalytic treatment progresses
5. Investigate the interaction of naphthenic acids and heavy metals in industrial OSPW
    - Observe the reduction of NA toxicity to *Lemna minor* using photocatalysis
    - Observe the interaction of NAs and heavy metals in raw and treated OSPW
    - Observe the interaction of NAs and heavy metals in raw and treated OSPW after the addition of EDTA

1.3 Thesis Outline

This thesis is composed of seven chapters: the introduction, a literature review, four experimental research-based chapters, and a final chapter summarizing the conclusions and recommendations for future research directions.

Chapter 1 introduces the setting of the thesis, current industrial challenges, research hypothesis, and specific research objectives.

Chapter 2 reviews the literature regarding the fundamentals of OSPW and possible mechanisms of NA toxicity in wildlife. Different water treatment methods considered for OSPW remediation including photocatalysis over TiO_2 are investigated. This chapter also explores various aquatic model organisms used for toxicity testing, their fit in overall aquatic toxicology, and the advantages and disadvantages of each with a focus on the freshwater macrophyte *Lemna minor*.

Chapter 3 explores the different components present in OSPW provided by Industry A such as the organics fraction, heavy metals, and oxidative compounds, and the effects of each to *L. minor*. The effect of exposing the nanopowder photocatalyst P25 TiO_2 and floating photocatalysts to *L. minor* is also explored.

Chapter 4 determines the IC_{50} of synthetic NAs to *L. minor* for the two conventional endpoints and shows that the IC_{50} differs depending on which endpoint was chosen to evaluate NA toxicity. As part of determining the IC_{50}, the research also determines which concentrations of synthetic NAs are considered non-toxic to *L. minor*.

Chapter 5 expands on the findings of Chapter 3 and focuses on showing that heavy metals, and not NA degradation by-products, are responsible for the residual toxicity of Industry A's OSPW to *L. minor*. In addition, Chapter 5 serves as a proof-of-concept that photocatalysis degrades organic compounds such as NAs, hence this chapter utilizes synthetic OSPW instead of industrial OSPW. This chapter also demonstrates that OSPW salts interact with NAs and this interaction changes the toxicity of NAs to *L. minor*. Chapter 5 also demonstrates that NAs are acutely and chronically toxic to *L. minor* although the definition for the toxicity is dependent on the chosen endpoint.

Chapter 6 shows the most recent research focusing on the interaction of NAs and heavy metals in an industrial OSPW sample provided by Industry B. This chapter shows that there is some interaction between NAs and heavy metals, but it is recommended that this experiment be repeated using a synthetic OSPW with known components so that it eliminates any interfering effects from the rest of the complex OSPW matrix found in industrial samples.

Chapter 7 highlights the overall conclusions drawn from the research in the previous four chapters and presents recommendations for future research based on these findings. These recommendations include the improvement of evaluation criteria for toxicity to *L. minor*, the focus on the interactions of OSPW components with one another and the changes in toxicity to *L. minor* resulting from these interactions.

Chapter 2
Literature Review

2.1 Summary

Oil sands process-affected water (OSPW) is a toxic by-product generated from the oil sands mining industry. It is currently stored in tailings ponds as there is no established method of reducing the toxicity for environmental release; however, mining companies are required to treat and return the OSPW into the landscape upon the completion of mining operations. Photocatalysis is a promising method for reducing the toxicity of OSPW for environmental return, and it can be coupled with constructed wetlands for remediation in a hybrid treatment to meet the industry's water return and landscape reclamation requirements. In this chapter, we review the fundamental concepts of OSPW toxicity, photocatalysis a promising tool for OSPW treatment and the organisms commonly used in aquatic toxicity testing with a focus on the freshwater macrophyte *Lemna minor*.

2.2 Oil sands process-affected water (OSPW)

The Canadian oil sands mining industry produces 2.9 million barrels of oil per day and production is predicted to increase to 4 million barrels per day by 2030[13]. Oil sands are a mixture of bitumen, a viscous tar-like material, and sand particles[14]. Surface mining is used to remove oil sands from the ground, and bitumen is extracted from oil sands using an alkaline hot-water extraction process which produces oil sands process-affected water (OSPW) as a toxic by-product[9,14,15]. OSPW can be reused in the extraction process for a limited number of times before being stored on-site in active tailing ponds as per industry regulations[1,5,14]. There is an estimated 1 billion m^3 of OSPW stored in tailing ponds covering approximately 170 km^2[6,7]. There is currently no universal or standardized treatment of OSPW to render it safe for environmental release. However, oil sands companies are required to treat and incorporate OSPW into the landscape upon completion of mining operations as part of their landscape reclamation goals to restore the disturbed land to the "equivalent landscape ability" of the pre-mined landscape[6,12,15]. Potential leakage, drainage, or leaching of tailings into the aquatic environment have raised concerns on the effects to wildlife and human health[16].

OSPW is a complex saline mixture of residual bitumen, dissolved organics, clays, trace heavy metals, and other inorganic compounds[1,7,9,17]. The dissolved organics fraction of OSPW is composed of asphaltenes, alkanes, polycyclic aromatic hydrocarbons, benzene, toluene, ethylbenzene, and xylenes

(BTEX), and naphthenic acids (NAs)[8,9]. NAs occur naturally in petroleum deposits and extraction water, and multiple reuses of extraction water concentrates NAs in OSPW[14]. The acid-extractable organics (AEO) and base-neutral extractable organic fractions of OSPW both contain NAs and are associated with much of the toxicity in OSPW[9].

NAs are a structurally diverse group of aliphatic, alicyclic, and aromatic carboxylic acids traditionally defined using the formula $C_nH_{2n+z}O_2$, where "n" is the number of carbon atoms and "z" represents zero or a negative even integer indicating the hydrogen deficit caused by cyclization[1,8–10]. Ring structures may be fused or bridged, and acyclic NAs may be straight-chain or highly branched[18]. Recently, heteroatomic species containing additional oxygen, nitrogen and sulfur atoms and diamondoid structures in naphthenic acids have been identified, and the aforementioned formula can be modified to $C_nH_{2n+z}O_\alpha N_\beta S_\gamma$ to accommodate these additional elements where "α", "β" and "γ" refer to the number of oxygen, nitrogen and sulfur atoms, respectively[2,17,19].

OSPW is acutely and chronically toxic to wildlife, however the mechanism for toxicity is not well understood[1,5]. It has been proposed that multiple components in OSPW contribute to oxidative stress in aquatic organisms[1,20]. NAs and heavy metals in OSPW are both capable of causing oxidative stress with the former interfering with cell membrane permeability and the latter disrupting protein structures when high concentrations are present in cells [17,21,22]. Oxidative stress damages cell components and leads to general cellular dysfunction and apoptosis[20]. Lacaze et al. (2014) exposed rainbow trout (*Oncorhynchus mykiss*) hepatocytes to several NAs and two commercial NA mixtures for 18 h and demonstrated that exposure to NAs led to DNA damage using the comet assay[16]. In fathead minnow (*Pimephales promelas*) embryos, genes for glutathione S-transferase and superoxide dismutase are upregulated in response to oxidative stress, and He et al. (2012) showed that fathead minnow embryos exposed to OSPW had an abundance of these gene transcripts compared to embryos in a control group[23]. Alberts et al. (2019) demonstrated that exposing *Arabidopsis thaliania* (Arabidopsis) root hairs to the acid-extractable organic (AEO) fraction caused H_2O_2 to accumulate in the cytosol, thus leading to oxidative stress[1].

NAs are speculated to cause cell dysfunction by disrupting the cell membrane and membrane structures of organelles such as mitochondria, peroxisomes, the endoplasmic reticulum, and the Golgi apparatus[1,17], although the structural complexities of NAs such as aromaticity and heteroatom content also suggests other mechanisms for toxicity[17,24]. In the mitochondria, NAs can potentially disrupt the membrane permeability and uncouple oxidative phosphorylation (OXPHOS) using their carboxylic

acid moiety, eventually leading to oxidative stress[17]. Electrons move through the electron transport system and generate a proton gradient, which fuels the production of ATP, and complete uncoupling of OXPHOS involves the dissipation of the proton gradient without it being used for ATP production[17]. Mild uncoupling, where the electron transport system is inhibited, increases the production of reactive oxygen species (ROS)[17]. Excess ROS can lead to oxidative stress and cause mitochondria to release cytochrome *c*, resulting in apoptosis[17]. To support this hypothesis, resin acids in pulp and paper effluent share a structural similarity with NAs and were also found to disrupt the mitochondrial membrane, uncouple OXPHOS, and decrease ATP production[25,26]. Rundle et al. (2018) demonstrated that NAs uncoupled OXPHOS and increased ROS production in mitochondria isolated from rainbow trout hepatocytes[17]. As for the other organelles, Alberts et al. (2019) reported that exposing the endoplasmic reticulum of Arabidopsis root epidermal cells to NAs and the AEO fraction resulted in morphology changes and breakdown of much of the reticulate structure[1]. They also demonstrated that exposing NAs and the AEO fraction to both Arabidopsis root epidermal cells and onion epidermal cells effectively eliminated movement of mitochondria, peroxisomes, and the Golgi apparatus, suggesting that changes in organelle membrane dynamics interfere with inter-organelle interactions[1]. When the same two plant cell lines were exposed to NAs and AEO fractions, both cell lines suffered negative effects on the cytoskeleton from the disruption of microtubule and actin filament integrity[1]. In summary, the complexity of the OSPW matrix and the large structural variety of NAs contribute to acute and chronic toxicity by a variety of mechanisms[1,17,20–22,24] such as, but not limited to: inducing oxidative stress[1,17,25,26], changing the permeability of cell membranes, altering inter-organelle interactions[1], and interfering with the cytoskeleton[1].

2.3 OSPW remediation methods

As mentioned above, oil sands mining companies must achieve reclamation goals after the completion of mining operations, however there is currently no universal or standardized treatment rendering OSPW safe for environmental release[6,12,15]. There are a variety of NA remediation methods that are being explored such as microbial biodegradation, flocculation, membrane filtration, advanced oxidation processes (AOPs), and remediation using constructed wetlands[12,19,27,28]. It has been suggested by Afzal et al. (2012) that different treatment methods such as UV/H_2O_2 and microbial degradation are combined in order to achieve a more comprehensive detoxification of OSPW[29]. Martin et al. (2010) demonstrated that ozonation can be complemented with microbial degradation to achieve further OSPW remediation than using either treatment method by itself[30]. In this study, OSPW microorganisms accelerated the

biodegradation of the remaining NAs in reintroduced ozonated OSPW[30]. Flocculation should be used as a pretreatment step to remove silt and suspended solids prior to membrane filtration to prevent membrane fouling[31–33]. Another possible hybrid method could be coupling photocatalysis with phytoremediation in constructed wetlands as the latter is capable of treating problematic constituents such as metals and organics[6,12,15].

2.3.1 Microbial degradation

Certain microbes such as *Acinetobacter anitratum*, *Alcaligenes faecalis*, *Pseudomonas putida,* and *Pseudomonas florescens* are capable of degrading NAs, often resulting in partial or complete degradation[34–38]. However, this process is slow, and it has been shown in tailings ponds 7-11 years after the last input of OSPW that there are still residual NAs[3,11,39]. Aged tailings that are several decades old have been found to have a high concentration of 19 mg/L NAs, demonstrating that there are persistent NAs that natural degradation has difficulties mineralizing[40]. Lower molecular weight (MW) NAs are more easily biodegraded by microbial populations than larger MW NAs, and it is suggested that the persisting toxicity in older tailings is due to higher MW NAs[3,11,39,41]. Multiple rings and alkyl branching in high MW NAs create further steric hindrance, thus making them more resistant to microbial degradation[11,39,41].

Microbes can use β-oxidation (βOX), a combination of α-oxidation (αOX) and βOX, or aromatization pathways to degrade aliphatic and alicyclic NAs[41–44]. Most microorganisms degrade aliphatic and alicyclic carboxylic acids using βOX, hence it is likely that NAs are biodegraded using this mechanism[41,44]. In noncyclic NAs, the presence of a tertiary carbon at the β position will prevent βOX, and the presence of a quaternary carbon at either the α or β position will prevent βOX[41,42]. If there is only a tertiary carbon at the α position and there is no branching at the β position, βOX can still occur albeit slowly due to steric hindrance[41,42].

Alicyclic and aromatic compounds containing side chains with an odd number of carbons are much more labile than those containing side chains with an even number of carbons as the ring structure can be cleaved using βOX [41,45]. For example, if an *n*-alkyl-substituted cyclohexane has an odd number of carbons in the alkyl chain, βOX degrades the compound into cyclohexyl carboxylic acid which can then undergo further βOX to be mineralized[41]. If the alkyl chain has an even number of carbons, βOX of the *n*-alkyl-substituted cyclohexane produces cyclohexylacetic acid, which requires a combination of α- and βOX to be degraded further as βOX by itself is inhibited by the tertiary carbon on the ring[43,46].

Some microbes such as *Alcaligenes* sp. PHY 12 are capable of combining α- and βOX together to degrade even carbon numbered n-alkyl-substituted cyclohexanes as long as the there are no tertiary or quaternary carbons present to inhibit αOX[41,43].

In addition to α- and βOX, cyclic NAs can also be degraded by aromatization where hydroxylation occurs at the *para* position, however, aromatization will be prevented if the *para* position is occupied by an alkyl group or another ring[36,41,44]. The presence of quaternary carbons at any position of the ring will also prevent aromatization from occurring, providing evidence of why branched alkanes add to the recalcitrance of some NAs[36,41]. Other bacteria are capable of degradation pathways such as *ortho* and *meta* aromatic degradation[47,48]. Some *P. putida* strains such as *P. putida* KT2440 have the enzymes for the *ortho* aromatic degradation pathway[47] which is involved in metabolizing protocatechuates and catechols[49]. Bacteria with the TOL plasmid have the enzymes for the *meta* pathway[48]. The *meta* pathway is less abundant, but has a wider substrate specificity than the *ortho* pathway and is able to use substituted aromatic compounds and polyaromatic hydrocarbons[49–51]. Johnson et al. (2011) identified *Burkholderia*, *Pseudomonas*, and *Sphingomonas* spp. which may participate in aromatic alkanoic acid degradation[38]. There is not much information on aromatic alkanoic acid metabolism although Johnson et al. (2011) sought to determine the degradation mechanism of (4'-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA)[38]. Johnson et al. (2011) determined that the first two steps involve βOX of the carboxyl side chain followed by carboxylation of the alkyl chain via a monooxygenase, and further oxidation of the alkyl side chain and ring cleavage was proposed[38]. In a further study, Johnson et al. (2013) investigated the ability of *P. putida* KT2440 to degrade *n*-BPBA and the highly recalcitrant (4'-t-butylphenyl)-4-butanoic acid (*t*-BPBA), and while *P. putida* KT2440 was able to completely degrade *n*-BPBA via βOX after 14 days, it was unable to degrade *t*-BPBA even after 49 days[45].

While certain bacteria that are capable of degrading NAs have been identified, bacterial populations in tailings ponds are complex and more research is needed in order to identify bacteria that might be involved in NA degradation[38] and study the genetics involved with NA metabolism[45]. It is also recommended that microorganisms with the TOL plasmid are identified and implemented for OSPW microbial degradation[45].

2.3.2 Coagulation/Flocculation

The oil sands extraction process produces fine tailings which are stored in tailings ponds, and over time the tailings form a stable suspension containing around 30 wt% solids[52]. The accumulation and storage

of tailings is both an environmental and economical concern, so oil sands mining companies are exploring methods to reduce these tailings[52]. Coagulation/flocculation (CF) refers to the process where suspended particles collide and aggregate to form flocs which can be separated from solution by sedimentation or flotation[53]. The process has two steps: transport and attachment[53]. The transport step leads to the collisions between particles and is achieved by Brownian motion, mixing, or the differences in sedimentation velocities of the particles[53]. The second step depends on attraction forces such as electrostatic attraction, which are dependent on the particle surfaces themselves and causes charge neutralization and instability in aqueous solution[52–55]. The transport and attachment steps form microflocs, and the electrostatic attraction is usually between the compound of interest and a polyelectrolyte[52,54,55]. The microflocs then agglomerate into flocs via macrophase interactions such as bridging[56]. CF is often used for removing the fine solids in OSPW such as silt and colloidal solids[31–33], but it has been shown by Pourrezaei et al. (2011) and Bjornen (2011) to be effective at removing NAs as well[57,58]. Pourrezaei et al. (2011) used alum and the highly cationic polymer polydiallyldimethylammonium chloride (polyDADMAC) for CF in OSPW, and showed the process reduced NA concentrations by 37%, oxidized NA by 86%, and other pollutants present in OSPW such as vanadium and barium by 67-78% and 42-63%, respectively[57]. Higher MW NAs were also preferentially removed; this can be explained by the higher number of rings decreasing the solubility of these NAs in OSPW, thus allowing for easier separation[57]. However, Pourrezaei et al. (2011) demonstrated that the flocculant must be carefully chosen as the cationic polymer they used proved to be toxic to the benthic invertebrate *Chironomus dilutus*[57]. Sasaki et al. (2014) described a CF process removing NAs using iron oxide particles and polyacrylic acid, and they achieved the reduction of more than 90% of NAs in an OSPW with an initial NA concentration of 220 mg/L[59]. After removing the NAs, the floc was separated from the aqueous solution magnetically[59]. On the contrary, Wang et al. (2015) was unable to remove NAs from OSPW using polyaluminum chloride, but they demonstrated that flocculation can be used to remove metal ions from OSPW[60]. More than 90% of Fe, Al, Ga, and Ti cations were complexed after lowering the pH of OSPW to 6.9-8.1, and Na, Ca, Mg, and Ni cations had reduction rates from 0-40%[60]. While CF processes are relatively easy to maintain and are often energy efficient, they require a high operating costs as an excess of chemical flocculants is needed to effectively remove dissolved pollutants[61]. Furthermore, the large volumes of sludge produced from CF requires additional processing for disposal[62]. With regards to applications in remediating tailings, the salinity of OSPW can also affect the efficacy of CF as monovalent salts might interfere with electrostatic interactions between the coagulant/flocculant and the pollutant[19].

2.3.3 Membrane filtration

Membrane filtration refers to a process where a fluid is passed through an immobilized porous material and solutes are selectively removed[61]. The removal mechanism is usually through electrostatic interactions between the solute and membrane or size exclusion[63]. Due to its high throughput, membrane filtration may accelerate the remediation process[64]. Membrane fouling is an issue faced by membrane filtration technologies, but cleaning procedures such as backwashing or chemical treatment can restore the membrane[31,64]. However, membrane restoration would require the additional use of water or solvents which would generate more waste needing proper disposal[31–33]. Chemical modifications to the membrane can improve permeate flux, reduce fouling, and improve reversibility, as Kim et al. (2013) demonstrated by adding multi-walled carbon nanotubes modified with hydroxyl and carboxyl functional groups for added hydrophilicity[65]. Alpatova et al. (2014) investigated the use of a ceramic ultrafiltration membrane (TAMI Industries) with a size cutoff of 1000 g/mol to remove both dissolved organics and inorganics from OSPW[31]. Alum was added to the OSPW to prevent irreversible fouling of the membrane and to force suspended solids and colloidal matter to precipitate [31]. However, NA concentrations were not reduced using alum alone[31]. After the flocculation step, the ultrafiltration membrane was only able to remove 12.4% of NAs, and this was attributed to the small molecular size of NAs relative to the membrane pore size[31]. While an ultrafiltration membrane might not be able to significantly lower NA concentrations, it greatly reduced turbidity and silt in the filtrate and was thus suggested to be used as a pretreatment of OSPW prior to nanofiltration[52]. Peng et al. (2004) compared polymeric nanofiltration membranes Desal 5 (GE Osmonics), NF45 (Dow Chemical), and NF50 (Dow Chemical) on removing NAs and determined that while the membranes removed >95% of NAs in OSPW, the Desal 5 membrane performed the best due to its ability to maintain permeate flux[64]. Due to membrane fouling, CF should be used as a pretreatment step to remove silt and suspended solids prior to membrane filtration[31–33].

2.3.4 Advanced oxidation processes (AOPs)

Of the various methods proposed to treat OSPW, AOPs have been shown to effectively degrade NAs and lessen OSPW toxicity[9]. AOPs involve the oxidative degradation of chemical compounds using hydroxyl radicals[19,66]. Hydroxyl radicals can oxidize virtually any organic compound via hydrogen abstraction, and further oxidation and radical propagation eventually results in degradation, although the reaction rate varies from compound to compound[19,67]. The *in situ* production of hydroxyl radicals is promoted by ozonation, UV light[39,68], or a transition metal catalyst[19,69]. In OSPW, NA degradation

rates are slowed by the radical scavenging ions present such as carbonate and chloride[67]. While they have been shown to effectively degrade NAs, implementing AOPs for full-scale applications would require a relatively high energy usage, high capital, and high operating costs[14,70]. Hence, there is more interest in AOPs that are partially driven by solar energy[70].

Ozonation involves the generation of hydroxyl radicals from the decomposition of molecular ozone[29,30,39]. This AOP is already used for treating drinking water and industrial wastewaters, and thus has potential to be used in treating OSPW by partially or completely mineralizing toxic organic compounds[39]. At the natural pH of OSPW (i.e. pH 8-9), ozone is unstable and decomposes into highly reactive hydroxyl radicals that abstract hydrogens from tertiary carbons, and further radical propagation leads to degradation of NAs[30,39]. Ozonation of OSPW is capable of degrading NAs, but there is still some fraction remaining[30,39]. Pérez-Estrada et al. (2011) hypothesized that formation of oxidized NAs and short chain carboxylates during ozonation compete with NAs for hydroxyl radicals, hence there are NAs remaining after the reaction[39]. While ozonation is capable of degrading NAs and lowering OSPW toxicity, implementing this AOP faces difficulties due to the large volume of stored tailings[14], the relatively high cost of generating ozone[14], and the high ozone dosage required[68].

Solar UV light can be combined with H_2O_2 to degrade NAs as a pretreatment step to produce readily biodegradable wastewater for biological treatment or as a tertiary treatment for effluent remediation [29,70]. Photolysis of H_2O_2 generates hydroxyl radicals which then oxidizes virtually any organic compound[67]. Afzal et al. (2012) found that the UV/H_2O_2 process preferentially degraded higher MW NAs[29], which suggests that the structure-reactivity of cyclic compounds is determined by molecular ozone or hydroxyl radicals[29]. UV/H_2O_2 can be used to restore bioluminescence in *Aliivibrio fischeri* (i.e. reduce the toxicity) in a treated OSPW sample after 90 min of exposure[29]. While it does produce hydroxyl radicals, oxidation by H_2O_2 as a lone treatment is not effective if high concentrations of recalcitrant compounds are present due to low reaction rates at reasonable H_2O_2 concentrations[69]. Other than pairing with H_2O_2, UV light can also be paired with chlorine to generate hypochlorite ions to treat toxic organics in OSPW[70]. The structure-dependent reactivity of NAs was found to be similar to that of other hydroxyl-driven AOPs by Shu et al. (2014)[70]. However, unlike UV/H_2O_2, a drawback to UV/chlorine would be chlorination byproducts adding to the acute toxicity of OSPW[70]. Another drawback to UV/chlorine would be its dependence on sufficient sunlight to drive the photochemical decay of chlorine[70]. Other than pairing UV light with H_2O_2 or chlorine, UV light can also be combined with a transition metal catalyst such as Fenton's reagent to generate hydroxyl radicals[69]. Fenton's

reagent is a mixture of Fe^{2+} and H_2O_2, and the two react to form Fe^{3+}, hydroxide ions, and hydroxyl radicals[69]. The combination of Fenton's reagent with UV light is called the photo-Fenton reaction, and UV light allows for increased production of hydroxyl radicals and further breakdown of short-chain organic compounds complexed with iron[66,71]. Fenton's reagent has been proven to be able to degrade phenols and herbicides in wastewater[66]. Zhang et al. (2016) reported that the model NA cyclohexanoic acid was degraded by a modified Fenton reaction where the addition of aminopolycarboxylic acids as chelators prevented iron from forming complexes at a basic pH[72]. Ruppert and Bauer (1999) studied the effect of structure on the mineralization of organic compounds and discovered that aromatic compounds such as phenol, hydroquinone, 4-chlorophenol, and 4-chloroaniline were highly degraded after several hours[73]. By contrast, alicyclic compounds such as cyclohexanol and cyclohexanone did not undergo much degradation[73]. This could possibly be due to the hydroxyl radical being unable to attack conjugated C=C double bonds in alicyclic compounds, whereas in aromatics compounds, hydroxyl radicals open up the ring to allow further degradation[73]. While Fenton's reaction is particularly attractive due to its near-stoichiometric generation of oxidant from Fe^{2+} and H_2O_2[74], this reaction requires strict pH control (pH 2.7-2.8)[69], and the addition of additional chemicals to prevent complexation in wastewaters with a basic pH[72]. Furthermore, sludges can be formed by reaction byproducts which would then present additional disposal problems[66].

