TOXICITY, MORPHOLOGICAL CHANGES, AND DISSIPATION OF OIL SANDS NAPHTHENIC ACIDS IN *CHLAMYDOMONAS REINHARDTII*

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By

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ABSTRACT

Naphthenic acids fraction components (NAFCs) are a toxic byproduct of oil sands extraction and refining in the Athabasca region of Alberta, Canada, accumulating with other contaminants in the large volumes of oil sands process water produced. NAFCs are incredibly complex mixtures identified as a contaminant of concern across a wide variety of taxa, but the reasons for their acute and chronic toxicity are poorly understood.

Studies were conducted to help ascertain the toxic effects of NAFCs on the unicellular green algae *Chlamydomonas reinhardtii* wild type (WT) and two cell wall mutants: CC-400, which retains the innermost and outermost glycoprotein wall layers, and CC-3395, believed to be completely naked. The presence of the cell wall was strongly linked to NAFC toxicity. WT cells were most susceptible to NAFC toxicity (growth reduced at 10 mgL^{-1}, but growing even at 100 mgL^{-1}), followed distantly by CC-400 (decreased growth at 100 mgL^{-1}) while CC-3395 was unaffected.

Microscopy experiments (visible light, confocal laser scanning, and Fourier-transform infrared) and thin-layer chromatography of lipids were carried out to observe physiological effects. Exposure to NAFCs induced changes in cell surface protein structure and protein confirmations in WT and CC-400, and altered the diversity and composition of their phospholipid and lipid pools. CC-3395 had minor changes in phospholipid/lipid pools. Exposed WT cells showed evidence of decreased uptake of environmental macromolecules and palmelloid induction. Exposed CC-400 cells exhibited loss of phospholipids and showed some evidence of altered internal membrane and protein structures. CC-3395 showed increased active transport/export after exposure. All exposed cultures had an increase in the size and roundness

of cells, as well as increased presence of vacuoles and granules and indicators of osmotic stress or metabolic leakage. These changes were consistent with surfactant exposure, a theory supported by the differences in toxic impacts between cell lines, as surfactant effects vary greatly between similar species and similar compounds.

Studies were also conducted to determine the potential of NAFC biotransformation by *C. reinhardtii*. Changes in NAFC composition were observed, and were highly specific to compound class, structure, and algal strain. WT and CC-400 were capable of removing classical O_2 NAFCs, while modification of O_2S and O_3 composition were mediated by all three algae.

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PREFACE

This thesis contains two general sections, Chapters 1 and 5, which are respectively a general introduction and discussion. Chapters 2, 3, and 4 were prepared as manuscripts for submission to scientific journals, and as such there is some repetition in the introduction, methods, and materials sections. Chapter 2 has been submitted to *Science of the Total Environment*, Chapter 3 is prepared for submission to *Environmental Science and Technology*, and a modified version of Chapter 4 is in preparation for submission to *Planta*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Amide I
AII	Amide II
A750	Spectrophotometric absorption at 750 nanometres
$A750^{T}_{0}$	Spectrophotometric absorption at 750 nanometres at time zero
$A750^{T}_{x}$	Spectrophotometric absorption at 750 nanometres at time point *x*
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
BaF_2	Barium fluoride
°C	Degrees Celsius
$°Cmin^{-1}$	Degrees Celsius per minute
ConA	Concanavalin A
CC-400	*Chlamydomonas reinhardtii* cell wall mutant *cw15* mt+
CC-3395	*Chlamydomonas reinhardtii* cell wall mutant *arg7-8 cwd* mt-
CE	Cholesterol ester
Cl	Chlorine
CLS	Canadian Light Source
CLSM	Confocal laser scanning microscopy
cm^{-1}	Wavenumber
D_2O	Deuterium oxide/heavy water
day^{-1}	(Unitless) rate of growth per day

DB	Double bonds
DBE	Double bond equivalents in naphthenic acid fraction components, representing the number of rings present in a compound plus the number of double bonds to carbon atoms
df	degrees of freedom
DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration (50% affected)
ESI	Electrospray ionization
ESI-LRMS	Low resolution electrospray ionization mass spectrometry
FFA	Free fatty acids
FTIR	Fourier-transform infrared
GC	Gas chomatorgraphy
h	Hour
HCl	Hydrochloric acid
HRMS	High resolution mass spectrometry
IR	Infrared
km^2	Square kilometers
L^{-1}	Per litre
LRMS	Low resolution mass spectrometry
m	Minute
m^3	Cubic metre
mgL^{-1}	Milligrams per litre
mL	Millilitre

mm	Millimetre
mM	Millimoles
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
n	Sample size
n	Number of carbon atoms in the molecular formula for classical naphthenic acids $C_nH_{2n+Z}O_2$
N	Normality
N_2	Nitrogen (gas)
n3	Unsaturated lipid in omega 3 configuration
n6	Unsaturated lipid in omega 6 configuration
NA	Naphthenic acid
NAFC	Naphthenic acid fraction components
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NH_4OH	Ammonium hydroxide
nm	Nanometre
O_2	Classical naphthenic acid species, $C_nH_{2n+z}O_2$
O_2S	Non-classical species of the naphthenic acid fraction components containing O_2S
O_3	Non-classical species of the naphthenic acid fraction components containing O_3

O_4	Non-classical species of the naphthenic acid fraction components containing O_4
OSPW	Oil sands process water
p	p-value (probability)
PAH	Polycyclic aromatic hydrocarbon
pH	The inverse logarithmic representation of the hydrogen proton [H^+] concentration
RCF	Relative centrifugal force
RGB	Red-green-blue
rpm	Rotations per minute
S	Sulfur
s	second(s)
s/n	Signal-to-noise ratio
S9	SYTO 9
SPE	Solid phase extraction
TAG	Triacylglycerol
TAP	Tris-acetate phosphate nutrient media
TLC	Thin-layer chromatography
µg	Microgram
µL	Microlitre
µm	Micrometre, micron
µmol photons $m^{-2}s^{-1}$	Micromol of photons per metre squared per second
v/v	Volume per volume

W1-W7	Cell wall layers 1 through 7 in *Chlamydomonas reinhardtii*
WT	Wild type *Chlamydomonas reinhardtii* $1B^{-}$
w/v	Weight per volume
Z	Number of hydrogen atoms lost due to increased cyclicity in the chemical formula for classical naphthenic acids $C_nH_{2n+Z}O_2$
1-2 DAG	1-2 diacylglycerol
1-3 DAG	1-3 diacylglycerol

CHAPTER 1
1.0 INTRODUCTION

1.1 Background

1.1.1 Athabasca Oil Sands

Extraction and processing of the Athabascan oil sands in northern Alberta is of great economic and environmental interest and concern. Canadian oil sands account for an excess of 95% of globally known in-place oil sands volumes, with the majority located in three regions of northern Alberta: Cold Lake, Peace River, and Athabasca (Chalaturnyk et al., 2002). Combined, these three deposits underlie more than 140,200 km^2 and account for more than 85% of in-place bitumen (Government of Alberta, 2012, accessed April 5th 2012; Greene et al., 2006). Reserves of bitumen are estimated at 1.7 trillion barrels, with 173 billion estimated to be economically recoverable (Energy Resources Conservation Board, 2009). As increased global demand depletes conventional sources of oil, development of unconventional petroleum sources is set to increase. Further, rising gas prices and developing technology increase the proportion of reserves that are economically recoverable. Current operations provide more than half of all Canadian petroleum (Canadian Association of Petroleum Producers, 2011), an almost three-fold increase from 20% in 2001 (Leung et al., 2001).

Oil sands consist of a complex of bitumen -- the most dense and viscous form of petroleum – mixed with water and sand or clay. In order to form the economically relevant product, crude oil, bitumen must be extracted from the oil sands mixture using a modification of the Clarke caustic hot water method, and upgraded using a catalyzed heat and pressure process.

The procedure requires large volumes of water, on the scale of two to four barrels of water for every barrel of oil produced (Government of Alberta, 2009). This oil sands process water (OSPW) is contaminated with a large number of complex and widely varied toxicants, as well as being highly saline and alkaline. Companies in the oil sands industry operate under a zero discharge policy, retaining extraction wastes in on-site tailings ponds, with more than 10^9 m^3 currently accumulated (Han et al., 2009).