Solar photocatalysis over TiO_2 is a promising method for treating OSPW as it is able to completely mineralize NAs[9]. TiO_2 is a semiconductor, and the lone electron in its outer orbital allows it to induce a series of reductive and oxidative reactions on its surface[75]. When photon energy is equal to or greater than the bandgap energy of TiO_2 (3.2 eV and 3.0 eV for anatase and rutile, respectively), the lone electron is photoexcited to the electron band, leaving an unfilled valence band called a "hole"[75]. The electron can reduce oxygen to the $O_2^{\bullet-}$ radical and the "hole" can oxidize water to the hydroxyl radical[75]. These radicals can then degrade chemical bonds in organic compounds and eventual mineralize these compounds to CO_2[19,75]. This AOP is economically viable as the photocatalyst is inexpensive, earth-abundant, chemically stable, and is reusable over multiple treatments[2,4,9]. Furthermore, solar photocatalysis uses sunlight, a free and renewable resource, and oil sands tailing ponds have large surface areas that are exposed to sunlight[4]. TiO_2 NPs can be immobilized on buoyant hollow glass microspheres to maximize their photocatalytic activity at the water-air interface[4]. These combined benefits make solar photocatalysis using TiO_2 particularly promising for treating OSPW as it does not require the consumption of water or electrical power, the modification of existing infrastructure, or the addition of extra chemicals to fully oxidize all recalcitrant NA fractions[2,4]. A

drawback to solar photocatalysis over TiO_2 is that the reaction is dependent on sufficient sunlight[70]. Since sufficient sunlight is dependent on daytime, season, and location, higher latitude areas such as the Alberta oil sands might not receive enough sunlight to drive photocatalysis in the winter[70]. Another concern of photocatalysis over TiO_2 is the environmental fate of the photocatalyst, as there are conflicting results in the literature as to whether TiO_2 is damaging to wetland plants[76,77].

2.3.5 Constructed wetlands

Oil sands companies are required to treat and incorporate OSPW into the landscape upon completion of mining operations to achieve both water return and landscape reclamation goals, and constructed wetland treatment systems (CWTS) are being considered for its ability to treat problematic constituents such as metals and organics[6,11,12,15,78,79]. Wetland systems offer many transformation processes including sedimentation, biotransformation by plants and microbial communities, nitrification, denitrification, photolysis, and hydrolysis to address problem constituents[80–82]. Wetlands control water runoff, resulting in longer periods of time for water filtration and groundwater recharge, and wetland soils harbor natural microbial communities that aid with water purification by sequestering, transforming, and isolating many pollutants in the water column[78]. Plants have widespread root systems that are in contact with large volumes of soil and support large bacterial populations in the rhizosphere, and phytoremediation involves rhizobacteria transforming contaminants for plant uptake[40,83].

CWTS can be categorized into surface flow and subsurface flow wetlands[81]. The surface flow wetland is open-water and uses a gravel substrate to support wetland plants such as common reed (*Phragmites australis*), cattails (*Typha* spp.), or bulrush (*Scirpus* spp.)[81]. Subsurface flow wetlands focus on directing wastewater through the gravel substrate where the pollutants encounter plant root zones and microbial communities[81]. The variable redox environment offered in the gravel substrate promotes the removal of pollutants[81]. Constructing wetlands for phytoremediation is not without its challenges as wetland treatment technologies rely on the interactions between biological, chemical, and physical processes to deplete pollutant concentrations[12,27,28]. The chemical conditions of the post-mined landscape include a significantly higher water salinity, a higher dissolved oxygen concentration, a lower oxidative-reductive potential, and the sediment of constructed wetlands has significantly less total nitrogen and organic content than natural wetlands[28]. Physical properties such as hydrology and basin morphology must be finely tuned to support biological and ecological requirements of wetland plants, thus the initial vegetation composition in a CWTS would be different from that of a natural

wetland as CWTS created from a post-mined landscape will exhibit atypical hydrology and basin morphometry[28,84–86]. CWTS wetlands for phytoremediation of OSPW must be created from vegetation and hydrosoil compatible with OSPW to treat specific problem components, thus leading to an initial vegetation composition atypical of natural regional wetlands[12,28]. On a structural level, CWTS wetlands are less complex than natural wetlands, offering differences in biogeochemical cycling, available food sources, and shelter for wildlife[28,87–89].

Wetlands can be effective at removing toxicants from OSPW, however the treatment performance is variable[81]. It has been shown by Toor et al. (2013) and McQueen et al. (2017) that an aerobic wetland environment is capable of reducing total concentrations of NAs over time when OSPW is passed through laboratory microcosms[11,12]. Although the aerobic wetland environment by itself is unable to completely remove the persistent NAs[11,12], this drawback can be eliminated by first treating the OSPW using photocatalysis over TiO_2 as photocatalysis is capable of fully mineralizing even the most recalcitrant NAs[2,4]. Anderson et al. (2012) showed that compared to fresh OSPW, reclaimed OSPW did not affect the survival and growth of the benthic invertebrate *Chironomus dilutus*[90], however there was still reduced pupation and emergence observed in reclaimed OSPW[90]. This indicates that despite a long period of natural biodegradation, there is a fraction of toxicity left in the reclamation pond, thus more aggressive OSPW treatment methods are necessary[90].

Suncor and Syncrude have both established experimental reclamation ponds to investigate the effectiveness of remediation using CWTS[40], but few vegetated wetlands > 4 ha have been created on post-mined sites, and no large-scale CWTS (>100 ha) have been constructed despite ongoing research into CWTS and post-mining reclamation[78]. There is limited clear and consistent reclamation instructions and few standards by which to gauge reclamation success issued by the regulatory agencies[78], however, further advances in CWTS research might provide improvements[28]. Roy et al. (2016) showed that wetland zone surveys conducted with submersed plants provided an improved method of distinguishing between wetlands contaminated with tailings and those without, and they recommended that submersed plants be used in addition to plants of other zones for CWT monitoring[28]. Despite some regulatory limitations, CWTS for reclamation remain attractive to oil sands mining companies for environmental and economic reasons: they have a low environmental impact, should not produce waste that requires additional disposal, and appropriately designed CWTS can have low operating costs[81].

2.4 Aquatic toxicity testing model organisms

Test species for aquatic toxicity testing are chosen based on criteria such as available historical data, specimen availability, sensitivity to the toxicant, the relationship of the test species to the assessment endpoint, and how representative that species is for others in the ecosystem[91–93]. Model organisms for testing are also chosen to represent those inhabiting certain trophic levels or those with certain feeding strategies[94].

2.4.1 Fish

Fish are advantageous to use in aquatic toxicity as they play a vital ecological and environmental role in the food webs by acting as energy carriers from lower to higher trophic levels, and they are ubiquitous in aquatic environments[95]. Fish bioassays generally show good sensitivity and allow for real-time analysis[96]. Due to their location in the food web, fish can be used to study bioaccumulation of toxicants[97]. A limitation to using fish as test organisms is that between different species, there is considerable variation in basic physiological features and in the responsiveness of select biomarkers towards toxic stresses[95]. Fish assays also time consuming, require large sample volumes, specialized equipment, and skilled operators, and there are problems with standardization[96,98].

Rainbow trout (*Onchorynchus mykiss*) is used as a model to study the effect of toxicants on fish due to its universal presence in freshwaters worldwide as well as the magnitude of toxicological data available[99–101]. Furthermore, it is commonly reared in hatcheries and aquaculture, hence there is an appreciable amount of culture maintenance data available[99]. Alsop et al. (2016) used rainbow trout to study the bioaccumulation of waterborne and foodborne lead[97]. It has been shown by Lari et al. (2017, 2019) that OSPW impairs the olfactory system of rainbow trout and this impairment increases with chronic exposure[100,101]. Rainbow trout cell lines can also be used to study toxicity effects *in vitro*[16,102]. Gagné et al. (2012) and Lacaze et al. (2014) both showed that NAs in OSPW cause genotoxicity in rainbow trout cell lines[16,102]. Lacaze et al. (2014) showed that the chemical structures of individual NAs influenced their genotoxicity using the comet assay[16].

Fathead minnow (*Pimephales promelas*) can be used to study toxic effects on the embryonic, larval, and early juvenile periods of fish development, and are advantageous to use as the usage of embryos terminating at hatch allows for whole-organism testing without coming into conflict with animal-welfare regulations[103]. Toxicity tests using embryos also require only very small volumes of test samples compared to running tests on fish that have reached the exogenous feeding stage of

development[103]. Marentette et al. (2015) showed that the NA fractions of OSPW caused mortality, abnormal heart rates two days post-fertilization, and severe cardiovascular abnormalities such as edemas and hemorrhages by 5-7 days post-fertilization in fathead minnow embryos[104]. Bauer et al. (2017) showed that edema resulting from the grouping of the yolk sac and pericardial enema are the most common abnormality in fathead minnow exposed to the AEO of OSPW[24]. In addition to physical abnormalities, He et al. (2012) showed that genes for glutathione S-transferase and superoxide dismutase were found to be upregulated in fathead minnow embryos exposed to OSPW compared to embryos in a control group[23].

2.4.2 *Aliivibrio fischeri*

Traditional aquatic toxicity tests involve fish, crustaceans, and algae, and these often require long exposure times as well as large sample volumes[96]. Bacteria are an integral part of the ecosystem, hence the popularity of bacterial assays[96]. Despite the differences in toxicity mechanisms for various organisms, toxic effects are often similar, hence a toxic effect on a bacterium can predict toxic effects on higher organisms[96,105]. The marine bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*/*Photobacterium phosphoreum*) emits light which is a result of central metabolic activity[106,107]. As the reduction of bioluminescence is linked to reduced metabolic activity, the reduction of bioluminescence is also proportional to test sample toxicity[106,107]. The *A. fischeri* toxicity test can be conducted on aqueous, soil, and sediment samples, and can test for both acute and chronic toxicity[96,107–110]. The test is rapid, simple, sensitive, cost-effective, and requires very little sample volume[96,106,111]. While the test usually takes 5-30 min, the assay can accommodate longer hour-based time points, and multiple time-based endpoints can be obtained from the same experiment[96,107–110]. Another advantage to using *A. fischeri* for toxicity tests is that it allows animal testing to be minimized[107]. The disadvantages of using *A. fischeri* for toxicity tests is that coloured or turbid samples cause uncertainty in light output measurements and further calculations must be performed to correct the data[108]. Certain chemicals samples might not be ideal for testing with *A. fischeri* either, as hydrophobic compounds require carrier solvents, and these can affect toxicity measurements even after the carrier solvent has been diluted to < 1.5% using 2% NaCl[96,112]. Another disadvantage is that this bacterium is native to a marine habitat so freshwater samples require a salinity adjustment to 2% NaCl prior to the introduction of *A. fischeri*[108]. Hence, *A. fischeri* might not be representative for freshwater bacteria as well as the freshwater environment[108].

2.4.3 *Daphnia magna*

Zooplankton inhabit the pelagic zone of an aquatic system and occupy a central position in the aquatic food web, and thus are influenced by bottom-up and top-down processes[113]. Due to the homogeneity of the pelagic zone with respect to temperature and water volume, chemical properties of abiotic factors do not fluctuate wildly and are more predictable when compared to the chemical properties of abiotic factors in terrestrial or benthic systems[113]. The short life cycles of zooplankton allow for studies on reproductive rates and mortalities [113].The zooplankton habitat, biological features, and position in the food chain make them suitable for testing hypotheses for general ecology[113]. The cladoceran *Daphnia magna* is a pelagic freshwater zooplankton grazer that occupies a central position in the aquatic food web, and it is commonly used as a standard toxicity test organism due to its short life cycle, easy culture maintenance, and the wealth of existing information on its response to a wide range of toxicants[114,115]. *D. magna* is sensitive to many toxicants found in aquatic environments and can exhibit a variety of physiological, anatomical, and behavioural changes in response to toxic stresses[116,117]. *D. magna* can be used for observations on changes to chemosensory function due to toxicant exposure[115,118,119]. The impairment to *D. magna* chemosensory systems provides useful information on possible toxicity to other aquatic animals as aquatic animals rely on chemosensory systems to locate food and avoid predators[120,121]. Toxicity tests involving *D. magna* and OSPW show that chronic exposure resulted in inhibited feeding behaviour, reduced reproductive output, disrupted endocrine balance, suppressed growth, impaired chemosensory function, and reduced fitness overall[122,123].

2.4.4 Algae

Aquatic plant toxicity tests are conducted to determine the potential impact of toxicants to primary producers[124]. Algae and higher plants play important roles in ecosystems as they serve as primary energy sources as well as habitat and shelter for other aquatic wildlife, and are responsible for oxygen production, sediment stabilization, and water quality control[91]. Aquatic plants used for toxicity testing include algal and macrophytic species from a variety of habitats[91]. Freshwater algae have been used for most phytotoxicity testing and the species chosen for testing is dependent on its availability, culturing requirements, and ease of use[91]. The species commonly used include the green algae *Selenastrum capricornutum* and *Scenedesmus* sp.[91]. Other types of algae such as diatoms and blue-green algae are used less often due to slow growth rates and fastidious culturing requirements[91]. Algae are grown in test samples for 72-96 h, and after the test period, algal numbers are assessed using a

particle counter[98]. The inhibition of growth is used as benchmark for toxicity[98]. The main disadvantages of algal assays are difficulties in culturing and lack of reproducibility[98].

2.4.5 *Lemna minor*

Macrophyte species occupy a key position in aquatic environments by providing food, shelter and substrate to other aquatic organisms[125]. Macrophytes are also used in toxicity testing, although less frequently than algae[91]. However, it is recommended that vascular plants are used in toxicity testing as they can provide toxic stress response information that algae cannot[91]. Hence, tests should be run with both algae and macrophytes to generate a more comprehensive understanding on toxicity[91]. There is question to whether aquatic plants can be sufficiently represented by algae alone as there are considerable differences in sensitivity among species[126]. Duckweeds are a group of aquatic plants from the genera *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*[91], and they inhabit a variety of aquatic environments such as ponds, lakes, quiet streams, stagnant waters, and estuaries across a wide range of temperatures, and thus are ubiquitous in nature[127]. The duckweeds *Lemna minor* and *Lemna gibba* have both been extensively studied, hence they are recommended by OECD guidelines as macrophyte-based toxicity test organisms[128].

Lemna minor is a ubiquitous floating freshwater macrophyte that inhabits the surfaces or slightly below the surfaces of relatively still waters across a wide range of temperatures[127,129,130]. *L. minor* is structurally simple and consists of two parts: frond and root[131]. The plants form aggregates of two or more fronds in a colony[131]. *L. minor* has a single rootlet, but instead of absorbing nutrients through a central root system, each whole plant absorbs nutrients directly from the water[129,131]. This feature combined with the buoyancy of plants on the water surface exposes them to compounds present in the test solution as well as surface-active compounds, thus causing the plants to be susceptible to heavy metals, dissolved compounds, hydrophobic compounds, and surfactants[127,130,132,133]. Therefore, *L. minor* is commonly used for aquatic toxicity testing due to its ease of culturing, structural simplicity, rapid growth rate, vegetative reproduction, and test simplicity[127,129,130,133–135]. As the plants reproduce vegetatively, clonal lineages can be established, allowing for genetically identical plants to be used in tests[125]. The plant size is small enough that it does not require large laboratory spaces for culturing and testing, but the size is sufficiently large enough to allow for easy visual observation[125,131]. The test itself does not require large volumes of test solution which allows for easier disposal after test completion[131]. Advantages of using *L. minor* over algae for toxicity testing include the ability to renew test solutions daily and the removal of plants to assess frond production or chlorophyll amounts[130,131].

Furthermore, coloured and turbid samples are better assessed using *L. minor* than algae as the degree of water transparency affects algal growth[136]. Conventional endpoints such as frond number and biomass are easy to assess, but other endpoints such as root length, ^{14}C uptake, total Kjeldahl nitrogen, and chlorophyll amount have all been used to evaluate toxicity[127,130,137].

Apart from its use in toxicity screening, *L. minor* can be used to monitor aquatic toxicity[138]. Roy et al. (2018) demonstrated that submersed and floating plants can serve as better monitors of wetland conditions compared to plants of other zones[28]. *L. minor* has the potential to serve as a means for both phytoremediation and wetland assessment as their recovery from the wetland environment is generally considered non-invasive and does not require extensive filtration[27,139]. Compared to other macrophytes, *L. minor* is more resistant to low temperatures and can grow in water temperatures as low as 5-7°C and thus can be used year-round for wetland monitoring[129,135]. *L. minor* is also capable of growth across a wide pH range; the typical pH range for growth is 4.5-7.5, although growth does not become inhibited until the pH is greater than 10[129]. As mentioned above, *L. minor* accommodates flow-through or renewal of test solutions in toxicity tests, and it also accommodates coloured or turbid samples, therefore allowing it to be used effectively for effluent biomonitoring[131,136]. However, with regards to applications in environmental risk assessment, the conditions of the standard toxicity test in the laboratory such as artificial lighting and standardized testing solutions are vastly different than those encountered in the environment[136,140]. This could lead to problems in the interpretation of the results of toxicity tests and their applications in environmental risk assessment[136,140].

A disadvantage of using *L. minor* for aquatic toxicity testing is that it is considered less sensitive to toxicants compared to animal test species[133,136,141]. Kenaga & Moolenaar (1979) reported that *L. minor* was less sensitive to toxic substances compared to fish and daphnids[141]. There is conflicting information in the literature regarding the sensitivity of *L. minor* as a toxicity test organism, however; Wang (1986) showed that *L. minor* collected from an unpolluted water source was sensitive to heavy metals, particularly cadmium and nickel[138], and Blinova (2004) demonstrated that the sensitivity of animal and plant species to individual toxicants and to complex mixtures of toxicants was unpredictable, and that there was no reason to consider plant species less sensitive to animal species for aquatic toxicity testing[136]. *L. minor* might also be less sensitive to toxicants as it is used to remove toxicants and nutrients from wastewater[134,136], and due to their fast growth rate, *L. minor* may adapt and/or develop resistance quickly to sublethal toxicant concentrations[131].

Another disadvantage of the *L. minor* toxicity test is the choice of endpoints[131]. The conventional endpoints used are frond number and dried biomass as their determination is quick, simple, and non-destructive[127,131]. Frond count is irrelevant to frond size or biomass, and all frond sizes are considered equal despite the frequent observations that plants under toxic stress produce small buds that may be less than 5% of the biomass of a healthy frond[131]. In addition, frond count does not distinguish whether the plants are alive or dead[131]. Thus, frond count grossly underestimates toxicity to *L. minor*[131]. Using dried biomass as the endpoint is a slight improvement over frond number as it takes into account the change in biomass as a toxic stress response, but like frond number, it does not distinguish between live or dead plants[131]. Physiological activity-based endpoints are potentially more sensitive and accurate, however these require more training to evaluate compared to counting fronds or measuring dried biomass [131]. Endpoints such as pigment content[137,142], lipid peroxidation[142], catalase activity[143], peroxidase activity[143], and the alkaline comet assay have been used to determine phytotoxic and genotoxic toxicity in *L. minor*[142]. Despite the potential of increased sensitivity and accuracy of physiology-based endpoints, classical endpoints are still used as it is understood that visible damage to *L. minor* is linked to exposure to a toxicant[144].

Chapter 3

Effect of Organic Compounds and Heavy Metals on *Lemna minor*

3.1 Summary

Oil sands process-affected water (OSPW) is a by-product generated from the oil sands bitumen extraction process and it is currently not approved for environmental release due to industrial regulations. Thus, OSPW is currently stored in tailings ponds. Oil sands mining companies are required to treat OSPW and return it into the environment upon mine closure, but there is no standardized treatment method rendering OSPW safe enough to be incorporated back into the landscape.

OSPW is a complex and saline mixture of clays, residual bitumen, dissolved organics, trace heavy metals, and other inorganic compounds. Toxic compounds such as naphthenic acids in the organic fraction can be mineralized by photocatalysis, thus making this a promising method for treating OSPW. However, due to the intricate composition of OSPW, photocatalysis is one of many methods needed to treat OSPW.

To take full advantage of sunlight for photocatalysis, the TiO_2 NPs must be buoyant. This can be achieved by immobilizing TiO_2 NPs onto glass microspheres to make floating photocatalysts (FPCs). Since FPCs are added directly onto the surfaces of tailing ponds, they will inevitably end up in the ecosystem through wastewater runoff, and it is inevitable that the TiO_2 NPs will eventually detach from surface of the glass microspheres from repeated usage. The environmental effect of the TiO_2 NPs and FPCs must be evaluated before photocatalysis over TiO_2 can be deployed as a method for treating OSPW.

The objective of this work is to demonstrate the photocatalytic degradation of the organic fraction in an OSPW sample provided by Industry A. The extent of photocatalysis on treating Industry A's OSPW is evaluated using *Lemna minor* toxicity tests with frond number as the endpoint. Another purpose of this work is to examine the effects of exposing P25 TiO_2 and FPCs to *L. minor*, and this was evaluated using a *L. minor* 7-day toxicity test with frond number and biomass as the endpoints.

3.2 Experimental

3.2.1 Materials

Raw OSPW was provided by Industry A. Potassium chloride was purchased from ACP Chemicals Inc. Boric acid, sodium chloride, and anhydrous magnesium sulfate was purchased from EMD Chemicals Inc. P25 titanium dioxide Aeroxide™ was purchased from Acros Organics. All other chemicals and reagents were purchased from Sigma Aldrich. The floating photocatalyst (FPC) used for the photocatalytic treatments in this chapter was synthesized using a process described by Leshuk et al., (2018)[4]. FPCs with various photocatalytic capacities were also used to test for toxicity to *L. minor*; these were synthesized using a process described by Leshuk et al., 2018[4]. A 50 g/L $Na_2EDTA{\cdot}2H_2O$ stock solution in deionized water (Millipore, >15 MΩ cm^{-1}) was prepared according to the protocols provided by Huang & Hao (1989), Di Palma et al., (2003), and McQueen et al., (2017)[145–147]. A 25 g/L sodium thiosulfate stock solution in deionized water (Millipore, >15 MΩ cm^{-1}) was prepared following the protocol described by Andersen (2005)[148]. A 10X synthetic Industry A OSPW saline solution was also provided; ion concentrations in Industry A's OSPW were measured according to standard methods by ALS Environmental (Waterloo, ON, Canada), and the preparation method for this synthetic salt solution can be found in Appendix A.