Despite these goals, it is expected that some discharge will occur through a mix of leaching, runoff, and other environmental processes. Recent reports indicate that environmental contamination of water systems around the Athabasca oil sands is more extreme than previously thought (Kelly et al., 2009). Even where a true zero-discharge state was obtained, sites would require eventual reclamation. As such, it is essential to obtain an understanding of the nature and toxic behaviours of oil sands process water, as well as the ways in which it may be remediated.

1.1.2 Tailings Ponds

Tailings ponds contain OSPW, a complex mixture of residual bitumen, fine sediments (silts and clays), ions, and various organic and inorganic products. Of greatest concern are high levels of salts, and dissolved organics (Energy Resources Conservation Board, 2009). This process water has been found to be chronically and acutely toxic, with reports of toxicity to rainbow trout (*Oncorhynchus mykiss*) during initial development of the area (Allen, 2008); all fathead minnows (*Pimephales promelas*) exposed to fresh tailing tailings pond water died within 48 hours (Lai et al., 1996), and reproduction and development were impaired even in aged OSPW (Kavanagh et al., 2011). Later fish work has indicated histopathological changes in the gills and livers of yellow perch (*Perca flavescens*) and gold fish (*Carassius auratus*) (Nero et al., 2006) and gill lesions and increased disease in yellow perch (van den Heuvel et al., 2000). In

constructed and natural wetlands, OSPW reduces seed germination and plant species diversity, as well as impacting recruitment and colonization of aquatic and terrestrial plants (Crowe et al., 2002), decreases or prevents development of toad (*Bufo boreas*) and frog (*Rana sylvatica*) tadpoles (Pollet and Bendell-Young, 2000), and has been found to elevate thyroid hormone levels in tree swallow nestlings (*Tachycineta bicolor*) (Gentes et al., 2007). However, other studies have found no impact on tree swallow reproductive success, immune function, or nestling growth rate (Smits et al., 2000).

1.2 Naphthenic Acids

1.2.1 Structure and Toxicity

The complex mixtures of OSPW contain numerous compounds that have been identified as of particular interest, including salinity, pH, and dissolved organics (Energy Resources Conservation Board, 2009); of these, naphthenic acids (NAs) have been identified as a contaminant class of concern (Clemente and Fedorak, 2005; Han et al., 2009; Headley and McMartin, 2004). Tailings pond waters contain NAs in average concentrations of 40-120 mgL^{-1}, (Holowenko et al., 2002; Quagraine et al., 2005) and they have been found to have adverse effects on various organisms including algae, microorganisms, invertebrates, fish, and mammals (summarized in Quagraine et al., 2005). These compounds form a poorly understood complex mixture of alkyl-substituted mono- , poly-, and a-cyclic carboxylic acids that have the general chemical formula $C_nH_{2n+Z}O_2$, where the carbon number is *n* and ring number is a zero or a negative integer represented by *Z* (Figure 1.1). Numerous possible isomers exist for each combination of *n* and *Z* allowing for an incredible array of compounds (reviewed in Headley et al., 2009b).

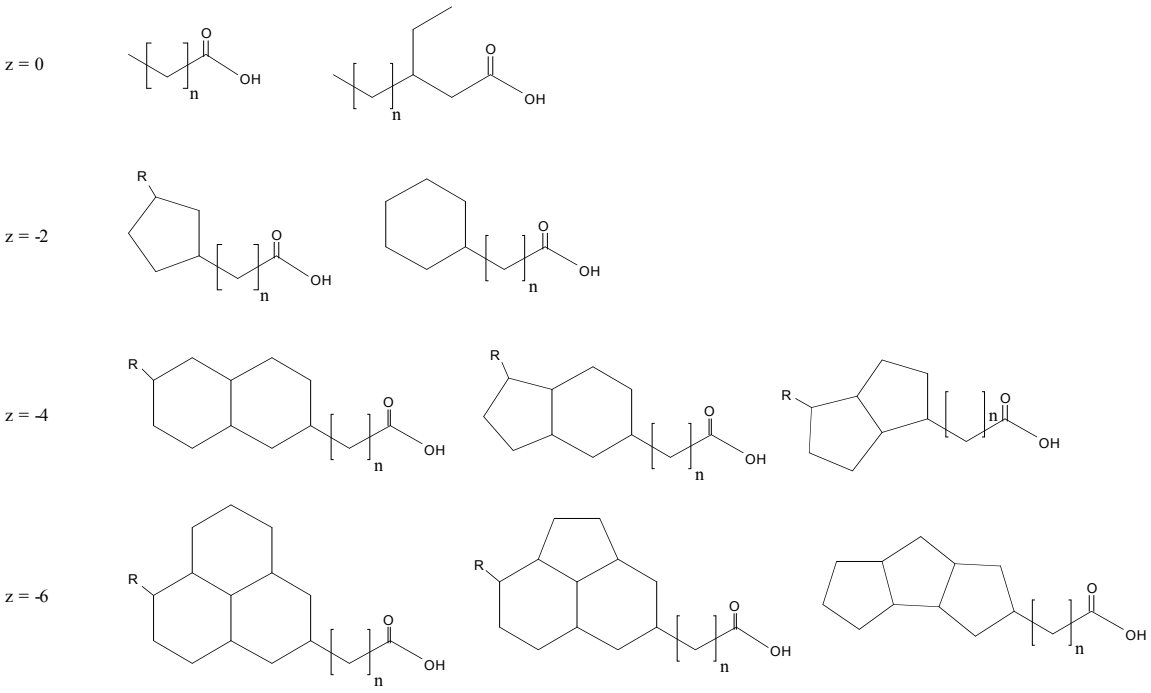

Figure 1.0**:** Theoretical representative structures for classical NAs (O_2 species) grouped according to *z*-series. The *z*-series represents the number of hydrogen atoms lost with increased cyclicity; *n* represents the number of carbon atoms. Adapted after Headley et al. (2007).

Studies have isolated NAs and their sodium naphthenate salts as possible primary agents of toxicity (Boerger et al., 1986; Dokholyan and Magomedov, 1983; Frank et al., 2008). Further work has been done on their toxicity, both as isolated compounds and as complex mixtures, using both NAs extracted from OSPW and model compounds of classical NAs. Their toxicity is poorly understood, but their amphipathic nature suggests the possibility of surfactant toxicity: surfactants are compounds that contain both hydrophilic and hydrophobic groups, making them capable of lowering surface tensions and disrupting water-air and water-lipid interfaces, and suggesting interactions with cell membranes as a possible basis for acute toxic response (Clemente and Fedorak, 2005; Frank et al., 2009; Rogers et al., 2002a; Rogers et al., 2002b). Classical NAs are anionic surfactants; the impact of environmental factors and co-toxins on anionic surfactant toxicity is difficult to predict and varies based on individual mixtures, as well

as the exposed species of interest. Anionic surfactants may or may not act synergistically with metals to increase toxicity; one of the most consistent trends noted, however, is that mixtures of oil and surfactants are consistently more toxic than predicted by individual mixture components (Lewis, 1992). Only a few studies have been done on the impact of salinity on surfactant toxicity, but they indicate increasing toxicity with increasing salinity (Lewis, 1992). Though this is also of interest, given the high salinity of OSPW – and increased gill histopathology with the addition of $1gL^{-1}$ of salt (Nero et al., 2006). It is important again to note that surfactant toxicity varies greatly depending upon the individual compound, and NAs are a highly complex mixture.

In addition, there is growing evidence that the acid extractable fraction referred to as oil sands naphthenic acids contains a variety of non-classical naphthenic acids in addition to classical NAs and other compounds (Headley et al., 2011b; Headley et al., 2011c), and is perhaps more correctly referred to as oil sands naphthenic acid fraction components (NAFCs) to distinguish it from purely classical or commercial NAs. While both NAs and NAFCs induce toxic response, there is evidence of variability in their toxicity. Both induce abnormal embryonic development in yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*), but exposure to NAFCs resulted in a greater response than equivalent levels of NAs (Peters et al., 2007). Both cause modification of immune response and gene expression in mice, but the timing of the impact, as well as the pathways and genes altered differed between NAs and NAFCs (Garcia-Garcia et al., 2011). Work with emergent macrophytes indicated lower toxicity from NAFCs than NAs, as well little evidence for the uptake of NAFCs or NAs (Armstrong et al., 2008). The broader body of work thus indicates an inconsistent variation in response to NAs and NAFCs. Worst-case exposure to NAFCs in small mammals is not predicted to cause acute toxicity, but repeated exposure may cause stress and adverse health effects (Rogers et al.,

2002b). In wetlands receiving OSPW, NAFCs have been shown to be the driving factor altering microbial community structure and decreasing population diversity (Hadwin et al., 2006). Similar effects have been shown with phytoplankton communities, though increased salinity was also correlated (Leung et al., 2001, 2003). As a whole, these differences suggest that while commercial NA mixtures may be much better characterized than NAFCs, the results obtained with model compounds cannot be extrapolated to environmental effects.