Axenic *Lemna minor* 490 stock cultures were obtained from the Canadian Phycological Culture Centre following the protocol of Gopalapillai et al., (2013)[149], and the axenic *L. minor* plants were cultured in modified Hoagland's E+ medium and subcultured weekly according to the Environment Canada standardized protocol[127]. Modified Hoagland's E+ stock solution A contains 59.00 g/L calcium nitrate tetrahydrate, 75.76 g/L potassium nitrate, 34.00 g/L potassium phosphate monobasic, and 6 mL of 6N HCl. Modified Hoagland's E+ stock solution B contains 3.00 g/L tartaric acid. Modified Hoagland's E+ stock solution stock solution C contains 1.21 g/L iron (III) chloride hexahydrate, 3.35 g/L disodium EDTA dihydrate, and 1.2 mL of 6N KOH. Modified Hoagland's E+ stock solution D contains 50.00 g/L magnesium sulfate heptahydrate. Modified Hoagland's E+ stock solution E contains 2.86 g/L boric acid, 0.22 g/L zinc sulfate heptahydrate, 0.12 g/L sodium molybdate dihydrate, 0.08 g/L copper (II) sulfate pentahydrate, and 3.62 g/L manganese (II) chloride tetrahydrate. 1 L Modified Hoagland's E+ medium was created by adding 20 mL stock solution A, 1 mL stock solution B, 20 mL stock solution C, 10 mL stock solution D, 1 mL stock solution E, 10 g sucrose, 0.10 g yeast extract, and 0.6 g tryptone to 900 mL of deionized water (Millipore, >15 MΩ cm^{-1}). The pH was adjusted to 4.4-4.8 using 1M NaOH and 1M HCl and the final volume was brought to 1 L using

deionized water (Millipore, >15 MΩ cm^{-1}). The modified Hoagland's E+ medium was autoclaved at 121°C and 1.1 bar for 20 min (Priorclave) before use.

American Public Health Association (APHA) stock solution A contains 25.5 g/L sodium nitrate, 15.0 g/L sodium bicarbonate, 1.04 g/L potassium phosphate monobasic, and 1.01 g/L potassium chloride in deionized water (Millipore, >15 MΩ cm^{-1}). APHA stock solution B contains 4.41 g/L calcium chloride dihydrate, 12.17 mg/L magnesium chloride hexahydrate, 0.4149 g/L manganese (II) chloride tetrahydrate, and 0.16 g/L iron (III) chloride hexahydrate in deionized water (Millipore, >15 MΩ cm^{-1}), and the solution was acidified to pH 2 with HCl. APHA stock solution C contains 14.7 g/L magnesium sulfate heptahydrate, 0.186 g/L boric acid, 7.26 mg/L sodium molybdate dihydrate, 3.27 mg/L zinc chloride, 1.4 mg/L cobalt (II) chloride hexahydrate, and 15 µg/L copper (II) chloride dihydrate in deionized water (Millipore, >15 MΩ cm^{-1}). Each APHA stock was added to the test sample solution to produce a final concentration of 1 v/v% of each APHA stock solution (i.e. after the addition of the three APHA stocks, the test sample solution has a final concentration of 97%). Each test solution requires the addition of APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth. The control and acclimation solutions both contain the same components, and both are made using deionized water (Millipore, >15 MΩ cm^{-1}) with each APHA stock added to produce a final concentration of 1 v/v% of each APHA stock solution. As with the test solutions, both the control and acclimation solutions are spiked with the three APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth.

The photocatalytic treatment using FPCs on Industry A's OSPW was conducted using a custom built photoreactor according to the method described by de Oliveira Livera et al., (2018)[2], and 300 mL of filtered treated Industry A OSPW of the following time points were provided for *L. minor* toxicity testing: 0 h, 24 h, 48 h, 96 h, 144 h, and 192 h. A second photocatalytic treatment using FPCs on Industry A OSPW was conducted according to the same protocol described above[2], and 300 mL of treated and filtered Industry A OSPW of the following time points were provided for *L. minor* toxicity testing: 0 h, 25 h, 50 h, 115 h, and 217 h. A third photocatalytic treatment using P25 TiO_2 was conducted using the same aforementioned protocol[2] to treat Industry A's OSPW for 123 h, and the treated sample was filtered and provided for *L. minor* toxicity testing. A raceway pond photocatalytic treatment was conducted based on the methods provided by de Oliveira Livera et al., (2018) and Leshuk et al., (2018)[2,4], and this employed FPCs to treat Industry A's OSPW for 167 h. The treated sample was filtered and provided for *L. minor* toxicity testing.

3.2.2 *Lemna minor* toxicity tests

Since some of the treated OSPW time points were generated around a week after the first time point and the recommended storage limit for water samples is 3 days in a 4°C fridge, three separate *L. minor* toxicity tests were run to accommodate all the time points from the first photocatalytic test.

Axenic *Lemna minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights to acclimate for 18-24 hours before the start of the toxicity test.

For the first toxicity test, five replicates were used for the control, which was created by adding 5 mL of each APHA stock solution to 485 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The following time points were tested: 0 h (Industry A's untreated OSPW), 24 h, and 48 h. Each of the above samples were tested with three replicates. For the 0 h, 24 h, and 48 h samples, 3 mL of each APHA stock solution was added to 291 mL of the respective test solution for a total volume of 300 mL. All of the test samples were aerated for at least an hour to stabilize the pH. In the biosafety cabinet, the 0 h sample was sterilized using 0.8 µm and 0.2 µm syringe filters (Acrodisc). The 24 h sample was sterilized using 0.8 µm and 0.2 µm syringe filters (Acrodisc) and the 48 h sample was sterilized using 0.45 µm and 0.2 µm syringe filters (Acrodisc). The test samples with 300 mL were evenly divided into three 250 mL Erlenmeyer flasks, and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were stoppered with sterile Identi-plugs® foam plugs (Jaece Industries Inc.) to prevent evaporation of test solutions and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 14 days. On Day 14, the number of fronds were counted in each flask.

For the second toxicity test, four replicates were used for the control, which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The 96 h and 144 h time points were tested with three replicates each. Each APHA-spiked test sample was prepared by adding 3 mL of each APHA stock solution to 291 mL of test solution, and the spiked solutions were aerated for at least an hour to stabilize the pH. In the biosafety cabinet, each test solution was sterilized using 0.45 µm and 0.2 µm syringe filters (Acrodisc), and each test solution was evenly divided into three 250 mL Erlenmeyer flasks. Two 3-frond *L. minor* plants

from the acclimated culture were transferred into each flask. The flasks were stoppered with sterile Identi-plugs® foam plugs (Jaece Industries Inc.) to prevent evaporation of test solutions and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 11 days. On Day 11, the number of fronds were counted in each flask.

For the third toxicity test, four replicates were used for the control, which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. Another 192 h test solution was tested, this time with three replicates. To create the APHA-spiked 192 h test solution, 3 mL of each APHA stock solution was added to 291 mL of the filtered 192 h sample, and this spiked solution was aerated for at least an hour. In the biosafety cabinet, the test solution was sterilized using 0.45 µm and 0.2 µm syringe filters (Acrodisc) and evenly divided into three 250 mL Erlenmeyer flasks. Two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were stoppered with sterile Identi-plugs® foam plugs (Jaece Industries Inc.) to prevent evaporation of test solutions and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 9 days. On Day 9, the number of fronds were counted in each flask.

For the test samples generated from the second photocatalytic test, five test samples were involved with three replicates each: FPC-treated 25 h, FPC-treated 50 h, FPC-treated 115 h, FPC-treated 217 h, and a synthetic saline solution made from salts found in Industry A's OSPW. Four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. For each of the FPC-treated time points, they were first filtered using 47 mm diameter glass microfiber filters (Whatman) to remove the floating photocatalyst and clays from the OSPW. Afterwards, 3 mL of each APHA stock solution was added to 291 mL test solution, and the test solution was aerated for at least 20 min to stabilize the pH. For the APHA-spiked synthetic Industry A saline solution, 30 mL of the 10x synthetic OSPW saline solution was added to 270 mL deionized water (Millipore, >15 MΩ cm^{-1}) to create the 1x saline solution. 3 mL of each APHA stock solution was added to 291 mL of the 1x saline solution. The solution was aerated for at least 20 min to stabilize the pH. In a biosafety cabinet, 100 mL of each test sample was transferred into 250 mL Erlenmeyer flasks, and two 3-frond plants from the acclimated *L. minor* culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent

evaporation and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds was counted for each flask.

The third photocatalytic test generated four test samples with three replicates each: P25-treated OSPW, P25-treated OSPW spiked with thiosulfate, P25-treated OSPW spiked with EDTA, and a synthetic Industry A saline solution. Four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. To prepare the APHA-spiked P25-treated OSPW, 3 mL of each APHA stock solution was added to 291 mL of the treated OSPW in a clean 600 mL beaker. The contents of this beaker were aerated for at least 20 min to stabilize the pH. To prepare the sodium thiosulfate-spiked treated OSPW, 3 mL of each APHA stock solution was added to 291 mL of the P25-treated OSPW in a clean 600 mL beaker, and 1.2 mL of the 25 g/L sodium thiosulfate stock solution was added to this solution. The contents of this beaker were aerated for at least 20 min to stabilize the pH. To prepare the EDTA-spiked treated OSPW, 3 mL of each APHA stock solution was added to 291 mL of the P25-treated OSPW in a clean 600 mL beaker, and 0.3 mL of the 50 g/L $Na_2EDTA{\cdot}2H_2O$ stock solution was added. The contents of the beaker were aerated for at least 2 hours to stabilize the pH. For the APHA-spiked synthetic Industry A saline solution, 30 mL of the 10x synthetic OSPW saline stock solution was added to 290 mL deionized water (Millipore, >15 MΩ cm^{-1}) to create the 1x saline solution. 3 mL of each APHA stock solution was added to 291 mL of the 1x saline solution. The solution was aerated for at least 20 min to stabilize the pH. In a biosafety cabinet. 100 mL of each test sample was transferred into a 250 mL Erlenmeyer flask, and two 3-frond plants from the acclimated *L. minor* culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds was counted for each flask.

From the raceway tub photocatalytic test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least an hour to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. This toxicity test involved four test samples, and each was vacuum filtered using 47 mm diameter glass microfiber filters (Whatman) to remove the floating photocatalyst and clays from the OSPW. Five replicates were used for both raw Industry A OSPW and EDTA-spiked raw Industry A OSPW samples. The raw Industry A OSPW was prepared by adding 5 mL of each

APHA stock solution to 485 mL of test sample. This was aerated for at least 20 min to stabilize the pH. The EDTA-spiked raw Industry A OSPW was prepared by adding 5 mL of each APHA stock solution to 485 mL of test sample and adding 0.5 mL of the 50 g/L $Na_2EDTA{\cdot}2H_2O$ stock solution. This was aerated for at least 20 min to stabilize the pH. Three replicates were used for both the FPC-treated OSPW and EDTA-spiked FPC-treated OSPW. The FPC-treated OSPW was prepared by adding 3 mL of each APHA stock solution to 291 mL of test sample. This was aerated for at least 20 min. The EDTA-spiked FPC-treated OSPW was prepared by adding 3 mL of each APHA stock solution to 291 mL of test sample, and 0.3 mL of the 50 g/L $Na_2EDTA{\cdot}2H_2O$ stock solution. This was aerated for at least 20 min to stabilize the pH. In a biosafety cabinet, 100 mL of each test sample was transferred into a 250 mL Erlenmeyer flask, and two 3-frond plants from the acclimated *L. minor* culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds was counted for each flask.

To prepare the acclimation culture for testing the toxicity of the photocatalysts, axenic *Lemna minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights to acclimate for 18-24 hours before the start of the toxicity test.

1.6 L modified APHA medium was prepared by adding 16 mL of each APHA stock solution and adding deionized water (Millipore, >15 MΩ cm^{-1}) to the 1.6 L mark. The modified APHA medium was aerated for at least 1 h and the pH was adjusted to 8.3 afterwards using 1M NaOH. 400 mL of the prepared modified APHA medium was saved for the control and the remaining 1.2 L was saved for the following three test conditions.

The P25 TiO_2 test solution was created by adding 0.2 g P25 TiO_2 to 400 mL modified APHA medium and stirred to disperse the particles. The contents of the beaker were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). 100 mL of the sonicated liquid was added to each of four 250 mL Erlenmeyer flasks. Two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solution. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the

number of fronds were counted in each flask and the plants and their roots were transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of dried *L. minor* biomass was determined the next day.

The FPC mixture test condition tests the toxicity of a blend of FPCs with different catalytic abilities altogether. The FPC mixture was created by weighing out an equal amount of each FPC and thoroughly mixing them together in a 20 mL glass vial. 0.25 g of this FPC mixture and 100 mL modified APHA medium were added to each of four 250 mL Erlenmeyer flasks. Two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solutions. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants and their roots were transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of dried *L. minor* biomass was determined the next day.

0.25 g FPC and 100 mL modified APHA medium were added to each of four 250 mL Erlenmeyer flasks. Two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solution. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants and their roots were transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of dried *L. minor* biomass was determined the next day.

For the *L. minor* toxicity tests, the percentage of growth inhibition for frond number was calculated using the Equation 1 where % GI is the percent of growth inhibition, C_{fc} is the control frond count, and T_{fc} is the test sample frond count.

$$\% \, GI = 100 \times \frac{C_{fc} - T_{fc}}{C_{fc}} \qquad \text{Equation 1}$$

The percentage of growth inhibition for dried biomass was calculated using Equation 2 where % GI is the percent of growth inhibition, C_{db} is the control dried biomass in grams, and T_{db} is the test sample dried biomass in grams.

$$\% \, GI = 100 \times \frac{C_{db} - T_{db}}{C_{db}} \qquad \text{Equation 2}$$

3.3 Results and Discussion

3.3.1 Photocatalysis can treat organics but not heavy metals

Figure 1A shows the 9-day toxicity response of *Lemna minor* exposed to Industry A's OSPW that was subjected to increasing lengths of photocatalytic treatment. Toxicity was evaluated using frond number as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

Figure 1B shows the 7-day toxicity response of *L. minor* exposed to Industry A's OSPW that was subjected to increasing lengths of photocatalytic treatment. Toxicity was evaluated using frond number as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

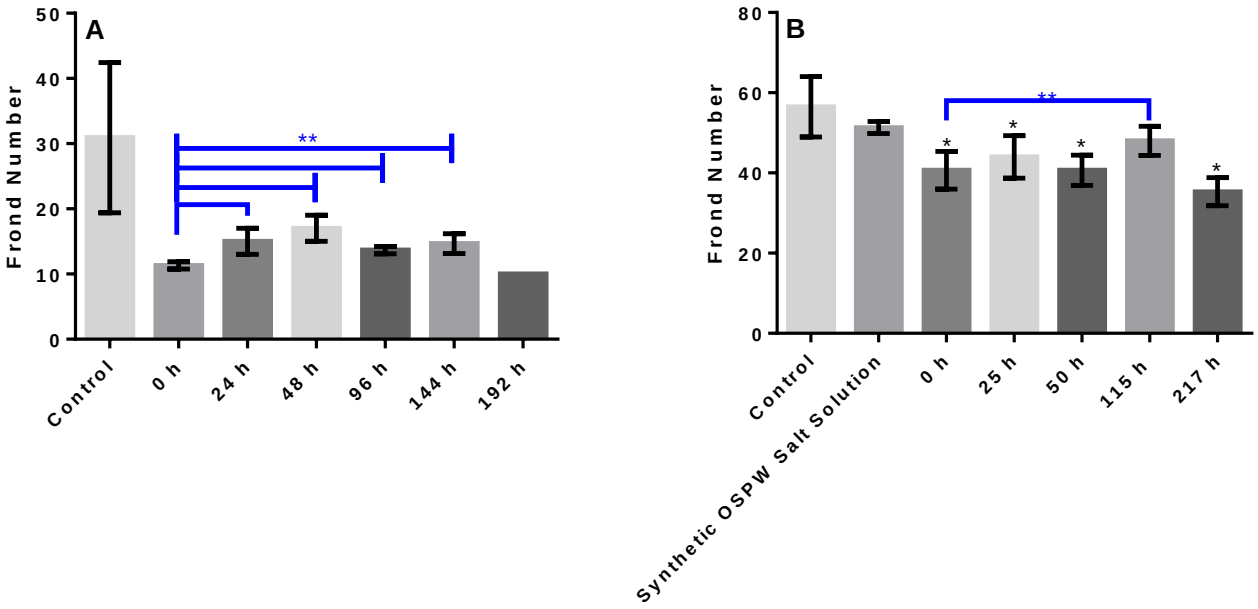

Figure 1. ***Lemna minor*** **growth on Industry A's OSPW in response to increasing lengths of photocatalytic treatment: (A) 9-day toxicity response, mean values ± SD (*n* = 13 for the control, *n* = 3 for test samples). Each test condition was significantly different from the control ($p < 0.05$). Treatment time mean values that are significantly different from the 0 h treatment ($p < 0.05$) are indicated in blue (**). (B) 7-day toxicity response, mean values ± SD (*n* = 4 for the control, *n* = 3 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). Treatment time mean values that are significantly different from the 0 h treatment ($p < 0.05$) are indicated in blue (**).**

Tables 1 and 2 below show the growth inhibition for frond number corresponding to the test samples shown in Figure 1A and Figure 1B, respectively.

Test Sample	% Growth Inhibition
Control	0
0 h	63.4
24 h	51.5
48 h	45.0
96 h	55.8
144 h	52.6
192 h	67.7

Table 1. Growth inhibition of *L. minor* exposed to Industry A's OSPW which was subjected to increasing lengths of photocatalytic treatment using FPCs

Test Sample	% Growth Inhibition
Control	0
Synthetic OSPW Salt Solution	9.1
0 h	24.5
25 h	22.1
50 h	28.0
115 h	15.0
218 h	37.5

Table 2. Growth inhibition of *L. minor* grown on Industry A's OSPW which was subjected to increasing lengths of photocatalytic treatment using FPCs

Figure 1A and Table 1 both show the results from the three separate toxicity tests all pooled together. As each toxicity test was run for different lengths, the pooled results were taken at Day 9, which was the latest shared date between the three tests. Regardless of the treatment length, the difference between the control mean and the means of the 0 h treatment and each of the treatment time points were

significant. Thus, all photocatalytic treatments failed to treat Industry A's OSPW to the level of the control, indicating that there are other components within the OSPW causing toxicity to *L. minor*. The (**) shows that the 24 h, 48 h, 96 h, and 144 h treatments were significant when the means were compared to that of the 0 h treatment, showing that the organics fraction contributed to a portion of the toxicity to *L. minor* and that photocatalytic treatment could treat the toxicity from the organics fraction in the OSPW. It is possible that the partial photocatalytic treatment generated more dissolved organic compounds that sequestered some of the trace heavy metals present in the OSPW, thus resulting in reduced toxicity. Interestingly, the toxicity to *L. minor* returned with the longest photocatalytic treatment of 192 h. A possible reason for the toxicity returning is that most of the organic compounds present have mineralized by this time point so there are very few organic compounds available to sequester the metals and reduce their bioavailability.

Figure 1B and Table 2 both show the results from the second photocatalytic test using FPCs. Although it is not a photocatalytic treatment time point, a solution of synthetic Industry A OSPW salts was tested to ensure that salinity was not a significant contributor to the overall toxicity to *L. minor*. Although the saline solution did slightly reduce the *L. minor* frond count, a one-tailed t-test showed that the difference between the means of the control and synthetic OSPW salts was not significant. Thus, OSPW salts were not a significant contributor to the overall toxicity to *L. minor*.

The (*) in Figure 1B also shows that regardless of the treatment length, the difference between the control mean and the means of the 0 h treatment and each of the treatment time points were significant. Thus, all photocatalytic treatments failed to treat Industry A's OSPW to the level of the control, indicating that there are other components within the OSPW causing toxicity to *L. minor*. The (**) shows that the 115 h treatment was significant when the means were compared to that of the 0 h treatment. This indicates that while the photocatalytic treatment could treat the OSPW, it was only capable of remediating a portion of the toxicity to *L minor*. It is possible that the partial photocatalytic treatment generated more dissolved organic compounds that sequestered some of the trace heavy metals present in the OSPW, resulting in some reduced toxicity. Interestingly, the toxicity to *L. minor* returned with the longest photocatalytic treatment time point (217 h), reproducing the result shown in Figure 1A. This supports the reasoning that after long treatment lengths, most of the dissolved organic compounds have mineralized and there are very few left to sequester the metals and reduce their bioavailability to *L. minor*.

Dissolved organic matter (DOM) has been known for decades to be involved with the mobility and biogeochemical cycling of trace metals in aquatic environments[150,151], and DOM forms a portion of the complex matrix in OSPW[9]. In the earlier stages of photocatalytic treatment, the smaller organic compounds generated from degrading NAs can interact with and sequester trace metals to change their bioavailability[150]. The change in trace metal bioavailability can partially explain the slight reduction of toxicity of the treatment samples when compared to the 0 h treatment. Another portion of the toxicity can also stem from the photocatalytic reaction itself as sunlight is known to excite the DOM in a DOM-metal complex, which in turn acts as chromophore and participates in photochemical reactions[150]. These photochemical reactions can generate highly reactive intermediates such as ROS or organic radicals which can damage biomolecules in plants and cause general cellular dysfunction[20,150]. Furthermore, plants are more susceptible to photochemical reactions and the resulting damage as they rely on increased light intensities for photosynthesis[137]. In order to investigate whether the remaining toxicity stems from trace heavy metals, raw and treated OSPW samples were compared to raw and treated OSPW samples spiked with the metal chelator EDTA. If the toxicity is caused by heavy metals, the addition of EDTA would remedy the toxicity to *L. minor* and increased frond counts should be observed.

Figure 2A shows the 7-day toxicity response of *L. minor* to raw and FPC-treated Industry A OSPW, and raw and FPC-treated Industry A OSPW spiked with EDTA. The toxicity response was evaluated using frond number as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

Figure 2B shows the 7-day toxicity response of *L. minor* to raw and P25 TiO_2-treated Industry A OSPW, and treated Industry A OSPW spiked with EDTA. The toxicity response was evaluated using frond number as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

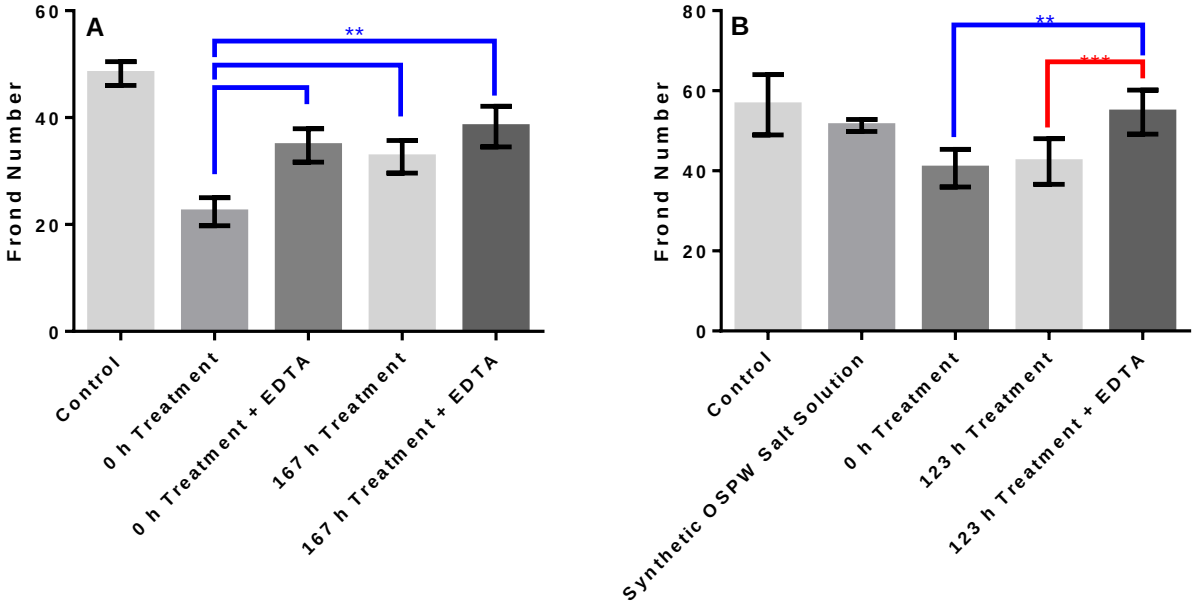

Figure 2. (A) 7-day toxicity response to FPC-treated OSPW, mean values ± SD (*n* = 4 for the control, *n* = 5 for the 0 h treatment, *n* = 3 for the rest of the treatments). All photocatalytic treatment mean values are significantly different from the control ($p < 0.05$). Treatment time mean values that are significantly different from the 0 h treatment ($p < 0.05$) are indicated in blue (**). (B) 7-day toxicity response to P25 TiO_2-treated OSPW, mean values ± SD (*n* = 4 for the control, *n* = 3 for test samples). All photocatalytic treatments are significantly different from the control ($p < 0.05$). Treatment time mean values that are significantly different from the 0 h treatment ($p < 0.05$) are indicated in blue (**). Treatment time mean values that are significantly different from the 123 h treatment ($p < 0.05$) are indicated in red (***).