Research has indicated that constituents of NAFC mixtures exhibit differential toxicity; Jones et al. (2011) determined the toxicity of 35 NAFC constituents on the gram negative bacteria *Vibrio fischeri*, with EC_{50}s ranging from 0.7 mM down to 0.004 mM. There are trends delineating toxicity – compounds with a lower molecular weight, lower proportion of multi-ring structures, and lower carboxylic acid content tend to exhibit greater toxicity (Frank et al., 2009; Lo et al., 2006). Overall, while there are many suppositions and possible explanations for NAFCs toxicity, the mechanisms are largely unknown.

1.2.2 Degradation

Field-based studies have noted decreased toxicity of OSPW as tailings pond water ages (Holowenko et al., 2002). The decreased toxicity of this weathered process water can be attributed at least in part to microbial degradation of NAFCs (Han et al., 2009; Herman et al., 1994); compounds which exhibit lower toxicity (low MW, low proportion of multi-ring structures, lower carboxylic acid content) are more easily degraded, while those recalcitrant to degradation (higher MW, higher proportion of multi-ring structures, increased branching, methyl substantiations) are more likely to exhibit lower toxicity (Herman et al., 1994; Lo et al., 2006). The presence of natural degradative pathways suggests the possibility of bioremediation as a tool to reclaim and detoxify waters, despite demonstrated acute and chronic toxicity to algal and

microbial communities. NAFCs are also more recalcitrant to bioremediation than NAs (Scott et al., 2005), and are less sequestered by some emergent macrophytes (Armstrong et al., 2008).

Due to weathering of oil sands-containing formations and soils, NAs are naturally present in surface waters of the area in concentrations of 1 – 2 mgL^{-1}, which suggests the possibility for community and species level adaptation over thousands of years (Leung et al., 2003). NAs and NAFCs are not the only contaminants in tailing water, however, and the effects of other contaminants (including salinity, metals, and other organic and inorganic compounds) must be accounted for. In addition, the bitumen released from upgrading facilities may naturally be weathered into NAs and other recalcitrant organic acids by the same organisms (Quagraine et al., 2005).

Despite these challenges, in situ biodegradation of contaminated waters remains a remediation technique of interest. Biodegradation has been explored in relation to oil spills (Macnaughton et al., 1999), petroleum contamination (Kirk et al., 2005), and crude oil (Whyte et al., 1996), and there is a growing body of work on its applications to tailing ponds (Clemente and Fedorak, 2005; Quagraine et al., 2005; Quesnel et al., 2011). *In situ* degradation of OSPW offers a convenient and important remediation tool if its efficacy can be shown and the mechanisms behind it elucidated.

1.3 Bioremediation

1.3.1 Background and Current Work

Microbial degradation is dependent upon growth and metabolism of cultures, which are impacted by a variety of geoenvironmental factors including temperature, dissolved oxygen levels, salinity, pH, redox potential, hardness of water, and composition of sediments (Conrad Environmental Aquatics Technical Advisory Group (CEATAG), 1998). Nutrients such as nitrogen or phosphorous may be limiting to microbial and algal communities involved in

bioremediation, as they are in fresh bodies of water (Herman et al., 1993). Salinity effects have been shown to alter the structure of phytoplankton communities (Leung et al., 2003) and may work to increase toxic effects of NAs by increasing osmotic stress (Quagraine et al., 2005). Other constituents of oil sands process water, such as bitumen, volatile organic compounds, ammonia, total dissolved solids, and trace metals cause complex environmental stress (Allen, 2008). The chemical structure of the compound to be degraded, as well as the species transforming it, both contribute to preferential break down of NAs (Herman et al., 1993). Contaminant fractions with larger structures, increased ring numbers and branching, or methyl substitutions have been found to be more persistent (Herman et al., 1993; Lo et al., 2006).

Bioremediation approaches may be gathered under three umbrellas:

1) Biostimulation: Viable native populations exist but require environmental alterations;
2) Bioaugmentation: Enhancement of native environments with suitable species; or
3) Intrinsic treatment: No large scale modifications are made to the community or the environment (King et al., 1992).

Most research to date on biotransformation of oil sands NAFCs has focused on the use of microbial populations or constructed wetlands. A number of bacterial groups have been found to occur naturally in tailings ponds, or have been isolated from enriched tailing waters, some of which degrade NAFCs and their related products (Quagraine et al., 2005). These tests have been conducted in water phase only, and often with isolated culture strains, neglecting substrate interactions and community effects. The use of constructed wetlands as bioreactors has also generated interest. Studies have involved degradation by emergent macrophytes such as cattails, reed grass, and bulrush, in addition to their associated microrhizal communities. However, it should be noted that these lab studies have focused on degradation by isolated species and

showed selective uptake and transformation by different species (Armstrong, 2008; Armstrong et al., 2008).

1.3.2 Algal Bioremediation

There is an extensive body of work centering about the biodegradation and bioremediation of petroleum hydrocarbons. These studies – and in particular those of NA bioremediation – have focused in large part on the role of bacteria and fungi (Quagraine et al., 2005). While some algal studies exist (Headley et al., 2008; Quesnel et al., 2011), published literature principally focuses on changes in community composition caused by exposure to NAs, NAFCs, and OSPW (Leung et al., 2001, 2003). These same studies have however identified a number of tolerant algal strains, as summarized in Table 1.1.

Table 1.1: Summary of algae found in oil sands tailings ponds by Leung et al. (2001, 2003). Species associated with greater than 20 mgL^{-1} NAs are indicated by **, those associated with 10 – 20 mgL^{-1} by *.

Green Algae	**Dinophytes**	**Golden Algae**
Botryococcus braunii **	*Ceratium hirundinella* **	*Chromulina* spp. **
Chlamydomonas frigida *	*Glenodinium* spp. **	*Chrysococcus rufescens* **
Chlorella spp. **	*Gymnodinium* spp. **	*Mallomonas* spp. **
Coccomyxa minor **	*Peridinium cinctum* *	*Ochromonas* spp. **
Cosmarium depressum **		
Gloeococcus schroeteri **	**Diatoms**	**Blue-Green Algae**
Gyromitus spp. **	*Diatoma vulgare* *	*Aphanizomenon* spp. *
Keratococcus spp. **	*Navicula cuspidata* **	*Merismopedia* spp. **
Lobomonas rostrata *	*Navicula radiosa* **	*Microcystics* spp. **
Monoraphidium convulutium *	*Nitzschia dissipata* *	
Oocystis crassa **	*Nitzschia linearis* *	**Other Groups**
Oocystis spp. **		*Euglena acus* **
Scourfieldia spp. *		*Rhodomonas lacustrus* *

The presence of tolerant species is the requisite first step for investigation of algal-mediated remediation. One study by Headley et al. (2008) has looked at the degradation of model and oil sands NAs by twelve species of diatoms, blue-green, and green algae in axenic

culture. It reported some transformation of model compounds by two species of diatoms, and the possibility of minor degradation of oil sands NAs by one species of green algae. Quesnel et al. (2011) identified the unicellular green algae *Dunaliella tertiolecta* as a degrader of model NAs. These results are not as dramatic as observed reductions mediated by bacteria or emergent macrophytes; they may however be taken as preliminary work regarding the interaction of a limited number of species with a limited number of compounds over a 14 day period. Algal species present in tailings ponds have had a large number of generations over which to adapt to their conditions and NAFCs as a carbon source.

Algae have the possibility to become an important part of a biodegradation program. Emergent macrophytes are restricted to the littoral zones, while algae occupy the entire epilimnion of a body of water. Photosynthetic reactions intrinsically release oxygen into water, aerating it and promoting aerobic environments. This suggests the possibility of synergistic environmental interactions for the degradation of NAFCs and other oil sands byproducts. In order to utilize both algal biodegradation and community level effects, however, a greater understanding must be reached of the interaction of algal communities and oil sands process water.