Tables 3 and 4 below show the growth inhibition for frond number corresponding to the test samples shown in Figure 2A and Figure 2B, respectively.

Test Sample	% Growth Inhibition
Control	0
0 h Treatment	53.6
0 h Treatment + EDTA	27.9
167 h Treatment	32.3
167 Treatment + EDTA	20.6

Table 3. Growth inhibition of *L. minor* exposed to raw and 167 h treated OSPW, and raw and 167 h treated OSPW spiked with EDTA

Test Sample	% Growth Inhibition
Control	0
Synthetic OSPW Salt Solution	9.1
0 h Treatment	28.0
123 h Treatment	25.1
123 Treatment + EDTA	3.2

Table 4. Growth inhibition of *L. minor* exposed to raw OSPW, 123 h treated OSPW, and 123 h treated OSPW spiked with EDTA

The (*) in Figures 2A and 2B show that despite the addition of EDTA into both raw and treated OSPW samples, the difference between the control means and the means of the test samples were significant. Thus, EDTA was unsuccessful in reducing the OSPW toxicity to the level of the control in *L. minor*. This suggests that there is another component in Industry A's OSPW that contributes to the toxic response of *L. minor* in addition to organic compounds and heavy metals.

In Figure 2A, the difference between the means of 0 h treatment and the EDTA-spiked 0 h treatment is significant, showing that the addition of EDTA did reduce a portion of its toxicity *to L. minor*. The

EDTA-spiked 0 h treatment is still quite toxic as it has not been treated using photocatalysis, therefore any toxicity from the organic fraction of OSPW is still present. The difference between the means of 0 h treatment and the treated OSPW is also significant, demonstrating that photocatalytic treatment can mineralize the organic compounds present in OSPW and remedy that portion of the toxicity. However, photocatalysis cannot treat heavy metals and reduce them into a less harmful form.

The difference between the means of the 167 h treated OSPW and EDTA-spiked 167 h treated OSPW in Figure 2A is not significant, although spiking the treated OSPW with EDTA did increase the *L. minor* frond number. This result hints at there being another cause of toxicity in OSPW to *L. minor* other than organics or heavy metals. This is further supported by comparing the difference between the means of the raw Industry A OSPW and the EDTA-spiked 167 h treated OSPW; the photocatalytic treatment and EDTA should have reduced the toxicity of the organic fraction and the heavy metals, respectively, and the frond number should be similar to that of the control, but this is not the case.

In Figure 2B, a solution of synthetic Industry A OSPW salts was tested to ensure that salinity was not a significant contributor to the toxicity. Although the saline solution did slightly reduce the *L. minor* frond count, a one-tailed t-test showed that the difference between the means of the control and synthetic OSPW salts was not significant. Thus, the OSPW salts were not a significant contributor to the toxicity of Industry A's OSPW.

The (*) in Figure 2B show that the differences between the mean of the control to that of the 0 h treatment and the P25-treated OSPW are still significant. Using P25-TiO_2 to treat Industry A's OSPW for 123 h did not reduce its toxicity; however, the addition of EDTA after photocatalytic treatment addressed the toxicity as the EDTA-spiked treated OSPW had similar frond counts to those of the control. A one-tailed t-test comparing the means of the control to that of the EDTA-spiked P25-treated OSPW demonstrated that the difference was not significant.

From these experiments, it can be concluded that although photocatalysis can mineralize toxic organic compounds, it cannot treat any heavy metals and reduce them into a less harmful form. The addition of metal chelators such as EDTA can address the portion of toxicity from heavy metals; however this also presents its own difficulties to remediating OSPW as the treatment would require both the toxic heavy metal as well as the chelating agent to be removed[74].

Figure 2A suggests that there is another contributor to the overall OSPW toxicity that was not addressed by photocatalysis or metal chelation. The photocatalytic reaction generates residual chlorine

species in a saline aqueous system such as OSPW[152–154], and it is possible that these oxidative compounds contribute to the overall toxicity of OSPW to *L. minor*. In order to investigate whether the remaining toxicity stems from oxidative compounds generated from photocatalysis, a treated OSPW sample was compared to a treated OSPW sample spiked with the reducing agent sodium thiosulfate. If the toxicity is caused by oxidative compounds, the addition of thiosulfate would remedy the toxicity to *L. minor* and increased frond counts would be observed.

3.3.2 Oxidative compounds generated from photocatalysis represent a very small portion of toxicity

While photocatalysis is a promising OSPW remediation method capable of mineralizing even the most recalcitrant NAs, the photocatalytic reaction itself generates oxidative compounds such as hypochlorous acid (HOCl), halogenated or partially oxidized organic compounds, and other residual chlorine species in a saline aqueous system such as OSPW[152–154]. The amount of residual chlorine generated is directly proportional to the salinity of the aqueous solution[154]. HOCl and residual chlorine generated from photocatalysis was shown to damage the cell walls of *Escherichia coli*[154]. Thus, it is possible that the oxidative nature of the residual chlorine and/or the halogenated organic compounds can cause toxicity to *L. minor*.

Figure 3 below shows that the toxicity response was evaluated using frond number as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

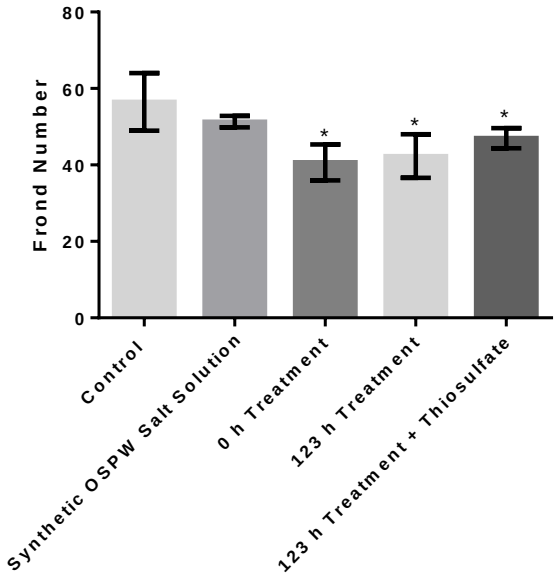

Figure 3. 7-day toxicity response of *L. minor* to P25 TiO_2-treated Industry A OSPW spiked with thiosulfate. Mean values ± SD (*n* = 4 for the control, *n* = 3 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*).

Table 5 below shows the growth inhibition for frond number corresponding to the test samples shown in Figure 3.

Test Sample	**% Growth Inhibition**
Control	0
Synthetic OSPW Salt Solution	9.1
0 h Treatment	28.0
123 h Treatment	25.1
123 h Treatment + Thiosulfate	16.8

Table 5. Growth inhibition of *L. minor* exposed to raw and P25-treated Industry A OSPW, and P25-treated Industry A OSPW spiked with thiosulfate

Once again, a solution of salts found in Industry A's OSPW was tested to ensure that salinity was not a significant contributor to the toxicity. Although the saline solution did slightly reduce the *L. minor* frond count, a one-tailed t-test showed that the difference between the means of the control and the OSPW salts was not significant.

One-tailed t-tests comparing the mean of the control to that of the other test samples showed that the differences were still significant. Using P25 TiO_2 to treat Industry A's OSPW for 123 h did not reduce its toxicity to *L. minor*, and a one-tailed t-test comparing the means of the 0 h treatment and 123 h treatment showed that the difference was negligible.

The difference between the mean of the 123 h treatment and that of the 123 h treatment spiked with thiosulfate was not statistically significant. Although the addition of thiosulfate reduced a small portion of the toxicity, the difference between the mean of the 0 h treatment and that of the 123 h treatment spiked with thiosulfate was not significant. Based on these results, residual chlorine or any halogenated organic compounds present in Industry A's OSPW represent a very small and negligible component of the total toxicity to *L. minor*. As OSPW is a complex matrix, it is possible that amines and NAs containing nitrogen atoms present in the organic fraction affected the equilibrium of HOCl and the hypochlorite ion[152]. The high pH of OSPW also disfavors this equilibrium, hence the production of residual chlorine and any halogenated compounds might not be enough to cause cellular damage via oxidation[152].

After investigating the origins of OSPW toxicity to *L. minor*, it can be concluded that although photocatalysis can mineralize toxic organic compounds, it cannot treat any heavy metals and reduce them into a less harmful form. The addition of metal chelators such as EDTA can address the portion of OSPW toxicity caused by heavy metals, and the addition of reducing agents such as sodium thiosulfate can treat the very small portion of toxicity stemming from oxidative compounds. However, there is still a portion of the toxicity to *L. minor* that is unexplained. The experiments described here investigated different OSPW components that contribute to the *L. minor* toxic stress response as separate entities, but the interactions between different OSPW components and the subsequent effects on *L. minor* growth have not been studied.

It is recommended that the interactions between DOM and heavy metals are investigated as DOM can alter the bioavailability of a metal by sequestration. While it is possible that DOM can reduce the toxicity of a heavy metal, it is also possible that these interactions can render a metal into a more bioavailable form that is toxic to *L. minor*.

3.3.3 The photocatalysts do not significantly inhibit *L. minor* growth

Although the floating photocatalysts (FPCs) used for OSPW treatment are recoverable for future treatments by skimming them off the surface, it is inevitable that some will end up in the environment when deployed for actual industrial usage[4]. It is also inevitable that some of the TiO_2 will detach from the glass microspheres over repeated FPC usage[4]. Elevated concentrations of TiO_2 nanoparticles (NPs) are predicted to accumulate in aquatic and terrestrial environments, and plants act an entry point into higher organisms as they serve as the basis of many food chains[76]. Plant cells have cell walls that can prevent larger diameter NPs from penetrating and entering the cells, but NPs have been shown to have toxic effects on plant cells without internalization being necessary[77]. The following studies aim to determine whether P25 TiO_2 or the FPCs will induce a toxic stress response in *L. minor*. In these studies, all of the test solutions are modified APHA medium with the photocatalyst added. The P25 TiO_2 was added at a concentration of 0.5 g/L, and floating photocatalysts (FPCs) were added at a concentration of 2.5 g/L to maintain the same TiO_2 concentration as the FPCs are 20% TiO_2 by mass.

Figure 4A describes the averaged frond number for *L. minor* grown under each test condition, and Figure 4B describes the averaged dried biomass for *L. minor* plants grown under each test condition. One-tailed t-tests were used to determine whether a difference between two means was significant.

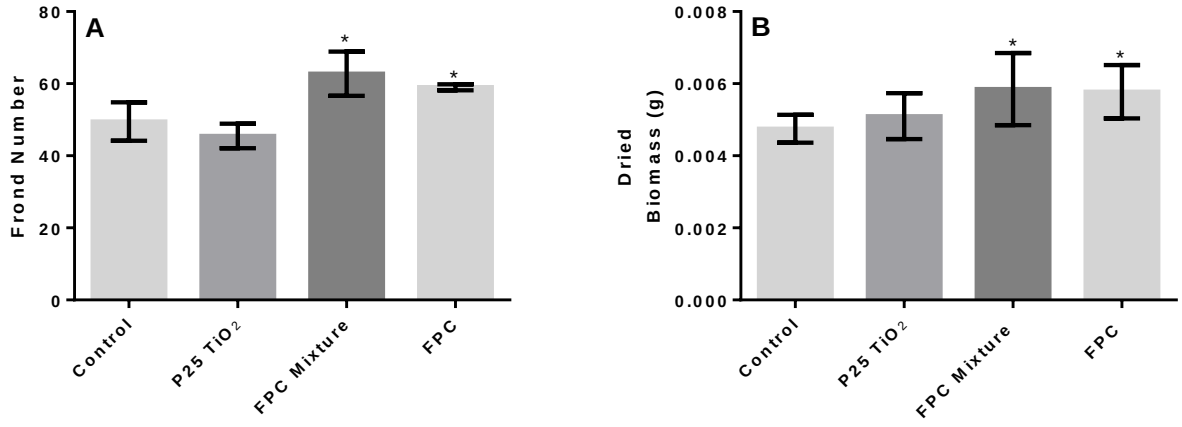

Figure 4. (A) 7-day toxicity response of *L. minor* to P25 TiO_2 and FPCs evaluated using frond number, mean values ± SD (*n* = 4). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). (B) 7-day toxicity response of *L. minor* to P25 TiO_2 and FPCs evaluated using dried biomass, mean values ± SD (*n* = 4).

Tables 6 below shows the growth inhibition for frond number and dried biomass corresponding to the test samples shown in Figure 4.

	% Growth Inhibition	
Test Sample	**Frond Number**	**Dried Biomass**
Control	0	0
P25 TiO_2	8.1	-7.4
FPC mixture	-26.8	-23.2
FPC	-19.2	-21.6

Table 6. Growth inhibition of *L. minor* exposed to different photocatalysts

While P25 TiO_2 did not significantly inhibit *L. minor* frond number or biomass growth, there was a significant stimulatory effect (hormesis) of FPCs incubated with *L. minor* for both frond number and dried biomass when compared to the control. The significance was determined using one-tailed t-tests comparing the mean of the control to that of the other test samples.

A possible reason explaining hormesis for the FPCs is that they are buoyant and float on the surface along with the plants for the entire duration of the test. As both FPCs tested are white, this can reflect more light and increase the light intensity around the plants, resulting in increased photosynthesis which in turn increased plant growth. Although the P25 TiO_2 is also white in colour, it is not buoyant and sank to the bottom of the flasks over the test duration which might explain why this effect was not observed for that test condition.

With regards to the physical appearance observed at the end of the 7-day testing period, *L. minor* fronds were green in the control and in all test conditions, there were no abnormally-sized fronds, and the roots of all plants appeared healthy. There was no loss of buoyancy, colony destruction, gibbosity or necrosis observed, hence these observations showed that the *L. minor* plants across all test conditions were healthy under all definitions. These results are consistent with those of Dolenc Koce (2017) where TiO_2 NPs were shown to have no effects or very minor inhibitory effects on *L. minor*[76]. Although Song et al. (2012) reported that TiO_2 NPs significantly inhibited *L. minor* growth at concentrations above 200 mg/L[77], this difference could be due to Song et al. using modified Steinberg medium instead of

modified APHA medium as the two test media have slightly different chemical compositions as well as different pH.

While TiO_2 NPs are considered to have no significantly harmful effects to *L. minor* growth, they can clog the pores of the cell wall resulting in reduced transport of water and minerals[76]. TiO_2 NPs can also accumulate in plant cells and interfere with microtubules, resulting in isotropic cell growth which differs from the trademark anisotropic cell growth for plants[76]. Due to these cellular effects of TiO_2 exposure, it is recommended that the long-term effects of TiO_2 NP exposure to plants are studied as TiO_2 NPs are currently manufactured on a large scale for industrial and household uses other than OSPW treatment, and thus are already accumulating in the environment through wastewater and urban runoff[77].

3.4 Environmental Significance

Unlike other AOPs, photocatalysis over TiO_2 is a promising method of treating OSPW as it is capable of mineralizing even the most recalcitrant NAs. However, OSPW is a complicated saline matrix of dissolved organic compounds, inorganic compounds, trace heavy metals, and silt. Compounds belonging in the other OSPW components may not respond well to photocatalysis, thus requiring other treatments to be used in conjunction with photocatalysis to further treat the OSPW.

A potential remediation method for OSPW is to partially treat it using photocatalysis and return it to wetlands for further bioremediation and phytoremediation. Investigating the toxicities of different OSPW constituents provides information on the capabilities of photocatalysis and the effects of these constituents to other wetland plants using *L. minor* as a proxy. OSPW with a high heavy metal content will need the addition of metal chelators such as EDTA in order to reduce their toxicity to *L. minor*. As there is still a significant portion of toxicity to *L. minor* that is not explained by organics, heavy metals, or oxidative compounds, interactions between DOM and heavy metals need to be further studied as DOM can change the bioavailability of heavy metals to *L. minor* and other wetland plants.

As for the photocatalysts themselves. TiO_2 NPs have been shown previously in literature to be relatively harmless to *L. minor*; however, the potential toxicities of the hollow glass microspheres as well as the floating photocatalysts have not been previously reported. Determining the effects of P25 TiO_2 and FPCs to *L. minor* provides information on whether solar photocatalysis over TiO_2 can be a safe method for treating OSPW especially since the materials used will inevitably end up in the environment and bioaccumulate in wetland plants. It is important that the photocatalyst itself is not

toxic to wetlands as that would defeat the purpose of OSPW remediation if additional toxic material is introduced into the remediation site.

Chapter 4
IC_{50} of Naphthenic Acids

4.1 Summary

Oil sands process-affected water (OSPW) is a by-product generated from the oil sands bitumen extraction process, and due to the toxicity from compounds such as naphthenic acids (NAs), it is not approved for release into the environment and thus is stored in tailing ponds. Oil sands mining companies are required to treat OSPW and return it into the landscape upon mine closure; however, there is no universal or standardized treatment method rendering OSPW safe for environmental release.

Photocatalysis has been shown to be a promising method for treating OSPW. A proposed method of remediating OSPW and incorporating it to the landscape is to first treat OSPW using photocatalysis to reduce the toxicity of the organics fraction, followed by the release of the partially treated OSPW into wetlands for bioremediation and phytoremediation. The objective of this work is to determine the 7-day half maximal inhibitory concentration (IC_{50}) of NAs to the freshwater macrophyte *Lemna minor*. The IC_{50} was evaluated using both frond number and dried biomass as the endpoints.

4.2 Experimental

4.2.1 Materials

Potassium chloride was purchased from ACP Chemicals Inc. Sodium chloride and boric acid were purchased from EMD Chemicals Inc. All other chemicals and reagents were purchased from Sigma Aldrich.

Axenic *Lemna minor* 490 stock cultures were obtained from the Canadian Phycological Culture Centre following the protocol of Gopalapillai et al., (2013)[149], and the axenic *L. minor* plants were cultured in modified Hoagland's E+ medium and subcultured weekly according to the Environment Canada standardized protocol[127]. Modified Hoagland's E+ stock solution A contains 59.00 g/L calcium nitrate tetrahydrate, 75.76 g/L potassium nitrate, 34.00 g/L potassium phosphate monobasic, and 6 mL of 6N HCl. Modified Hoagland's E+ stock solution B contains 3.00 g/L tartaric acid. Modified Hoagland's E+ stock solution stock solution C contains 1.21 g/L iron (III) chloride hexahydrate, 3.35 g/L disodium EDTA dihydrate, and 1.2 mL of 6N KOH. Modified Hoagland's E+ stock solution D contains 50.00 g/L magnesium sulfate heptahydrate. Modified Hoagland's E+ stock

solution E contains 2.86 g/L boric acid, 0.22 g/L zinc sulfate heptahydrate, 0.12 g/L sodium molybdate dihydrate, 0.08 g/L copper (II) sulfate pentahydrate, and 3.62 g/L manganese (II) chloride tetrahydrate. 1 L Modified Hoagland's E+ medium was created by adding 20 mL stock solution A, 1 mL stock solution B, 20 mL stock solution C, 10 mL stock solution D, 1 mL stock solution E, 10 g sucrose, 0.10 g yeast extract, and 0.6 g tryptone to 900 mL of deionized water (Millipore, >15 MΩ cm^{-1}). The pH was adjusted to 4.4-4.8 using 1M NaOH and 1M HCl and the final volume was brought to 1 L using deionized water (Millipore, >15 MΩ cm^{-1}). The modified Hoagland's E+ medium was autoclaved at 121°C and 1.1 bar for 20 min (Priorclave) before use.

A synthetic OSPW saline stock solution was created using 44.0572 g/L $NaHCO_3$, 13.1882 g/L NaCl), and 11.8294 g/L Na_2SO_4 in a volumetric flask filled to the mark using deionized water (Millipore, >15 MΩ cm^{-1}).

APHA stock solution A contains 25.5 g/L sodium nitrate, 15.0 g/L sodium bicarbonate, 1.04 g/L potassium phosphate monobasic, and 1.01 g/L potassium chloride in deionized water (Millipore, >15 MΩ cm^{-1}). APHA stock solution B contains 4.41 g/L calcium chloride dihydrate, 12.17 mg/L magnesium chloride hexahydrate, 0.4149 g/L manganese (II) chloride tetrahydrate, and 0.16 g/L iron (III) chloride hexahydrate in deionized water (Millipore, >15 MΩ cm^{-1}), and the solution was acidified to pH 2 with HCl. APHA stock solution C contains 14.7 g/L magnesium sulfate heptahydrate, 0.186 g/L boric acid, 7.26 mg/L sodium molybdate dihydrate, 3.27 mg/L zinc chloride, 1.4 mg/L cobalt (II) chloride hexahydrate, and 15 µg/L copper (II) chloride dihydrate in deionized water (Millipore, >15 MΩ cm^{-1}). Each APHA stock was added to the test sample solution to produce a final concentration of 1 v/v% of each APHA stock solution (i.e. after the addition of the three APHA stocks, the test sample solution has a final concentration of 97%). %). Each test solution requires the addition of APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth. The control and acclimation solution both contain the same components, and both are made using deionized water (Millipore, >15 MΩ cm^{-1}) with each APHA stock added to produce a final concentration of 1 v/v% of each APHA stock solution. As with the test solutions, both the control and acclimation solutions were spiked with APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth.

4.2.2 *L. minor* IC_{50} toxicity test

Axenic *Lemna minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The

flasks were set under wide-spectrum T8 32 W fluorescent grow lights to acclimate for 18-24 hours before the start of the toxicity test.

To estimate the 7-day IC_{50} of synthetic NAs in OSPW, eight NA concentrations were used including modified APHA medium as the control. A geometric dilution series was used where each successive concentration was half of the previous one. As the original NA solution was spiked with each APHA stock added to produce a final concentration of 1 v/v% of each APHA stock solution, this geometric series was 97% (of the original NA concentration), 48.5%, 24.3%, 12.1%, 6.1%, 3.0%, and 1.5% (or 485 mg/L, 242.5 mg/L, 121.3 mg/L, 60.6 mg/L, 30.3 mg/L, 15.2 mg/L, and 7.6 mg/L where the original NA concentration was 500 mg/L).

800 mL of the APHA-spiked 485 mg/L NA synthetic OSPW was created by adding 8 mL of the 50 mg/L NA stock solution in 1M NaOH, 20 mL of the synthetic OSPW saline stock solution (to give the synthetic OSPW 800 mg/L HCO_3^- (from $NaHCO_3$), 200 mg/L Cl^- (from NaCl), and 200 mg/L SO_4^{2-} (from Na_2SO_4), 8 mL of APHA stock solution A, 8 mL APHA stock solution B, 8 mL stock solution C, and topped up to 800 mL using deionized water (Millipore, >15 MΩ cm^{-1}). The pH of the solution was adjusted to 8.7 using nitric acid. 200 mL of this synthetic OSPW was added to each of four 250 mL Erlenmeyer flasks.

2.8 L modified APHA medium was created using deionized water (Millipore, >15 MΩ cm^{-1}) and 28 mL of each APHA stock solution. 100 mL of the modified APHA medium was added to the other twenty-eight 250 mL Erlenmeyer flasks. 100 mL from each of the 485 mg/L flasks was transferred into the 242.5 mg/L flasks, and 100 mL was transferred from the 242.5 mg/L flasks to the 121.3 mg/L flasks. 100 mL was transferred into the subsequent dilution set until each of the 7.6 mg/L flasks had volumes of 200 mL. From this last set of flasks, 100 mL was discarded for a final volume of 100 mL.

Two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solution and loss of volatiles. Each flask was set under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants and their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of dried *L. minor* biomass was determined the next day.

The growth inhibition for frond number and dried biomass are calculated as shown in Section 3.2.2 using Equation 1 and 2, respectively.

4.2.3 Statistical analysis

The sigmoidal growth inhibition curves for both dried biomass and frond number were fitted using non-linear curve fitting (GraphPad Prism). The error bars represent the 95% confidence limits. The sigmoidal log (inhibitor concentration) vs response model is given by the following equation where "Top" and "Bottom" refer to the plateaus in the units of the y-axis, and the Hill slope describes the steepness of the curve.

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{[(\log(IC_{50} - x))(Hill\ slope)]}} \qquad \text{Equation 3}$$

4.3 Results and Discussion

In these studies, the initial NA concentration is 485 mg/L and is diluted by a factor of 0.5 to 242.5 mg/L, 121.3 mg/L, 60.6 mg/L, 30.3 mg/L, 15.2 mg/L, and 7.6 mg/L. The first two NA concentrations in the dilution series are unlikely to be found in tailing ponds as the NA concentrations reported there are much lower and range from 20 to 130 mg/L[155,156].

Figure 5A below shows the dose-response curve of synthetic NAs on *L. minor* when frond number was used as the endpoint. Figure 5B shows the averaged frond number for each NA concentration. One-tailed t-tests were used to determine whether a difference between two means was significant.

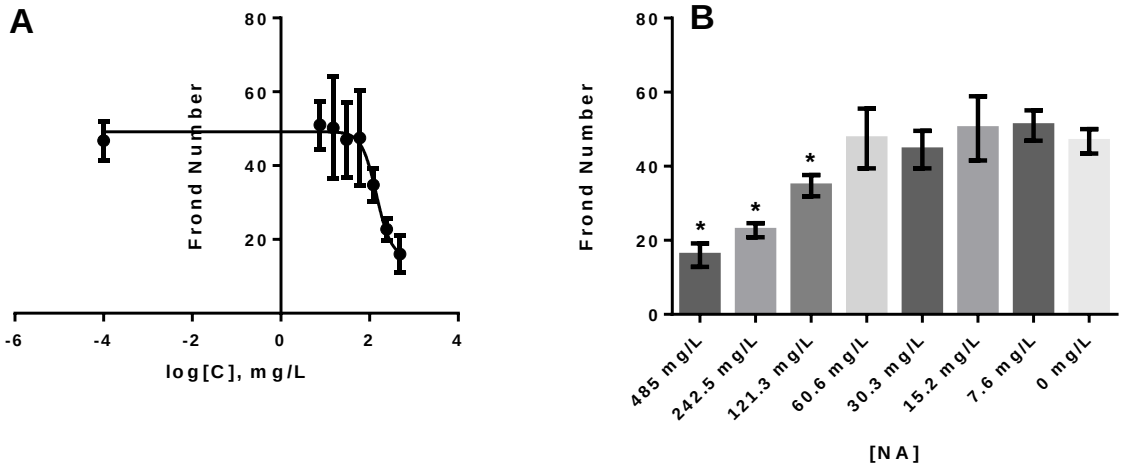

Figure 5. (A) 7-day growth inhibition curve of *L. minor* frond number in response to synthetic NAs, mean values ± 95% CI (*n* = 4). (B) 7-day toxicity response of *L. minor* to decreasing concentrations of synthetic NAs evaluated using frond number as the endpoint, mean values ± SD (*n* = 4). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*).

The equation below describes the sigmoidal growth inhibition curve for *L. minor* exposed to synthetic NAs using frond number as the endpoint. Here, the "Top" and "Bottom" values are 49.18 and 15.06, respectively, and subtracting the "Bottom" from the "Top" yields 24.12 in the numerator. The Hill slope is -2.607, and the IC_{50} is 142.9 mg/L.

$$y = 15.06 + \frac{24.12}{1 + 10^{[(\log(142.9\ \text{mg/L} - x))(-2.607)]}} \qquad \text{Equation 4}$$

Figure 6A below shows the dose-response curve of synthetic NAs on *L. minor* when dried biomass was used as the endpoint. Figure 6B shows the averaged dried biomass corresponding to each NA concentration. One-tailed t-tests were used to determine whether a difference between two means was significant.

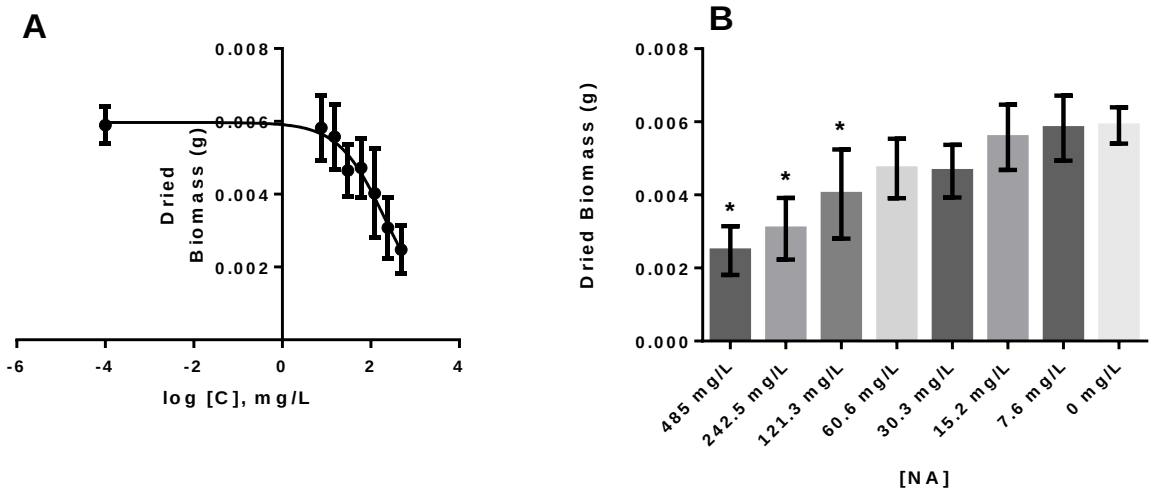

Figure 6. (A) 7-day growth inhibition curve of *L. minor* biomass exposed to synthetic NAs, mean values ± 95% CI (*n* = 4). (B) 7-day toxicity response of *L. minor* to decreasing concentrations of synthetic NAs evaluated using dried biomass as the endpoint, mean values ± SD (*n* = 4). Mean values that are significantly different from the control (p < 0.05) are indicated (*).

The equation below describes the sigmoidal growth inhibition curve for *L. minor* exposed to NAs using frond number as the endpoint. Here, the "Top" and "Bottom" values are 0.005971 g and 0.0007997 g, respectively, and subtracting the "Bottom" from the "Top" yields 0.0051713 g in the numerator. The Hill slope is -0.8386, and the IC_{50} is 198.1 mg/L.

$$y = 0.0007997\ \text{g} + \frac{0.0051713\ \text{g}}{1 + 10^{[(\log(198.1\ \text{mg/L} - x))(-0.8386)]}} \qquad \text{Equation 5}$$

Table 7 below shows the growth inhibition corresponding to Figure 5B and Figure 6B for frond number and dried biomass, respectively. The negative numbers shown in the table demonstrate that *L. minor* experienced a stimulatory effect from the test solution when compared to the control.

	% Growth Inhibition	
[NA] (mg/L)	**Frond Number**	**Dried Biomass**
485	65.8	58.1
242.5	51.3	47.9
121.3	25.7	31.8
60.6	-1.6	19.9
30.3	0.89	18.6
15.2	-10.5	2.8
7.6	-9.1	1.3
0	0	0

Table 7. Growth inhibition of *L. minor* subjected to the NA dilution series

The 7-day IC_{50} was determined to be 142.9 mg/L synthetic NAs when frond number was used as the endpoint. The concentrations of the dilution series at which the NAs were considered nontoxic are 60.6 mg/L and below; this was determined using Dunnett's multiple comparisons test comparing the average frond number of each test sample to that of the control. This shows that OSPW can be partially treated by photocatalysis until NA concentrations are 60.6 mg/L or lower and be released into the environment for further phytoremediation without being considered toxic to *L. minor*.

The 7-day IC_{50} was determined to be 198.1 mg/L synthetic NAs when dried biomass was used as the endpoint. The concentrations of the dilution series at which the NAs are considered nontoxic were 60.6 mg/L and below; this was determined using Dunnett's multiple comparisons test comparing the average dried biomass of each test sample to that of the control. This shows OSPW can be partially treated until NA concentrations are 60.6 mg/L or lower and be released into the environment for further phytoremediation without being considered toxic to *L. minor*.

A possible reason explaining the difference in IC_{50} values between frond number and biomass is that the frond number endpoint does not take frond size into consideration, whereas dried biomass only considers the total amount of plant growth over the course of the test period. For frond counts, a fully-grown frond and a visible bud are considered equal, and it is possible for *L. minor* plants to grow in higher NA concentrations and have many fronds albeit smaller ones when compared to those of healthier *L. minor* plants. Even if the plants grown in higher NA concentrations have a similar frond count to that of the control and the difference between the means is not significant, this does not mean that the plants are healthy as there are other factors used to gauge *L. minor* overall health.

According to Environment Canada (2007), the general appearance of healthy *L. minor* colonies show buoyant plants with fronds of variable shades of green, with newer fronds being a lighter green than grown fronds[127]. Healthy *L. minor* should not show the following characteristics: loss of buoyancy, colony destruction, loss of pigment on fronds (chlorosis), localized dead tissue on fronds (necrosis), humped appearance (gibbosity), yellow or abnormally-sized fronds, and root destruction[127].

With regards to the physical appearance observed at the end of the 7-day testing period, *L. minor* fronds were green across all tested concentrations. However, browning on the edges were observed for plants grown in synthetic OSPW containing 485 mg/L, 242.5 mg/L, and 121.3 mg/L NAs, and smaller fronds were observed for the 485 mg/L and 242.5 mg/L NAs. Gibbosity is described as fronds growing curving downwards into the test solution instead of across the test solution surface, and this was observed for plants grown in synthetic OSPW containing 485 mg/L, 242.5 mg/L, 121.3 mg/L, and 60.6 mg/L NAs. Plant overlap is described as *L. minor* plants growing on top of another instead of individual plant growth spreading across the liquid surface. This was also observed for plants grown in the synthetic OSPW containing 485 mg/L, 242.5 mg/L, 121.3 mg/L, and 60.6 mg/L NAs.

These observations on physical appearance demonstrated that *L. minor* plants would not be considered 'healthy' even when grown in NA concentrations that were considered nontoxic when evaluated using the conventional endpoints. As a result, other appearance-based endpoints that can be quantified such as chlorophyll concentration and/or amount of gibbosity should be considered alongside frond number and dried biomass when determining the IC_{50} of NAs on *L. minor*.

4.4 Environmental Significance

The IC_{50} for synthetic NAs has not been previously reported for *Lemna minor*. Determining the IC_{50} for both frond number and biomass provides information for which NA concentrations are expected to

be toxic to *L. minor*, and whether OSPW requires further photocatalytic treatment before being released into the environment.

Two characteristics that make *L. minor* specifically useful for OSPW toxicity testing is their excellent ability to accumulate metals and their susceptibility to surface-active and hydrophobic substances[127]. In the laboratory, its rapid growth, structural simplicity, and relative ease to culture make it a useful model organism for aquatic toxicity tests[127].

Naturally, *L. minor* inhabits the surfaces or slightly below the surfaces of relatively still waters such as ponds, lakes, quiet streams, stagnant waters, and estuaries across a wide range of temperatures, and thus is ubiquitous in nature[127]. Furthermore, submersed and floating plants are considered to be less susceptible to differences in wetland origin and can serve a better system of monitoring wetland health than plants of other zones[28]. The buoyancy of *L. minor* is an added benefit which allows its physical appearance, and therefore plant health, to be easily observed. Thus, *L. minor* can serve as a model aquatic plant representative for wetland health.

Chapter 5

Photocatalytic Degradation of Naphthenic Acids in Synthetic OSPW

5.1 Summary

Oil sands process-affected water (OSPW) is a by-product produced from the bitumen extraction process. Mining companies are forbidden from releasing OSPW into the environment as per industrial regulations, therefore OSPW is currently stored in tailings ponds. Upon mine closure, oil sands mining companies are required to treat OSPW and return it into the landscape, but currently there is no standardized method of treating OSPW for environmental return.

Since OSPW is a complex and saline matrix of clays, dissolved organic compounds, trace heavy metals and other inorganic compounds, there is no single contributor to the toxicity of OSPW. Hence for this work, a synthetic OSPW whose components and concentrations were known was used for two reasons: 1) show that the toxicity of Industry A's OSPW to *L. minor* in Chapter 3 was due to heavy metals, and 2) show that photocatalysis is effective at degrading NAs as a proof-of-concept.

The objectives of this work are to confirm that NAs can be degraded using photocatalysis in synthetic OSPW and demonstrate that the toxicity of Industry A's OSPW to *L. minor* after photocatalytic treatment resulted from heavy metals and not from NA degradation by-products. The effects of salts on NA toxicity to *L. minor* was also explored. Photocatalytic degradation of NAs was investigated using COD, TOC, and UV-Vis, and the corresponding reduction of toxicity was evaluated using *L. minor* toxicity tests where frond number, dried biomass, and gibbosity were used as the endpoints.

5.2 Experimental

5.2.1 Materials

Potassium chloride was purchased from ACP Chemicals Inc. Sodium chloride and boric acid were purchased from EMD Chemicals Inc. P25 titanium dioxide Aeroxide^{TM} was purchased from Acros Organics. All other chemicals and reagents were purchased from Sigma Aldrich.

Axenic *Lemna minor* 490 stock cultures were obtained from the Canadian Phycological Culture Centre following the protocol of Gopalapillai et al., (2013)[149], and the axenic *L. minor* plants were cultured in modified Hoagland's E+ medium and subcultured weekly according to the Environment Canada standardized protocol[127]. Modified Hoagland's E+ stock solution A contains 59.00 g/L

calcium nitrate tetrahydrate, 75.76 g/L potassium nitrate, 34.00 g/L potassium phosphate monobasic, and 6 mL of 6N HCl. Modified Hoagland's E+ stock solution B contains 3.00 g/L tartaric acid. Modified Hoagland's E+ stock solution stock solution C contains 1.21 g/L iron (III) chloride hexahydrate, 3.35 g/L disodium EDTA dihydrate, and 1.2 mL of 6N KOH. Modified Hoagland's E+ stock solution D contains 50.00 g/L magnesium sulfate heptahydrate. Modified Hoagland's E+ stock solution E contains 2.86 g/L boric acid, 0.22 g/L zinc sulfate heptahydrate, 0.12 g/L sodium molybdate dihydrate, 0.08 g/L copper (II) sulfate pentahydrate, and 3.62 g/L manganese (II) chloride tetrahydrate. 1 L Modified Hoagland's E+ medium was created by adding 20 mL stock solution A, 1 mL stock solution B, 20 mL stock solution C, 10 mL stock solution D, 1 mL stock solution E, 10 g sucrose, 0.10 g yeast extract, and 0.6 g tryptone to 900 mL of deionized water (Millipore, >15 MΩ cm^{-1}). The pH was adjusted to 4.4-4.8 using 1M NaOH and 1M HCl, and the final volume was brought to 1 L using deionized water (Millipore, >15 MΩ cm^{-1}). The modified Hoagland's E+ medium was autoclaved at 121°C and 1.1 bar for 20 min (Priorclave) before use.

A synthetic OSPW saline stock solution was created using 44.0572 g/L $NaHCO_3$, 13.1882 g/L NaCl), and 11.8294 g/L Na_2SO_4 in a volumetric flask filled to the mark using deionized water (Millipore, >15 MΩ cm^{-1}). A 50 g/L NA in 1M NaOH stock solution was created using 1 g NAs and adding 20 mL of 1M NaOH.

APHA stock solution A contains 25.5 g/L sodium nitrate, 15.0 g/L sodium bicarbonate, 1.04 g/L potassium phosphate monobasic, and 1.01 g/L potassium chloride in deionized water (Millipore, >15 MΩ cm^{-1}). APHA stock solution B contains 4.41 g/L calcium chloride dihydrate, 12.17 mg/L magnesium chloride hexahydrate, 0.4149 g/L manganese (II) chloride tetrahydrate, and 0.16 g/L iron (III) chloride hexahydrate in deionized water (Millipore, >15 MΩ cm^{-1}), and the solution was acidified to pH 2 with HCl. APHA stock solution C contains 14.7 g/L magnesium sulfate heptahydrate, 0.186 g/L boric acid, 7.26 mg/L sodium molybdate dihydrate, 3.27 mg/L zinc chloride, 1.4 mg/L cobalt (II) chloride hexahydrate, and 15 µg/L copper (II) chloride dihydrate in deionized water (Millipore, >15 MΩ cm^{-1}). Each APHA stock was added to the test sample solution to produce a final concentration of 1 v/v% of each APHA stock solution (i.e. after the addition of the three APHA stocks, the test sample solution has a final concentration of 97%). Each test solution requires the addition of APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth. The control and acclimation solution both contain the same components, and both are made using deionized water (Millipore, >15 MΩ cm^{-1}) with each APHA stock added to produce a final concentration of 1 v/v% of

each APHA stock solution. As with the test solutions, both the control and acclimation solutions were spiked with APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth.

5.2.2 Photocatalytic treatment

For the saline OSPW photocatalytic treatment, five treatment time points were used. For each beaker, 700 mL synthetic OSPW with [NA] = 150 mg/L, [HCO_3^-] = 800 mg/L. [Cl^-] = 200 mg/L, and [SO_4^{2-}] = 200 mg/L was prepared by adding 17.5 mL of the synthetic OSPW saline stock solution, 2.1 mL of the 50 g/L NAs in 1M NaOH stock solution, and deionized water (Millipore, >15 MΩ cm^{-1}) to the 700 mark. The pH was adjusted to 8.7 using nitric acid. 0.35 g P25 TiO_2 was added to each beaker and the beaker contents were stirred at 1000 rpm to disperse the particles. The contents of the beakers were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). Afterwards, the beakers were covered with aluminum foil and stirred at 350 rpm in the dark for at least an hour. With regards to the positions of the beakers on the multistirrer, beakers for time points 1, 2, 3, 4, and 5 were placed on spots 13, 3, 1, 15, and 8, respectively, and all beakers were stirred at 500 rpm. The UV light intensities of spots 1, 3, 8, 9, 13, and 15 were measured to be 1.97, 2.32, 2.69, 2.40, and 2.79 mW/cm^2, respectively, when the reaction was started. Time points 1, 2, 3, 4, and 5 were taken out after reacting for 3 h, 8 h, 11 h, 40 h, and 187 h, respectively. Each sample was vacuum filtered using 47 mm glass microfiber filters (Whatman) and stored in the fridge at 4℃.

For the no-salt OSPW photocatalytic treatment, five treatment time points were used. For each beaker, 700 mL synthetic OSPW with [NA] = 150 mg/L were prepared by adding 2.1 mL of the 50 g/L NAs in 1M NaOH stock solution and deionized water (Millipore, >15 MΩ cm^{-1}) to the 700 mark. The pH was adjusted to 8.7 using nitric acid. 0.35 g P25 TiO_2 was added to each beaker and the beaker contents were stirred at 1000 rpm to disperse the particles. The contents of the beakers were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). Afterwards, the beakers were covered with aluminum foil and stirred at 350 rpm in the dark for at least an hour. With regards to the positions of the beakers on the multistirrer, beakers for time points 1, 2, 3, 4, and 5 were placed on spots 13, 3, 1, 15, and 8, respectively, and all beakers were stirred at 300 rpm. The UV light intensities of spots 1, 3, 8, 9, 13,

and 15 were measured to be 2.09, 1.92, 2.15, 2.32, and 2.17 mW/cm^2, respectively, when the reaction was started. Time points 1, 2, 3, 4, and 5 were taken out after reacting for 1 h, 3 h, 5 h, 20 h, and 111 h, respectively. Each sample was vacuum filtered using 47 mm glass microfiber filters (Whatman) and stored in the fridge at 4°C.

5.2.3 Determining chemical oxygen demand (COD)

A 6 mL aliquot from each time point was syringe filtered into a 7 mL glass vial using 0.2 µm filters (Acrodisc). From these filtered samples, 2 mL from the time point 1, 2, 3, and 4 samples was added to the high-range COD vials (Hach) and 2 mL deionized water (Millipore, >15 MΩ cm^{-1}) was added to a high range COD vial to serve as the blank. 2 mL from the filtered time point 5 samples was added to the low range COD vials (Hach) and 2 mL deionized water (Millipore, >15 MΩ cm^{-1}) was added to a low range COD vial to serve as the blank. The contents of all the vials were mixed thoroughly and placed into the digital reactor (Hach, model number DRB200) to start the 2 h reaction. After the reaction, the vials were taken out of the reactor and placed on a rack to cool. To determine the COD for samples in high-range COD vials, the "HR COD" program in the portable colorimeter (Hach, model number DR900) was selected. The deionized water blank in the high-range vial was used to blank the colorimeter prior to reading the high range COD vials. To determine the COD for samples in low-range COD vials, the "LR COD" program in the portable colorimeter was selected. The deionized water blank in the low-range vial was used to blank the colorimeter prior to reading the low range vials.

5.2.4 Determining total organic carbon (TOC)

Total organic carbon (TOC, APHA 5310B, combustion temperature 800°C) for the saline OSPW was determined according to standard methods by ALS Environmental (Waterloo, ON, Canada). Total organic carbon (TOC, SM5310B, combustion temperature 800°C) was determined according to standard methods by Maxxam Analytics (Mississauga, ON, Canada).

5.2.5 Determining UV-Vis

From the filtered 6 mL aliquots, 300 µL of each sample was added to the wells of a clear 96-well plate in duplicate. 300 µL of deionized water (Millipore, >15 MΩ cm^{-1}) and 300 µL of the synthetic OSPW saline solution were added to the clear 96-well plate in duplicate. UV-Vis spectra were read from 230 nm to 400 nm using the Biotech Epoch spectrophotometer.

5.2.6 *L. minor* toxicity tests

Since some of the treated OSPW time points were generated around a week after the first time point and the recommended storage limit for water samples is 3 days in a 4°C fridge, two separate *L. minor* toxicity tests were run to accommodate all of the time points from the synthetic saline OSPW photocatalytic treatment. Axenic *L. minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights 18-24 hours to acclimate before the start of the toxicity test. For the first toxicity test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least 20 min to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The following time points were tested: 3 h, 8 h, 11 h, and 40 h. For each time point, 4 mL of each APHA stock solution was added to 388 mL, and each test solution was aerated for at least 20 min to stabilize the pH. In the biosafety cabinet, each test sample was evenly divided into four 250 mL Erlenmeyer flasks and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation of test solution and loss of volatiles. Each flask was set under wide-spectrum T8 32W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask and the plants with their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

The day before the second toxicity test, axenic *L. minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights 18-24 hours to acclimate. On the first day of the second toxicity test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least 20 min to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The following time points were tested: 0 h (untreated control) and 187 h. For each time point, 4 mL of each APHA stock solution was added to 388 mL test solution, and each test solution was aerated for at least 20 min to stabilize the pH. In the biosafety cabinet, each test sample was evenly divided into four 250 mL Erlenmeyer flasks and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation of test solution and loss of volatiles. Each flask

was set under wide-spectrum T8 32W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask and the plants with their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

Since some of the treated OSPW time points were generated around a week after the first time point and the recommended storage limit for water samples is 3 days in a 4°C fridge, two separate *L. minor* toxicity tests were run to accommodate all of the time points from the synthetic no-salt OSPW photocatalytic treatment. Axenic *L. minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights 18-24 hours to acclimate before the start of the toxicity test. For the first toxicity test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least 20 min to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The following time points were tested: 1 h, 3 h, 5 h, and 20 h. For each time point, 4 mL of each APHA stock solution was added to 388 mL test solution, and each test solution was aerated for at least 20 min to stabilize the pH. In the biosafety cabinet, each test sample was evenly divided into four 250 mL Erlenmeyer flasks and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation of test solution and loss of volatiles. Each flask was set under wide-spectrum T8 32W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants with their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

The day before the second toxicity test, axenic *L. minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights 18-24 hours to acclimate. On the first day of the second toxicity test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution was added to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least 20 min to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. The following time points were tested: 0 h (untreated control) and 111 h. For each time point, 4 mL of each APHA stock solution

was added to 388 mL, and each test solution was aerated for at least 20 min to stabilize the pH. In the biosafety cabinet, each test sample was evenly divided into four 250 mL Erlenmeyer flasks and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation of test solutions and loss of volatiles. Each flask was set under wide-spectrum T8 32W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants with their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

The day before the start of the toxicity test used to investigate the toxicity of OSPW salts, axenic *L. minor* were washed once with sterile deionized water (Millipore, >15 MΩ cm^{-1}) and transferred into 250 mL Erlenmeyer flasks each filled with 100 mL modified APHA medium. The flasks were set under wide-spectrum T8 32 W fluorescent grow lights 18-24 hours to acclimate. On the first day of the toxicity test, four replicates were used for the control which was created by adding 4 mL of each APHA stock solution was added to 388 mL deionized water (Millipore, >15 MΩ cm^{-1}). This solution was aerated for at least 20 min to stabilize the pH. Afterwards, the pH was adjusted to 8.3 using 1M NaOH and 1M HCl. Three synthetic OSPW test samples were created with the same NA concentration of 150 mg/L but varying salt concentrations: one sample had no OSPW salts added, one sample had reduced OSPW salts, and one sample had the regular amount of OSPW salts at 800 mg/L bicarbonate ions, 200 mg/L chloride ions, and 200 mg/L sulfate ions. The no-salt synthetic OSPW was created by adding 350 mL deionized water (Millipore, >15 MΩ cm^{-1}) to a 600 mL beaker, 1.2 mL of the 50 g/L NA in 1M NaOH stock solution, 4 mL of each APHA stock solution, and more deionized water (Millipore, >15 MΩ cm^{-1}) to the 400 mL mark. The pH was adjusted to 8.7 with nitric acid. The reduced salt synthetic OSPW was created by adding 350 mL deionized water (Millipore, >15 MΩ cm^{-1}) to a 600 mL beaker, 3 mL of the synthetic OSPW saline stock solution, 1.2 mL of the 50 g/L NA in 1M NaOH stock solution, 4 mL of each APHA stock solution, and more deionized water (Millipore, >15 MΩ cm^{-1}) to the 400 mL mark. The pH was adjusted to 8.7 with nitric acid. The regular salt synthetic OSPW was created by adding 350 mL deionized water (Millipore, >15 MΩ cm^{-1}) to a 600 mL beaker, 10 mL of the synthetic OSPW saline stock solution, 1.2 mL of the 50 g/L NA in 1M NaOH stock solution, 4 mL of each APHA stock solution, and more deionized water (Millipore, >15 MΩ cm^{-1}) to the 400 mL mark. The pH was adjusted to 8.7 with nitric acid. An OSPW salt blank was created by adding 350 mL deionized water (Millipore, >15 MΩ cm^{-1}) to a 600 mL beaker, 10 mL of the synthetic OSPW saline stock solution, 4 mL of each APHA stock solution, and more deionized water (Millipore, >15 MΩ cm^{-1}) to the 400 mL

mark. Each test solution was aerated for at least 60 min to stabilize the pH. In the biosafety cabinet, each test sample was evenly divided into four 250 mL Erlenmeyer flasks and two 3-frond *L. minor* plants from the acclimated culture were transferred into each flask. The flasks were covered with Petri dish lids to prevent evaporation of test solution and loss of volatiles. Each flask was set under wide-spectrum T8 32W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants with their roots were washed once with deionized water and transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

The growth inhibition for frond number and dried biomass are calculated as shown in Section 3.2.2 using Equation 1 and 2, respectively.

5.3 Results and Discussion

5.3.1 Photocatalysis is effective at degrading NAs

Photocatalytic treatments of synthetic saline OSPW with [NA] = 150 mg/L and synthetic no-salt OSPW with the same NA concentration were conducted, and the extent of mineralization is shown in Figure 7 and 8 for the synthetic saline OSPW and synthetic no-salt OSPW, respectively. Both synthetic OSPWs show that photocatalysis is capable of mineralizing high NA concentrations, as the NA concentration of 150 mg/L is higher than what would be expected in actual tailings ponds[156]. Although photocatalysis was successful in mineralizing NAs in both OSPW samples, the synthetic saline OSPW required a longer photocatalytic reaction time to fully mineralize the NAs when compared to the no-salt synthetic OSPW. This matches the results from the literature where the presence of inorganic salts has been shown to slow down the photocatalytic process by competing against adsorbed organic species to be photo-oxidized[157,158].

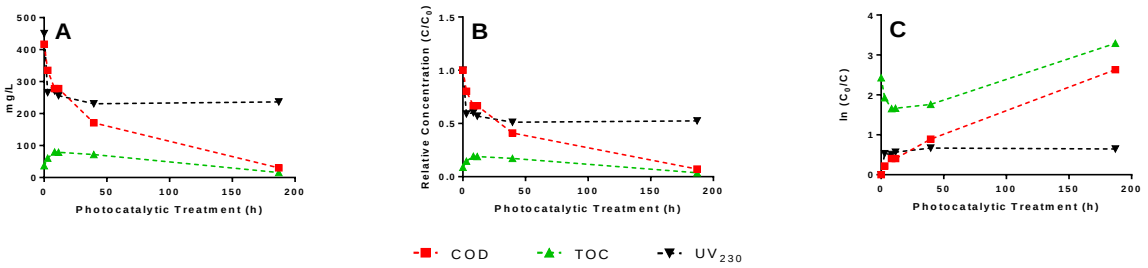

Figure 7. NA degradation kinetics in a synthetic saline OSPW

Figure 7 shows the photocatalytic degradation of NAs in a synthetic saline OSPW monitored using COD, TOC, and UV-Vis measured at 230 nm. Figure 7A shows the concentration for COD, TOC, and UV_{230} plotted against the length of photocatalytic treatment (h). Figure 7B shows the relative concentration plotted against the length of photocatalytic treatment (h), and C and C_0 represent the concentration and initial concentration, respectively. Figure 7B shows an exponential curve, suggesting that the degradation kinetics are heterogeneous in nature[4,9,41]. Figure 7C shows the linear transformation of the relative concentration vs time where ln (C_0/C) plotted against the length of photocatalytic treatment (h); the linearity of this plot indicates that the NA degradation kinetics follow pseudo-first order kinetics[4,9,41]. Figure 7B and 7C demonstrate that the degradation of NAs undergoes pseudo first-order kinetics, which match the results of other literature[4,9,41].

COD and UV-Vis both show that there is a decrease in concentration for organics and NAs, respectively, as the photocatalytic treatment progresses. The TOC shows a spike in carbon concentration above 100% TOC during the earlier photocatalytic treatment time points. A hypothesis of why this might occur is that as part of the TOC analysis, samples are acidified and purged to eliminate inorganic carbon from the sample as CO_2. This acidification step lowers the solubility of NAs, resulting in NAs attaching themselves to bubbles or the walls of the vessels instead of remaining in the test solution. When the instrument samples the water to test for TOC, it reads an artificially low organic carbon concentration as a fraction is adhering to the inside surface of the vessel. During early photocatalytic treatment, the NAs start losing their surfactant characteristics and are less vulnerable to acid precipitation, hence the compounds remain in solution and the instrument picks up a higher TOC reading. This “spike” above 100% was not observed for COD, and this can be explained by sampling and analysis methodology. Although COD involves the sample being digested for 2 h with dichromate in acidic conditions, there is no “spike” observed as measuring COD does not require sampling from

the COD vial for external quantification elsewhere. Even if the NAs in the COD vial displayed the same behavior as the NAs in the TOC vessels, COD is measured spectrophotometrically in the portable colorimeter where the whole vial is inserted, hence if there are any oxidized organic compounds adhering to the insides of the COD vials, this is accounted for spectrophotometrically. The "spike" above 100% was not observed for UV-Vis as this does not require an acidification step prior to the analysis.

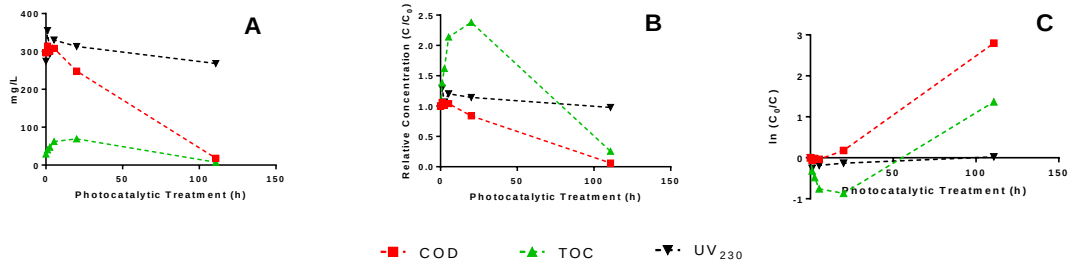

Figure 8. NA degradation kinetics in a synthetic no-salt OSPW

Although it is possible that the dissolved OSPW salts can change the solubility of NAs, it is unlikely that the dissolved salts were the major cause of the TOC "spike" as this "spike" was also observed in the partially treated synthetic no-salt OSPW. Figure 8 above shows the photocatalytic degradation of NAs in a synthetic no-salt OSPW monitored using COD, TOC, and UV-Vis. Figure 8A shows the concentration for COD, TOC, and UV_{230} plotted against the length of photocatalytic treatment (h). Figure 8B shows the relative concentration plotted against the length of photocatalytic treatment (h), and C and C_0 represent the concentration and initial concentration, respectively. Figure 8B shows an exponential curve, suggesting that the degradation kinetics are heterogeneous in nature[4,9,41]. Figure 8C shows the linear transformation of the relative concentration vs time where ln (C_0/C) plotted against the length of photocatalytic treatment (h); the linearity of this plot indicates that the NA degradation kinetics follow pseudo-first order kinetics[4,9,41].

Once again, COD and UV-Vis both show that there is a decrease in concentration for organics and NAs, respectively, as the photocatalytic treatment progressed, and the TOC shows a spike in carbon concentration above 100% TOC during the earlier photocatalytic treatment time points. Instead of OSPW salts interfering with the solubility of NAs, it is more likely that the acidification step in TOC lowered the solubility of NAs, thus resulting in NAs adhering to the walls of the vessels instead of remaining in the OSPW. During early photocatalytic treatment, the NAs started losing their surfactant

characteristics and were less vulnerable to acid precipitation, hence they remained in solution and the instrument picks up a higher TOC reading. This "spike" above 100% was not observed for COD, but this can be explained by the analysis methodology, as measuring COD does not require sampling from the COD vial for external quantification elsewhere, eliminating the chance of organic carbon being left behind on insides of the vials. The "spike" above 100% was not observed for UV-Vis as this does not require an acidification step prior to the analysis. Figure 8B and 8C further support that the degradation of NAs undergoes pseudo first-order kinetics, which match the results of other literature[4,9,41].

5.3.2 Salinity has a significant effect on OSPW toxicity to *L. minor*

For the synthetic OSPW sample, 150 mg/L was chosen as the NA concentration as this was slightly higher than the IC_{50} for frond number (142.9 mg/L), and this concentration was slightly higher than the NA concentrations likely to be found in tailing ponds as the NA concentrations reported range from 20 to 130 mg/L[155,156]. The final concentrations of OSPW salts in the synthetic OSPW were 800 mg/L bicarbonate ions, 200 mg/L chloride ions, and 200 mg/L sulfate ions. The purpose of this experiment is to treat this synthetic OSPW using photocatalysis, observe the NA degradation and reduction of toxicity to *L. minor*.

Figure 9A shows the 7-day toxicity response of *L. minor* to a synthetic saline OSPW with 150 mg/L NA using frond number as the endpoint. Figure 9B shows the 7-day toxicity response of *L. minor* to a synthetic saline OSPW with 150 mg/L NA using dried biomass as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

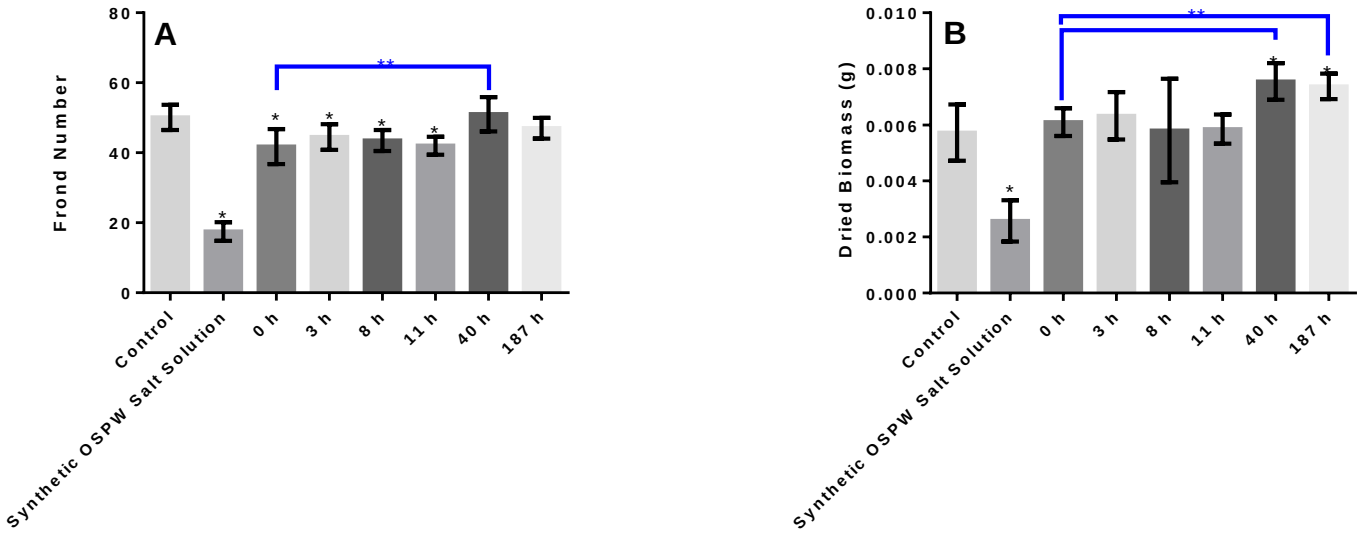

Figure 9. (A) 7-day toxicity response of *L. minor* to photocatalytic treatment of a synthetic saline OSPW with 150 mg/L NA evaluated using frond number, mean values ± SD (*n* = 8 for the control, *n* = 4 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). Mean values that are significantly different from the 0 h untreated time point ($p < 0.05$) are indicated in blue (**). (B) 7-day toxicity response of *L. minor* to photocatalytic treatment of a synthetic saline OSPW with 150 mg/L NA evaluated using dried biomass, mean values ± SD (*n* = 8 for the control, *n* = 4 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). Mean values that are significantly different from the 0 h untreated time point ($p < 0.05$) are indicated in blue (**).

Table 8 below shows the growth inhibition for frond number and dried biomass displayed in Figure 9A and Figure 9B, respectively. The negative numbers shown in the table demonstrate that *L. minor* experienced a stimulatory effect from the test solution when compared to the control.

	% Growth Inhibition	
Test Sample	**Frond Number**	**Dried Biomass**
Control	0	0
Synthetic OSPW Salt Solution	65.1	55.0
0 h	16.7	-6.6
3 h	11.2	-10.5
8 h	13.2	-1.3
11 h	16.2	-2.2
40 h	-1.7	-31.2
187 h	6.2	-28.8

Table 8. Growth inhibition of *L. minor* exposed to raw and treated synthetic saline OSPW with 150 mg/L NA for 7 days

One-tailed t-tests were used to evaluate whether a difference between two means was significant. The synthetic saline OSPW with 150 mg/L NAs was toxic if frond number was used as the endpoint, but surprisingly, this high concentration of NAs was not toxic to *L. minor* if dried biomass was used as the endpoint. However, the photocatalytic treatment was shown to be effective, as a treatment of 40 hours or more reduced the toxicity. Hormesis was observed in the 40 h and 187 h of treatment samples for both endpoints, and this can be due to organic compounds from the NA degradation serving as a nutrient source to *L. minor*. Contrary to the results from Chapter 3, OSPW salts by themselves were extremely toxic to *L. minor*. Curiously, the 0 h untreated time point and the other treatment time points did not show this extreme toxicity although each test sample other than the control all shared the same salt concentrations of 800 mg/L bicarbonate ions, 200 mg/L chloride ions, and 200 mg/L sulfate ions. Also, it is unlikely that the toxicity of the synthetic OSPW salt solution is a result of its high pH as the OSPW salt solution and the later photocatalytic treatment time points share a similar pH ~ 9. This suggests that there is an interaction between the OSPW salts and the NAs, and the presence of NAs reduced the toxicity from the salts.

To investigate the effect of salinity, synthetic OSPW samples were created where each sample had different salinities but each had the NA concentration kept constant at 150 mg/L. One sample had no OSPW salts added, one sample had reduced OSPW salts, and one sample had the regular amount of OSPW salts at 800 mg/L bicarbonate ions, 200 mg/L chloride ions, and 200 mg/L sulfate ions. The

sample with the reduced OSPW salts contained the amount of salts estimated at 150 mg/L NAs using Equation 4 in Section 4.3.

Figure 10A below shows the 7-day toxicity response of *L. minor* to synthetic OSPW with 150 mg/L NA and various salinities, and frond number as the endpoint. Figure 10B below shows the 7-day toxicity response of *L. minor* to synthetic OSPW with 150 mg/L NA and various salinities, and dried biomass was used as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

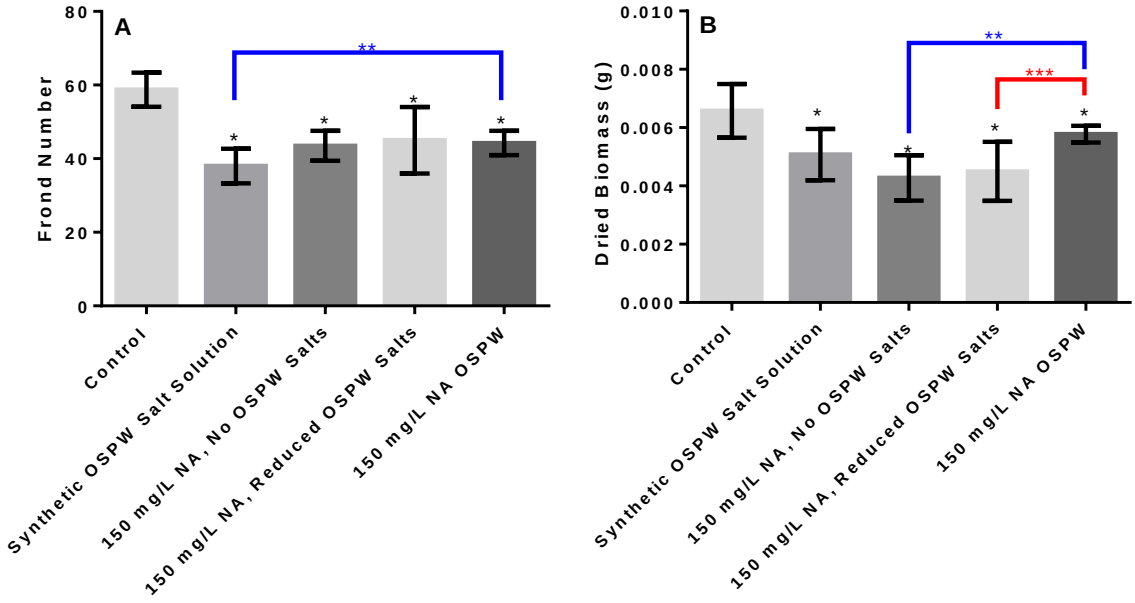

Figure 10. (A) 7-day toxicity response of *L. minor* to synthetic OSPW with increasing salinities evaluated using frond number, Mean values ± SD (n = 4). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). The significant difference ($p < 0.05$) between the mean values of the synthetic OSPW salt solution and the OSPW with regular salinity is indicated in blue (**). (B) 7-day toxicity response of *L. minor* to synthetic OSPW with increasing salinities evaluated using dried biomass, mean values ± SD (n = 4). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). The significant difference ($p < 0.05$) between the mean values of the OSPW with regular salinity and the no-salt OSPW is indicated in blue (**). The significant difference ($p < 0.05$) between the mean values of the OSPW with regular salinity and the reduced-salt OSPW is indicated in red (***).

Table 9 below shows the growth inhibition for *L. minor* frond number and dried biomass corresponding to Figure 10A and Figure 10B, respectively.

	% Growth Inhibition	
Test Sample	**Frond Number**	**Dried Biomass**
Control	0	0
Synthetic OSPW Salt Solution	35.3	22.8
150 mg/L NA, No OSPW Salts	26.0	35.0
150 mg/L NA, Reduced OSPW Salts	23.4	31.6
150 mg/L NA OSPW	23.4	12.2

Table 9. Growth inhibition of *L. minor* exposed to synthetic OSPW with 150 mg/L NA and increasing salinities

One-tailed t-tests were used to determine whether the difference between two means was significant. Both endpoints show that OSPW salts by themselves and the synthetic OSPW with various salinities are toxic to *L. minor* as the difference between the means of the control and each of the other test samples is significant.

It is interesting to note that for the frond number, the difference between the means of the synthetic OSPW salt solution and the synthetic OSPW with regular salinity is significant. Since the OSPW salt solution and the synthetic OSPW with regular salinity both have the same concentrations of salts, this suggests that there is some interaction between the NAs and the OSPW salts as the presence of NAs helped reduce some of the toxicity from the salinity.

For the dried biomass, the difference between the means of the synthetic OSPW with regular salinity and that of the no-salt OSPW is significant. In addition, the difference between the means of the synthetic OSPW with regular salinity and that of the OSPW with reduced salinity is also significant. These results suggest that the increase in salinity levels in OSPW helps to reduce the toxicity of NAs to *L. minor*.

Interaction plots between NAs and OSPW salts were thus created for both endpoints, and both show that an interaction between NAs and OSPW salts exists. These plots are shown in Figure 11 below.

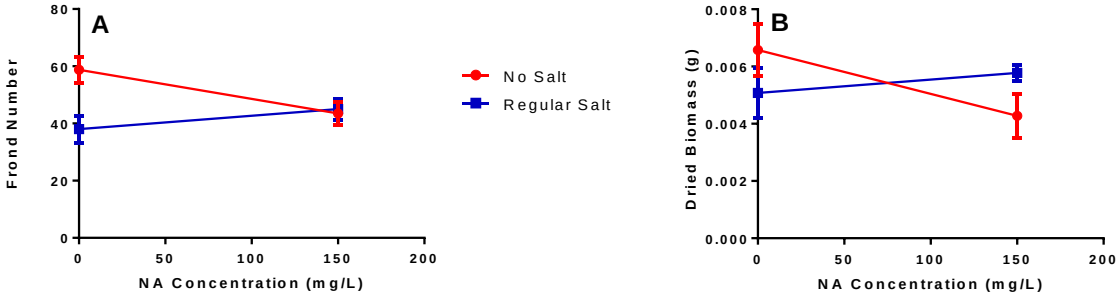

Figure 11. Interaction plot for NAs and OSPW salts evaluated with (A) frond number and (B) dried biomass

Interactions between OSPW salts and NAs exist, but the interactions themselves are poorly understood[159]. These salt-NA interactions have a wide range of effects from salts having no effect on the NAs, to salts enhancing of the solubility of some NA species, and to salts causing the formation of precipitates[159]. As OSPW salts can affect the solubility of NAs, this can in turn affect the bioavailability of NAs in aquatic environments[159]. In some freshwater aquatic systems, the elevated salinity characteristic of OSPW is associated with shifts in the composition of rooted plants, phytoplankton, and zoobenthos[159,160]. It is possible that the increase in salinity affected the solubility of NAs, thus reducing its bioavailability and reducing some of its toxicity to *L. minor*.

5.3.3 NAs are chronically toxic to *L. minor* when evaluated using frond number and dried biomass as the endpoints

As the effects of the OSPW salinity experiments showed that there is an interaction between OSPW salts and NAs, a synthetic no-salt OSPW was made with only 150 mg/L NAs in order to study the effect of only NAs to *L. minor*. Figure 12A below shows the 7-day toxicity response of *L. minor* to a synthetic no-salt OSPW with 150 mg/L NAs using frond number as the endpoint. Figure 12B below shows the 7-day toxicity response of *L. minor* to synthetic OSPW with 150 mg/L NA and no salt using dried biomass as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

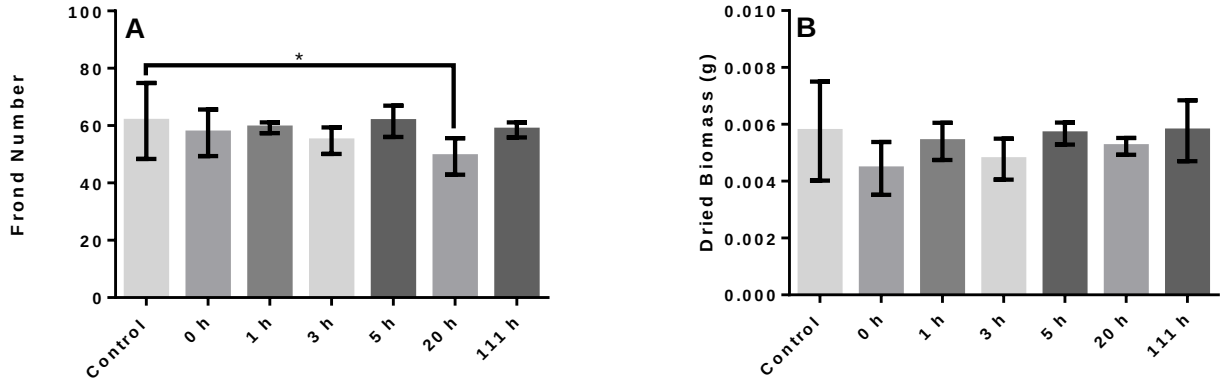

Figure 12. (A) 7-day toxicity response of *L. minor* to synthetic no-salt OSPW containing 150 mg/L NA evaluated using frond number, mean values ± SD (*n* = 8 for control, *n* = 4 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). (B) 7-day toxicity response of *L. minor* to synthetic no-salt OSPW containing 150 mg/L NA and no salt evaluated using dried biomass, mean values ± SD (*n* = 8 for control, *n* = 4 for test samples).

Table 10 below shows the growth inhibition for *L. minor* frond number and dried biomass corresponding to Figure 12A and Figure 12B, respectively.

	% Growth Inhibition	
Test Sample	**Frond Number**	**Dried Biomass**
Control	0	0
0 h	6.7	22.8
1 h	3.9	6.3
3 h	11.2	17.1
5 h	0.2	1.5
20 h	20.1	9.3
111 h	5.1	-0.2

Table 10. Growth inhibition of *L. minor* exposed to raw and treated synthetic no-salt OSPW containing 150 mg/L NA for 7 days

For Figure 12A, the difference between the means for the control and the 20 h treatment sample is significant unlike the other time points, but since this is the only treatment time point that is significant, it is more likely that this is an outlier. Overall, Figure 12 shows that a synthetic no-salt OSPW containing 150 mg/L NAs is not toxic to *L. minor*. Although the means for both frond number and biomass for 0 h are smaller than that of the control, the difference is not significant according to a one-tailed t-test.

While a 7-day toxicity test shown by Figure 12 demonstrated that NAs are not acutely toxic to *L. minor*, more toxic effects are apparent after a 14-day toxicity test. Figure 13 below shows the results of exposing *L. minor* to a synthetic no-salt OSPW containing 150 mg/L NAs for 14 days.

Figure 13A below shows the 14-day response of *L. minor* to a synthetic no-salt OSPW with 150 mg/L NA where frond number was used as the endpoint. Figure 13B below shows the 14-day response of *L. minor* to a synthetic no-salt OSPW with 150 mg/L NAs where dried biomass was used as the endpoint. One-tailed t-tests were used to determine whether a difference between two means was significant.

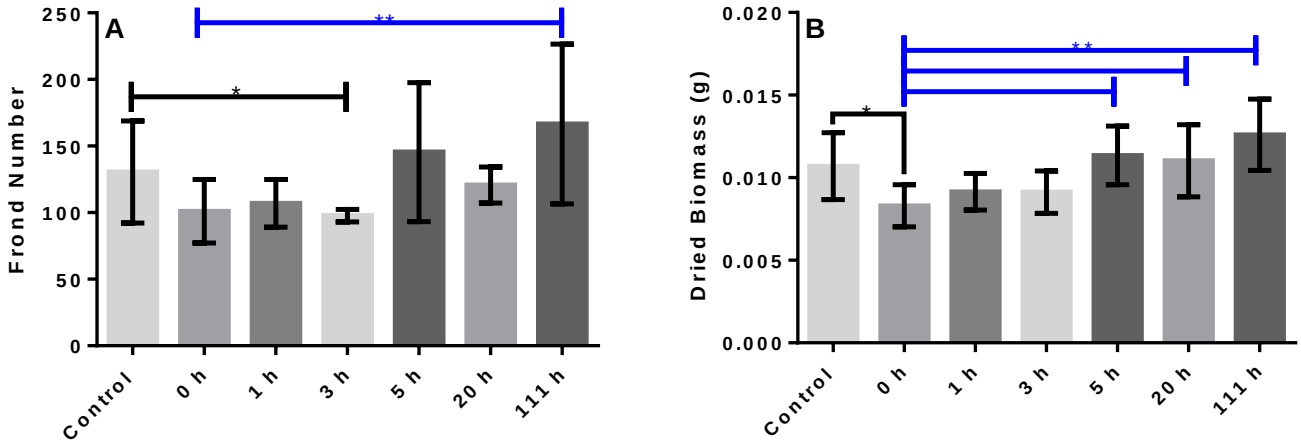

Figure 13. (A) 14-day response of *L. minor* to synthetic no-salt OSPW containing 150 mg/L NAs, evaluated using frond number, mean values ± SD (*n* = 8 for control, *n* = 4 for test samples). Mean values that are significantly different from the control ($p < 0.05$) are indicated (*). Mean values that are significantly different from the 0 h sample ($p < 0.05$) are indicated in blue (**). (B) 14-day response of *L. minor* to synthetic no-salt OSPW containing 150 mg/L NAs, evaluated

using dried biomass, mean values ± SD (*n* = 8 for control, *n* = 4 for test samples). Mean values that are significantly different from the control (p < 0.05) are indicated (*). Mean values that are significantly different from the 0 h sample (p < 0.05) are indicated in blue (**).

Table 11 below shows the growth inhibition for *L. minor* frond number and dried biomass corresponding to Figure 13A and Figure 13B, respectively.

	% Growth Inhibition	
Test Sample	**Frond Number**	**Dried Biomass**
Control	0	0
0 h	22.6	22.4
1 h	18.0	14.5
3 h	25.1	14.7
5 h	-11.5	-6.1
20 h	7.5	-3.0
111 h	-27.6	-17.8

Table 11. Growth inhibition of *L. minor* exposed to raw and treated synthetic no-salt OSPW containing 150 mg/L NA for 14 days

For the frond number, the difference between the means of the control and the 3 h treated sample is significant. Since this is the only test sample that is significantly different from the control, it is more likely that this sample had a lower frond count due to the placement of the flasks. Although the placement of the flasks is all randomized, it is possible that for this sample, the flasks happened to receive a lower light intensity than other flasks as some areas under the fluorescent lights have a higher light intensity than others. The difference between the means of the 0 h untreated sample and the 111 h treated sample is significant, showing that photocatalytic treatment is capable of degrading NAs and long-term exposure to 150 mg/L NAs in synthetic OSPW caused a more pronounced stress response in *L. minor*.

For the biomass, the difference between the means of the control and the 0 h untreated sample is significant, indicating that NAs are chronically toxic to *L. minor*. The differences between the means

of the 0 h untreated sample and the 5 h, 20 h, and 111 h treated samples are significant. This shows that photocatalysis is capable of degrading NAs in synthetic OSPW and reducing the toxicity to *L. minor*.

5.3.4 NAs are acutely toxic to *L. minor* when evaluated using gibbosity as the endpoint

The traditional endpoints for a *L. minor* toxicity test are frond number and dried biomass, but these endpoints might not be sufficient in evaluating OSPW toxicity as there are other characteristics of *L. minor* growth indicative of plant health. From the 7-day toxicity test illustrated in Figure 12, both frond number and dried biomass suggest that NAs in OSPW are chronically toxic to *L. minor*, but when plant surface area from the same toxicity test was measured using ImageJ software, the subsequent analysis suggested otherwise.

Normal and healthy *L. minor* colony growth should spread across the surface with the fronds flat against the air-water interface. Gibbosity refers to a humped appearance of *L. minor* colonies. In OSPW with higher NA concentrations, *L. minor* growth does not spread across the liquid surface, and the fronds grow curving downwards into the test solution. Figure 14 below shows the averaged frond area for each photocatalytic treatment time point after the 7-day *L. minor* toxicity test. One-tailed t-tests were used to determine whether a difference between two means was significant.

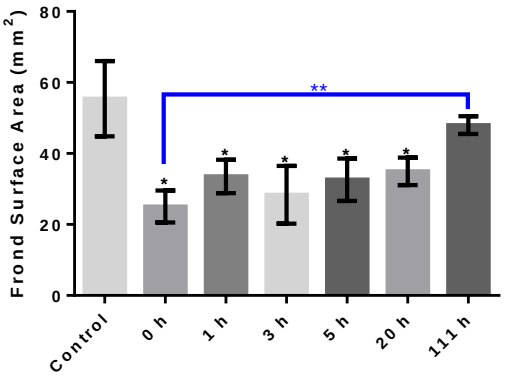

Figure 14. 7-day *L. minor* toxicity response to synthetic no-salt OSPW using gibbosity as the endpoint, mean values ± SD (*n* = 8 for control, *n* = 4 for test samples). Mean values that are significantly different from the control (p < 0.05) are indicated (*). Mean values of treatment time points that are significantly different (p < 0.05) from the 0 h time point are indicated in blue (**).

Table 12 below shows the growth inhibition for *L. minor* when gibbosity was used as the endpoint. This table corresponds to Figure 14 above.

Test Sample	**% Growth Inhibition**
Control	0
0 h	54.8
1 h	39.5
3 h	48.8
5 h	41.2
20 h	37.0
111 h	13.4

Table 12. Growth inhibition for *L. minor* using gibbosity as the endpoint

If gibbosity is used as the endpoint, photocatalysis is shown to be effective at reducing the toxicity of NAs to *L. minor*. This endpoint also shows that NAs are acutely toxic to *L. minor*. The differences

in NA toxicity results is dependent on the endpoint chosen. The frond number endpoint does not take frond size into consideration, and a fully-grown frond and a visible bud are considered equal. As shown in the previous results, it is possible for *L. minor* plants to grow in high NA concentrations and have a similar frond count to that of the control where a one-tailed t-test shows the difference between the means is not significant. However, the frond count does not consider that plants grown on OSPW have smaller fronds when compared to those of healthy *L. minor* plants. One qualitative criterion used to evaluate *L. minor* health is frond size, and according to Environment Canada (2007), healthy plants should not have abnormally-sized fronds[127].

Biomass might not be the best indicator for plant health either since *L. minor* can grow on OSPW with a high NA concentration. Even if the plants can grow in higher NA concentrations, this does not mean that the plants are healthy as there are other factors used to gauge *L. minor* overall health. According to Environment Canada (2007), healthy *L. minor* colonies should be buoyant, intact, and have fronds of variable shades of green, with newer fronds being a lighter green than grown fronds[127]. Healthy *L. minor* should not lose buoyancy, should not undergo chlorosis or necrosis on the fronds, and fronds should not be yellow or abnormally-sized[127]. It is recommended that other characteristics of *L. minor* health that can be quantified such as chlorophyll content should be used as endpoints in addition to the traditional endpoints to provide a more thorough understanding of NA toxicity to *L. minor*.

5.4 Environmental Significance

Photocatalysis is shown to be effective at mineralizing NAs which are one of the more toxic compounds found in OSPW. In an OSPW with mostly organics and minimal trace heavy metals, photocatalysis alone may be enough to reduce OSPW toxicity, allowing for its return into constructed wetlands for further bioremediation and phytoremediation.

The toxicity of OSPW salts was shown to be significant to *L. minor*. If *L. minor* is to be used as a representative for wetland health, this demonstrates that the salinity of OSPW might need to be adjusted after partial or full photocatalytic treatment prior to its return into the environment. In addition, there is an interaction between OSPW salts and NAs, and these can affect the bioavailability of NAs to freshwater organisms.

As NAs were shown to be both acutely and chronically toxic to *L. minor*, the choice of endpoint used to monitor wetland integrity should be carefully considered as the effects of the partially-treated OSPW

released into the environment might not be immediately apparent if certain endpoints are chosen as evaluation tools.

Chapter 6
Interactions between Naphthenic Acids and Heavy Metals

6.1 Summary

Oil sands process-affected water (OSPW) is a toxic by-product generated from the bitumen extraction process. Due to its acute and chronic toxicity to wildlife, it is not approved for environmental release and thus is stored on site in tailings ponds. Oil sands mining companies are required to treat OSPW and return it into the environment upon the completion of mining operations; however, there is no standardized treatment method rendering OSPW safe enough to be incorporated into the environment.

Due to the complexity of OSPW, there are many components that contribute to its overall toxicity. Naphthenic acids (NAs) are responsible for much of the toxicity in the dissolved organics fraction, and the trace amounts of heavy metals present are responsible for causing oxidative damage. While many studies have been conducted on the toxicity of NAs and the reduction of this toxicity using photocatalysis, there are few studies on the interactions of NAs and other OSPW components.

The objective of this work is to investigate whether there is a reduction of toxicity in an industrial OSPW sample with a low NA concentration. This was attempted by spiking the industrial OSPW with synthetic NAs to provide some residual organic compounds after the photocatalytic treatment to sequester heavy metals. After the photocatalytic treatment, the samples were also spiked with EDTA to chelate heavy metals.

6.2 Experimental

6.2.1 Materials

Raw OSPW was provided by Industry B. Potassium chloride was purchased from ACP Chemicals Inc. Sodium chloride and boric acid were purchased from EMD Chemicals Inc. P25 titanium dioxide Aeroxide^{TM} was purchased from Acros Organics. All other chemicals and reagents were purchased from Sigma Aldrich.

Axenic *Lemna minor* 490 stock cultures were obtained from the Canadian Phycological Culture Centre following the protocol of Gopalapillai et al., (2013)[149], and the axenic *L. minor* plants were cultured in modified Hoagland's E+ medium and subcultured weekly according to the Environment Canada standardized protocol[127]. Modified Hoagland's E+ stock solution A contains 59.00 g/L

calcium nitrate tetrahydrate, 75.76 g/L potassium nitrate, 34.00 g/L potassium phosphate monobasic, and 6 mL of 6N HCl. Modified Hoagland's E+ stock solution B contains 3.00 g/L tartaric acid. Modified Hoagland's E+ stock solution stock solution C contains 1.21 g/L iron (III) chloride hexahydrate, 3.35 g/L disodium EDTA dihydrate, and 1.2 mL of 6N KOH. Modified Hoagland's E+ stock solution D contains 50.00 g/L magnesium sulfate heptahydrate. Modified Hoagland's E+ stock solution E contains 2.86 g/L boric acid, 0.22 g/L zinc sulfate heptahydrate, 0.12 g/L sodium molybdate dihydrate, 0.08 g/L copper (II) sulfate pentahydrate, and 3.62 g/L manganese (II) chloride tetrahydrate. 1 L Modified Hoagland's E+ medium was created by adding 20 mL stock solution A, 1 mL stock solution B, 20 mL stock solution C, 10 mL stock solution D, 1 mL stock solution E, 10 g sucrose, 0.10 g yeast extract, and 0.6 g tryptone to 900 mL of deionized water (Millipore, >15 MΩ cm^{-1}). The pH was adjusted to 4.4-4.8 using 1M NaOH and 1M HCl, and the final volume was brought to 1 L using deionized water (Millipore, >15 MΩ cm^{-1}). The modified Hoagland's E+ medium was autoclaved at 121°C and 1.1 bar for 20 min (Priorclave) before use.

APHA stock solution A contains 25.5 g/L sodium nitrate, 15.0 g/L sodium bicarbonate, 1.04 g/L potassium phosphate monobasic, and 1.01 g/L potassium chloride in deionized water (Millipore, >15 MΩ cm^{-1}). APHA stock solution B contains 4.41 g/L calcium chloride dihydrate, 12.17 mg/L magnesium chloride hexahydrate, 0.4149 g/L manganese (II) chloride tetrahydrate, and 0.16 g/L iron (III) chloride hexahydrate in deionized water (Millipore, >15 MΩ cm^{-1}), and the solution was acidified to pH 2 with HCl. APHA stock solution C contains 14.7 g/L magnesium sulfate heptahydrate, 0.186 g/L boric acid, 7.26 mg/L sodium molybdate dihydrate, 3.27 mg/L zinc chloride, 1.4 mg/L cobalt (II) chloride hexahydrate, and 15 µg/L copper (II) chloride dihydrate in deionized water (Millipore, >15 MΩ cm^{-1}). Each APHA stock was added to the test sample solution to produce a final concentration of 1 v/v% of each APHA stock solution (i.e. after the addition of the three APHA stocks, the test sample solution has a final concentration of 97%). Each test solution requires the addition of APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth. The control and acclimation solution both contain the same components, and both are made using deionized water (Millipore, >15 MΩ cm^{-1}) with each APHA stock added to produce a final concentration of 1 v/v% of each APHA stock solution. As with the test solutions, both the control and acclimation solutions were spiked with APHA stock solutions to provide *L. minor* with the inorganic nutrients necessary for growth.

6.2.2 Photocatalytic tests

Two photocatalytic treatments were used to generate the treated Industry B OSPW and the treated NA-spiked Industry B OSPW samples, as one pair was used for the toxicity test run without EDTA and the second set was used for the toxicity test run with EDTA.

A raw Industry B OSPW sample and an NA-spiked raw Industry B OSPW sample were used as untreated controls for the samples generated from both photocatalytic treatments. Since two toxicity tests were run for a sample set without EDTA and a sample set with added EDTA, two raw Industry B OSPW sample beakers and two NA-spiked raw Industry B OSPW sample beakers were prepared. To prepare the raw Industry B OSPW samples, 450 mL of raw Industry B OSPW was vacuum filtered using 47 mm diameter glass microfiber filters (Whatman) and placed in the fridge. To prepare the NA-spiked raw Industry B OSPW samples, 1.35 mL of the 50 g/L NAs in 1M NaOH stock solution was added to 400 mL of raw Industry B OSPW, and the volume level was filled up to 450 mL afterwards using raw Industry B OSPW. The NA-spiked raw Industry B OSPW samples were vacuum filtered using 47 mm diameter glass microfiber filters (Whatman) and placed in the fridge.

To prepare the treated Industry B OSPW using the first photocatalytic test, 450 mL of raw Industry B OSPW and 0.225 g P25 TiO_2 were added to the beaker, and the contents were stirred at 400 rpm to disperse the particles. The contents of the beaker were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). The sides of the beaker were wrapped with aluminum foil and the beakers were sealed with Glad wrap. The beaker was stirred at 500 rpm in the dark for at least an hour. To prepare 450 mL of treated NA-spiked Industry B OSPW, 1.35 mL of the 50 g/L NAs in 1M NaOH stock solution was added to 400 mL of raw Industry B OSPW. The volume level was filled up to 450 mL afterwards using raw Industry B OSPW. 0.225 g P25 TiO_2 was added to the beaker and the contents were stirred at 400 rpm to disperse the particles. The contents of the beaker were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). The sides of the beaker were wrapped with aluminum foil and the beakers were sealed with Glad wrap. The beaker was stirred at 500 rpm in the dark for at least an hour. The beakers were placed on the multistirrer (Thermo Fisher) and stirred at 500 rpm. The treated Industry B OSPW and the treated NA-spiked Industry B OSPW beakers were placed on spots 5 and 11, respectively. The UV light intensities for

spot 5 and 11 at the start of the reaction were measured using a UVA/UVB meter (Sper Scientific) to be 2.30 and 2.24 mW/cm^2, respectively. The UV exposure was started, and the beakers were treated for 6 days 5 h, and 57 min. After the photocatalytic treatment, both beakers were vacuum filtered using 47 mm diameter glass microfiber filters (Whatman) and placed in the fridge.

To prepare the treated Industry B OSPW using the second photocatalytic treatment, 450 mL of raw Industry B OSPW and 0.225 g P25 TiO_2 were added to the beaker and the contents were stirred at 400 rpm to disperse the particles. The contents of the beaker were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). The sides of the beaker were wrapped with aluminum foil and the beakers were sealed with Glad wrap. The beaker was stirred at 500 rpm in the dark for at least an hour. To prepare 450 mL of treated NA-spiked Industry B OSPW, 1.35 mL of the 50 g/L NAs in 1M NaOH stock solution was added to 400 mL of raw Industry B OSPW. The volume level was filled up to 450 mL afterwards using raw Industry B OSPW. 0.225 g P25 TiO_2 was added to the beaker and the contents were stirred at 400 rpm to disperse the particles. The contents of the beaker were sonicated for 10 min at a 40% tip amplitude in 1 s on/off pulses, with the beaker submerged in an ice water bath and the flat probe tip submerged to ~30% of the depth of the liquid (Fisher Scientific Sonic Dismembrator Model FB505, 500 W, 20 kHz). The sides of the beaker were wrapped with aluminum foil and the beakers were sealed with Glad wrap. The beaker was stirred at 500 rpm in the dark for at least an hour. The beakers were placed on the multistirrer (Thermo Fisher) and stirred at 500 rpm. The treated Industry B OSPW and the treated NA-spiked Industry B OSPW beakers were placed on spots 5 and 11, respectively. The UV light intensities for spot 5 and 11 at the start of the reaction were measured using a UVA/UVB meter (Sper Scientific) to be 2.30 and 2.24 mW/cm^2, respectively. The pair of Industry B samples was treated for 6 days 15 h, and 33 min, and the samples were vacuum filtered using 47 mm diameter glass microfiber filters (Whatman) and placed in the fridge.

6.2.3 *L. minor* toxicity tests

To accommodate all of the samples, two toxicity tests were run, with one for the samples without EDTA and one for the samples with added EDTA. To run the toxicity test for the samples without EDTA, ~380 mL of each sample was syringe-filtered with 0.8 µm and 0.45 µm filters (Acrodisc), and 4 mL of each APHA stock solution was added into each sample beaker. Afterwards, each sample was topped up to 400 mL with the respective syringe-filtered test solution and aerated for at least an hour to stabilize

the pH. After aeration, each sample was syringe-filtered using 0.2 µm filters (Acrodisc) in the biosafety cabinet with 100 mL of sample going into each 250 mL Erlenmeyer flask. Two 3-frond plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solution and loss of volatiles. Each flask was placed under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants and their roots were transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

To run the toxicity test for the samples with added EDTA, ~380 mL of each sample was syringe-filtered with 0.8 µm and 0.45 µm filters (Acrodisc), and 4 mL of each APHA stock solution was added into each sample beaker. Each sample was topped up to 400 mL with the respective syringe-filtered test solution and 0.4 mL of the 50 g/L $Na_2EDTA{\cdot}2H_2O$ stock solution was added into each sample beaker. Each sample was aerated for at least an hour. After aeration, each sample was syringe-filtered using 0.2 µm filters (Acrodisc) in the biosafety cabinet with 100 mL of sample going into each 250 mL Erlenmeyer flask. Two 3-frond plants from the acclimated culture were transferred into each flask and the flasks were covered with Petri dish lids to prevent the evaporation of test solution and loss of volatiles. Each flask was placed under wide-spectrum T8 32 W fluorescent grow lights for a continuous period of 7 days. On Day 7, the number of fronds were counted in each flask, and the plants and their roots were transferred into labeled and pre-weighed weighing boats to dry overnight. The mass of the dried *L. minor* biomass was determined the next day.

6.3 Results and Discussion

For the following experiments, synthetic NAs were added into the Industry B OSPW at 150 mg/L as the raw Industry B OSPW does not have a high concentration of NAs by itself. Spiking the Industry B OSPW can provide some residual DOM after the photocatalytic treatment that might sequester any heavy metals present. EDTA was added to the raw and treated Industry B OSPW samples to investigate whether it can reduce the heavy metal toxicity to *L. minor*.

When comparing both EDTA treatment groups, the addition of EDTA to the control and each test condition had a significant stimulatory effect on *L. minor* growth ($p < 0.05$) as shown in Figure 15 below.

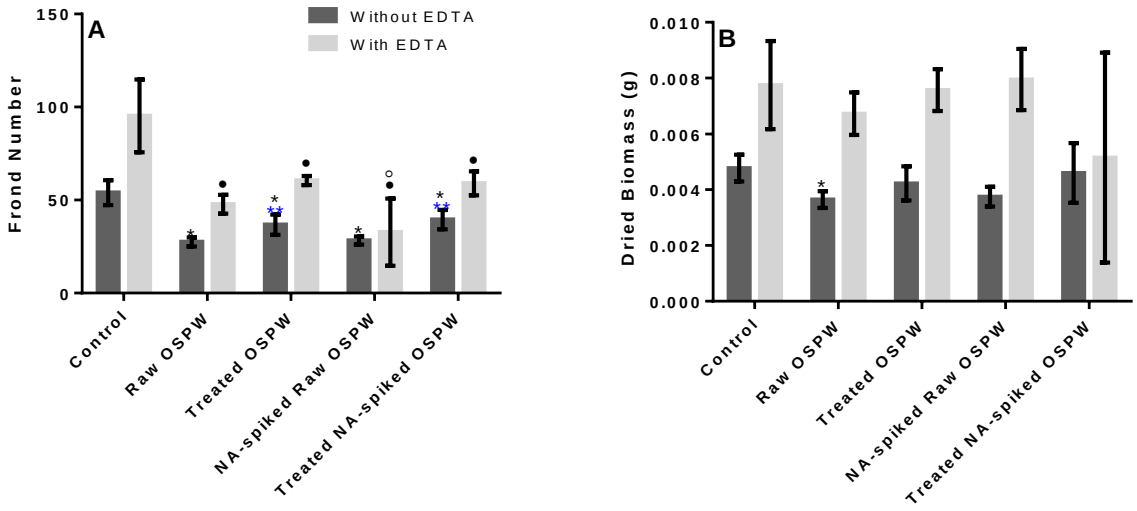

Figure 15. Comparison of the "With EDTA" and "Without EDTA" groups for (A) frond number, and (B) dried biomass. Mean values ± SD (*n* = 4 for test samples; *n* = 4 for control). In the "Without EDTA" group, mean values of OSPW test samples that are significantly different (p < 0.05) from the control are indicated (*), and mean values of other OSPW test samples that are significantly different (p < 0.05) from the raw OSPW are indicated in blue (**). In the "With EDTA" group, mean values of OSPW test samples that are significantly different (p < 0.05) from the control are indicated (●), and mean values of other OSPW test samples that are significantly different (p < 0.05) from the raw OSPW are indicated (o).

The main purpose of including both "With EDTA" and "Without EDTA" treatments in a double bar graph for frond number and dried biomass in Figure 15 is to show that the addition of EDTA into each test condition, including the control, had a significant stimulatory effect on *L. minor*. Figure 15 is separated into Figures 16 and 17 to individually observe the effects of not adding EDTA and adding EDTA, respectively. Figure 16 below shows the 7-day toxicity response of *L. minor* to raw and treated Industry B OSPW using frond number and dried biomass as endpoints. None of the test samples here have EDTA added in order to observe the full effects of heavy metal toxicity on *L. minor*.

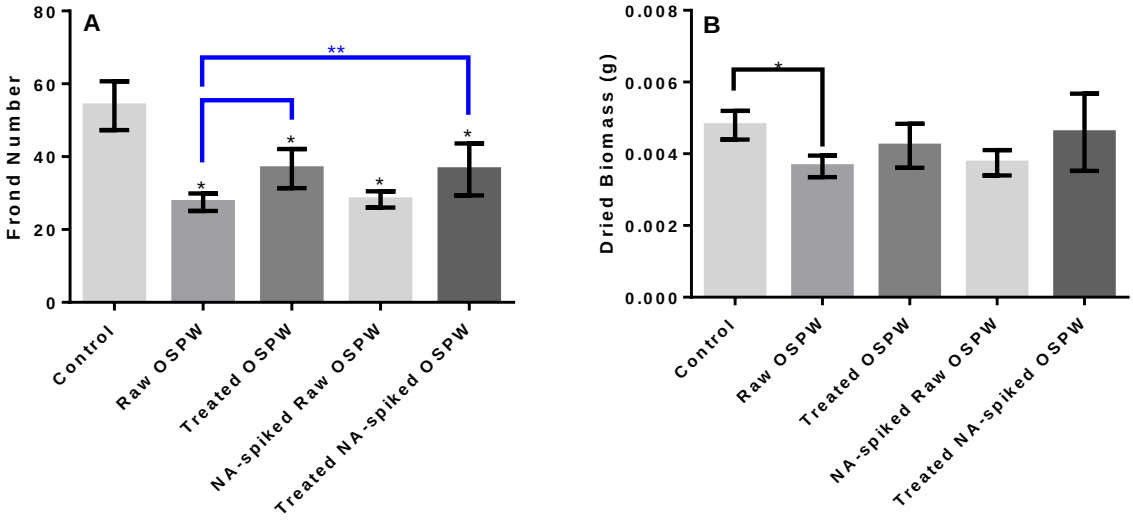

Figure 16. 7-day toxicity response of *L. minor* grown on various Industry B OSPW samples without added EDTA evaluated with frond number, mean values ± SD (*n* = 4 for test samples; *n* = 4 for control). Mean values of other OSPW test samples that are significantly different ($p < 0.05$) from the raw OSPW are indicated in blue (**). (B) 7-day toxicity response of *L. minor* grown on various Industry B OSPW samples without added EDTA evaluated with dried biomass, mean values ± SD (*n* = 4 for test samples; *n* = 4 for control). Mean values from that are significantly different from the control ($p < 0.05$) are indicated (*).

For frond number in Figure 16A, all OSPW samples are significantly different from the control, demonstrating that neither the photocatalytic treatment nor spiking the OSPW with NAs prior to the photocatalytic treatment reduced the toxicity to *L. minor* to the level of the control. However, there is a significant difference when the mean of the raw OSPW is compared to the means of both treated samples. In Figure 16B, the only significant difference found was between the control and the raw OSPW. Here, a photocatalytic treatment could reduce the toxicity of Industry B's OSPW as the treated OSPW was not toxic when compared to the control.

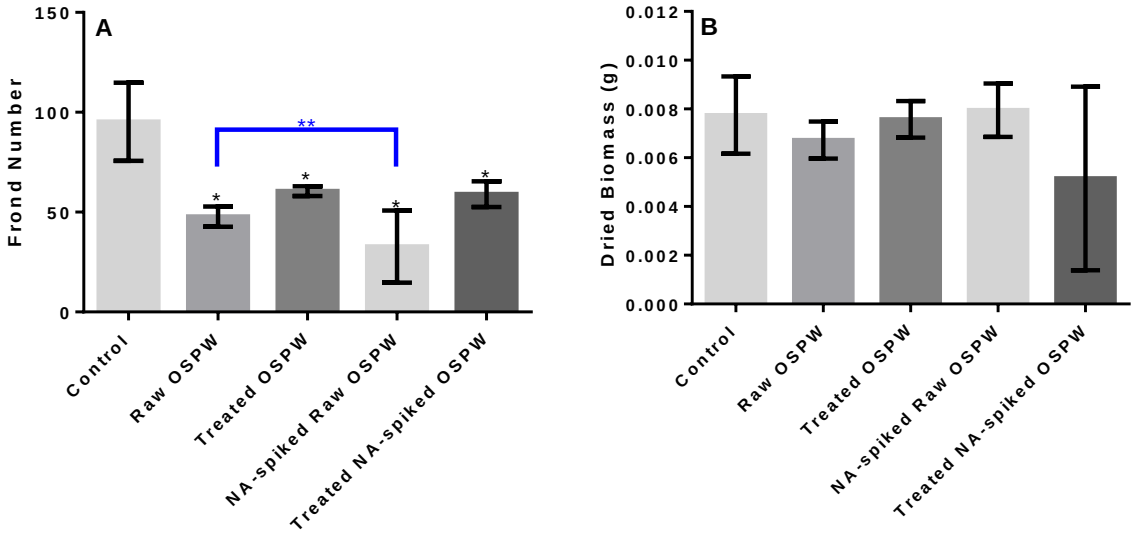

Figure 17. (A) 7-day toxicity response of *L. minor* grown on various Industry B OSPW samples with added EDTA evaluated using frond number, mean values ± SD (*n* = 4 for test samples; *n* = 4 for control). Mean values from that are significantly different from the control (p < 0.05) are indicated (*). Also, within this group of test samples, mean values of other OSPW test samples that are significantly different (p < 0.05) from the raw Industry B OSPW are indicated in blue (**). (B) 7-day toxicity response of *L. minor* grown on various Industry B OSPW samples with added EDTA evaluated using dried biomass, mean values ± SD (*n* = 4 for test samples; *n* = 4 for control).

Figure 16A and 17A demonstrate that while the photocatalytic treatment can reduce some of the Industry B OSPW toxicity in both the with and without EDTA groups, it is incapable of eliminating toxicity to the level of the control for *L. minor*. In Figure 17, it shows that adding EDTA to the raw and treated OSPW samples did not reduce the heavy metal toxicity to *L. minor*. Interestingly, spiking the raw OSPW with NAs and EDTA increased the toxicity to *L. minor*. If the added NAs interacted with the heavy metals and formed an NA-metal complex, the increased toxicity could be due to the NA-metal complex being more toxic than the heavy metals themselves. Another possible explanation for the increased toxicity could be due to NAs from the NA-spike interacting with EDTA so that EDTA cannot chelate the heavy metals present.

In Figure 17B, while there is growth inhibition for the raw OSPW compared to the control, the difference between the means is not significant. The treated OSPW and the NA-spiked raw OSPW both show increased biomass growth compared to the raw OSPW, however this difference between these two sets of means is not significant. These comparisons demonstrate that Industry B's OSPW is considered non-toxic to *L. minor* when dried biomass was used to measure toxicity. While the treated NA-spiked OSPW sample does show a decrease in biomass, this decrease is statistically not significant due to the large error bar.

Figure 18 below shows the interaction plots for NAs and heavy metals in raw Industry B OSPW. Figure 18A and 18B show the interaction plots for frond number and dried biomass, respectively.

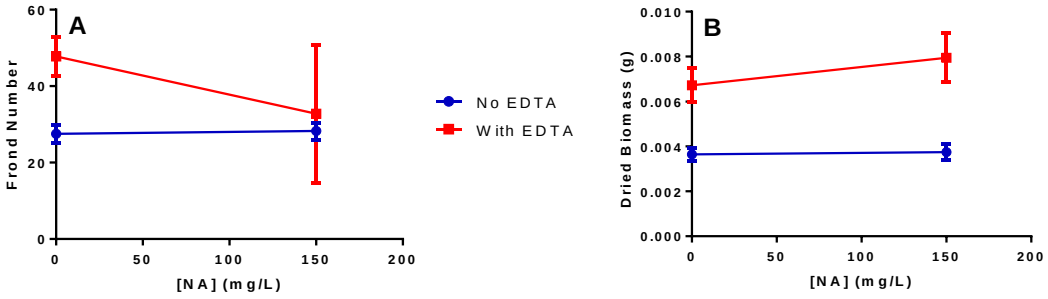

Figure 18. Interaction plots for NAs and heavy metals in raw Industry B OSPW evaluated using (A) frond number and (B) dried biomass

Since the lines intersect in Figure 18A, this plot shows that in raw Industry B OSPW, there is an interaction between the NAs and the heavy metals when frond number is used as the endpoint. In Figure 17B, this interaction is much less in raw Industry B OSPW when dried biomass is used to evaluate toxicity, and the presence of EDTA does not do much other than stimulating the growth of biomass. When comparing the raw OSPW and the NA-spiked raw OSPW in Figure 18, the addition of synthetic NAs into the raw OSPW negated any positive effects from EDTA. Although the addition of EDTA does stimulate *L. minor* growth, it seems that the addition of NAs negated any chelating ability and stimulatory effects of EDTA. It is also possible that this is due to the added NAs and EDTA interacting with each other, hence there is less EDTA available to reduce heavy metal toxicity.

Figure 19 below shows the interaction plots for NAs and heavy metals in treated Industry B OSPW. Figure 19A and 19B show the interaction plots for frond number and dried biomass, respectively.

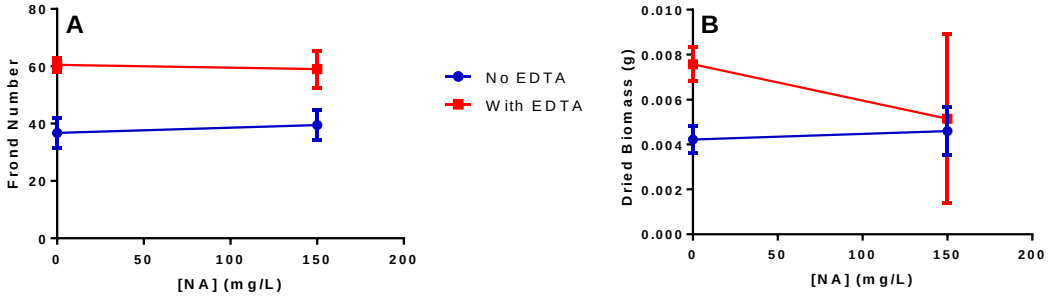

Figure 19. Interaction plots for NAs and heavy metals in treated Industry B OSPW evaluated using (A) frond number and (B) dried biomass

Unlike Figure 18 above, the results in Figure 19 are reversed. This shows that in the treated OSPW, there is very little interaction between the NAs and the heavy metals when frond number is used as the endpoint. However, when dried biomass is used as the endpoint, there is an interaction between the degraded NAs and heavy metals as the lines intersect. While Figure 19 shows the opposite result, the error bar is large for the treated NA-spiked OSPW sample with added EDTA, and therefore it is possible that the large error interfered with the overall observations. A repeat of the toxicity test with this sample can provide better information on whether there is an interaction between NAs and heavy metals in treated OSPW. A possible reason explaining why the addition of EDTA did not reduce the toxicity is that the NA degradation products can compete with EDTA to sequester heavy metals. Since the interaction between DOM and heavy metals can increase its toxicity, there is a portion of heavy metal toxicity that EDTA cannot treat as it is sequestered with DOM. As with Figure 18, it is also possible that an interaction between DOM and EDTA exists, hence there is less free EDTA available to remediate heavy metal toxicity to *L. minor*.

6.4 Environmental Significance

While photocatalysis is capable of completely mineralizing recalcitrant organic compounds in OSPW, it cannot reduce the toxicity to *L. minor* coming from heavy metals. While the results shown above are preliminary, they show that there is some interaction between NAs and their degradation products with heavy metals present in an industrial OSPW sample.

A potential remediation method of treating OSPW is to partially treat it using photocatalysis to lower the organic content and return it to wetlands for further phytoremediation. As photocatalysis cannot

address heavy metal toxicity to *L. minor*, another treatment method must be employed in conjunction with photocatalysis to address the heavy metal toxicity. OSPW with a high heavy metal content will need the addition of metal chelators such as EDTA to reduce their toxicity prior to environmental incorporation. Since it is known that DOM are responsible for cycling heavy metals through the environment, interactions between DOM and heavy metals need to be further studied as DOM in OSPW can change the bioavailability of heavy metals to *L. minor* and other wetland plants.

Chapter 7
Conclusions and Future Research Directions

7.1 Summary

This thesis illustrates new findings in the fields of water treatment, aquatic toxicology, chemical engineering, and nanotechnology. Solar photocatalysis over TiO_2, was found to degrade the organic fraction of OSPW and reduce its toxicity to *Lemna minor*. In an industrial OSPW sample, the heavy metals present caused a toxic stress response in *L. minor*. P25 TiO_2 and the floating photocatalyst used in the treatment process were found to be generally nontoxic to *L. minor*. The IC_{50} of synthetic NAs to *L. minor* was determined and the toxicity of NAs to *L. minor* was found to be dependent on the chosen endpoint. NAs were also demonstrated to interact with OSPW salts and heavy metals and change their bioavailability. Recommendations for future work are given towards improving the *L. minor* toxicity test protocol and focusing on researching the interactions between OSPW components and the effects of these interactions on aquatic toxicity.

7.2 Conclusions

Photocatalysis over TiO_2 was shown to be capable of reducing the organic fraction's toxicity in an industrial OSPW sample. Heavy metals present in the OSPW caused toxicity to *L. minor*, suggesting that a treatment targeting heavy metals must be used in conjunction with photocatalysis in order to further treat the OSPW. Oxidative compounds present in the industrial OSPW sample and those generated via photocatalytic treatment were shown to be a small and statistically insignificant contributor to the toxicity to *L. minor*. Even after addressing the toxicity from the organic, heavy metals, and oxidative compounds fractions, there was still a fraction of toxicity to *L. minor* that was still unaccounted for. This could be due to the components interacting with each other and thus modifying the bioavailability to *L. minor*. The photocatalyst P25 TiO_2 was confirmed with the literature to be generally non-toxic to *L. minor*, and the FPCs used to treat OSPW were also found to be nontoxic to *L. minor* and in fact had a stimulatory effect on *L. minor* growth.

The 7-day IC_{50} for synthetic NAs on *L. minor* differed depending on which endpoint was chosen to evaluate the toxicity. The 7-day IC_{50} for synthetic NAs was determined to be 142.9 mg/L for frond number and 198.1 mg/L for dried biomass. Based on these two endpoints, the concentration of NAs where the toxicity was statistically insignificant to *L. minor* was 60.6 mg/L.

Photocatalysis over TiO_2 was demonstrated to be capable of mineralizing NAs and detoxifying a synthetic OSPW sample containing 150 mg/L NAs, further supporting that the remaining toxicity to *L. minor* in the treated Industry A OSPW was not from NAs or from NA degradation by-products. OSPW salts were shown to interact with NAs and change their toxicity to *L. minor* in a synthetic OSPW sample with 150 mg/L NAs and varying OSPW salt concentrations. NAs were shown to be acutely toxic to *L. minor* when gibbosity was used as the endpoint, and NAs were shown to be chronically toxic when toxicity was evaluated using frond number and dried biomass. This demonstrates that the severity of NA toxicity to *L. minor* is dependent on the endpoint used to evaluate toxicity.

There is an interaction between NAs and their degradation products with heavy metals present in an industrial OSPW sample, but the results shown are preliminary and further experiments are needed to clarify that the interaction exists as well as its extent.

7.3 Recommendations for Future Research

The following research directions are suggested based on the conclusions drawn above to improve the understanding of OSPW aquatic toxicity and further advance proposed OSPW treatment methods:

1) Explore treatments for reducing heavy metal toxicity in OSPW. While photocatalysis could mineralize NAs and thus reduce the NA toxicity to *L. minor* as demonstrated in Chapter 5, it was incapable of lowering the toxicity of heavy metals present in industrial samples as demonstrated in Chapter 3. The addition of the metal chelator EDTA was shown in Chapter 3 and 6 to reduce the heavy metal toxicity, and while this demonstrates that photocatalysis combined with EDTA can be a more effective treatment method, usage of chelators must be carefully considered as some such as EDTA are not easily degraded and can be considered as persistent environmental pollutants[161].

2) Investigate interactions of NAs and heavy metals and the effect on toxicity to aquatic plants. As demonstrated in Chapter 3, there was some remaining toxicity to *L. minor* after the toxicities of organic compounds, heavy metals, and oxidative compounds have been addressed. As DOM is known to be involved in the biogeochemical cycling of trace metals in aquatic environments[150,151], it is possible that NAs and their degradation by-products influence the bioavailability of heavy metals by sequestration. To extend on the work shown in Chapter 6 and study possible interactions between heavy metals and NAs in OSPW, a synthetic OSPW can be prepared where there is a known concentration of NAs and a heavy metal. A photocatalytic treatment can be run to generate a partially-treated OSPW and a fully-treated OSPW, and the toxicity of these can be compared to that of an untreated control to determine whether the presence of NAs or degraded NAs can affect heavy metal toxicity to *L. minor*.

3) Investigate interactions between NAs and OSPW salts and the effect on NA toxicity to aquatic plants. Chapter 5 demonstrates that OSPW salts affect the NA bioavailability to *L. minor*, and NAs in synthetic OSPW with no OSPW salts or a low concentration of OSPW salts were more toxic to *L. minor* than NAs in a synthetic OSPW with the regular concentration of OSPW salts. As OSPW is saline, the salinity must be amended before environmental incorporation, and removal of salinity can affect the bioavailability of any NA-degradation by-products if partial photocatalysis is used to treat OSPW.

4) Evaluate toxicity using additional *L. minor* endpoints to achieve a more thorough understanding of NA toxicity. If *L. minor* is used as an aquatic plant representative of wetland health, the choice of endpoint used to monitor wetland integrity should be carefully considered as the effects of treated OSPW might not be immediately apparent if certain endpoints are chosen. Frond number and dried

biomass are conventionally used in the *L. minor* toxicity test as they are relatively simple to evaluate; however, these two endpoints are not the only qualities indicative of *L. minor* health. Healthy *L. minor* are buoyant, have intact colonies and fronds in variable shades of green, should not show gibbosity and should not have yellow or necrotic fronds and fronds of abnormal size[127]. As shown in Chapter 5, *L. minor* can have frond counts and or dried biomass like that of the control even in high NA concentrations, although these *L. minor* plants were not considered healthy by the other Environment Canada (2007) criteria. Also, the traditional endpoints might not be informative on the acute or chronic toxicity of a pollutant as demonstrated by using gibbosity as the endpoint in addition to frond number and dried biomass.

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Appendix A

10X synthetic salt solution derived from Industry A's OSPW

1. The following chemicals were measured using the Shimadzu AUW120D balance and added to a 250 mL glass jar:
   - Sodium bicarbonate ($NaHCO_3$): 1.2254 g
   - Sodium chloride (NaCl): 2.8700 g
   - Potassium chloride (KCl): 0.0496 g
   - Sodium fluoride (NaF): 0.0155 g
   - Magnesium sulfate ($MgSO_4$): 0.0802 g
   - Calcium sulfate ($CaSO_4$): 0.0652 g
   - Sodium sulfate (Na_2SO_4): 0.6298 g
   - Ammonium chloride (NH_4Cl): 0.0336 g
2. Added 200 mL deionized water (Millipore, >15 MΩ cm^{-1}) to the jar and the jar was shaken to dissolve the salts. This is a 10X synthetic salt solution derived from Industry A's OSPW.
3. To create 300 mL of the 1X synthetic salt solution, add 30 mL of the 10X salt solution to 270 mL deionized water (Millipore, >15 MΩ cm^{-1}).