1.4 Algae

1.4.1 Algal Selection

One of the algal species identified in OSPW is *Chlamydomonas frigida*, having been found in association with NAFC concentrations of 10 – 20 mgL^{-1} (Leung et al., 2003). While there is little information in the literature regarding this species, the genus is well-understood due to *C. reinhardtii*'s frequent use as a model organism. The genome has been fully sequenced (Merchant et al., 2007), and it is commonly used to study photosynthesis, movement, and response to light

and nutrition. In addition, they may be grown either phototrophically or heterotrophically, if provided with a complete nutrient media; their metabolism and required handling conditions are well characterized; and a wide variety of mutants are available (Harris, 2001). These factors make them ideal for the examination of NAFC toxicity, as the well-defined growth conditions and responses allows the removal of otherwise confounded variables. The availability of *C. reinhardtii* mutants facilitates the isolation of potential modes of toxic interaction.

The tolerance of *Chlamydomonas* spp. to NAs and NAFCs and their potential for biodegradation has not been examined. While work has not been done with regards to OSPW, species of Chlamydomonas have exhibited tolerance to many of the conditions in OSPW, including variable pH. *C. reinhardtii* is frequently a native inhabitant of acid mine tailings containing high amounts of metals (Das et al., 2009). In a 13 year study of acid mine drainage into a Canadian Shield lake, *Chlamydomonas* spp. were one of only two genera to be found in both the contaminated and control lakes, even as metal and acid pollution continued and water pH dropped to 3 (Kalin et al., 2006). Spain's River of Fire, with a pH of 2 and heavy metal contamination, possesses species of *Chlamydomonas* that remain extremely closely related to neighbouring, neutrophillic species (Amarak Zettler et al., 2002). A marine *Chlamydomonas* spp. has been found to grow at a pH of 10 (Sogaard et al., 2011).

C. reinhardtii in axenic culture has also been found to exhibit tolerance to and degradation of a variety of PAHs, cyclic, and substituted-cyclic compounds, including: phenol and catechol (Ellis, 1977), pyrene (Lei et al., 2002), and 2-amino-4-nitrophenol (Hirooka et al., 2006). While it has been found to be ineffective in the breakdown of benzo[a]pyrene (Warshawsky et al., 1995), it does degrade benzoic compounds substituted with N, S, and Cl (Gutenkauf et al., 1998). While these compounds are not naphthenic acids, they illustrate the potential of *Chlamydomonas*

spp. in the biodegradation of cyclic and substituted compounds such as the classical structures of NAs and NAFCs.

1.4.2 Mutant Selection

Wild type *C. reinhardtii* (WT [1B$^-$]) is a back crossed wild type strain (descended from lines obtained from Jean-David Rochaix, Department of Molecular Biology, University of Geneva). Two cell wall mutants, CC-400 (*cw15* mt+) and CC-3395 (*arg7-8 cwd* mt-) were obtained from the Chlamydomonas Genetics Center (University of Minnesota). The glycoprotein cell wall of *C. reinhardtii* is a likely route of toxic action as it moderates environmental influences. Previous studies of toxicity modification induced by cell wall loss include tolerance to hydrodynamic stress (Barbosa et al., 2003), ionic liquids (Sena et al., 2010), cancer drugs (Maucourt et al., 2002), and metals and heavy metals (Macfie et al., 1994; Macfie and Welbourn, 2000; Prasad et al., 1998). These studies have indicated that the wild-type, walled strain is more tolerant of toxic insult, excepting some ionic liquids used by Sena et al. (2010).

The seven-layered cell wall of *C. reinhardtii* is composed of hydroxyproline-rich glycoproteins rich in galactose, arabinose, mannose, and glucose (Harris, 2009, pgs 29-33). There is extensive O-glycosylation in their sugar bonds; O-glycosidic linkages in short-branched and short linear chain oligosaccharides and in polyprotein II confirmation of hydroxyproline sequences are stable only when glycosylated (Bollig et al., 2007; O'Neill and Roberts, 1981; Vallon and Wollman, 1995). In addition, the glycoprotein cell walls contain a wide variety of charged residues known to bind numerous compounds such as metals like cadmium, copper, cobalt, and nickel (Macfie et al., 1994) and act as a barrier to intracellular transport (Azencott et al., 2007).

Both cell wall mutants lack fully assembled cell walls; a full complement of cell wall precursor proteins is synthesized but fails to assemble, with unassembled wall precursor glycoproteins excreted into the media (Voigt et al., 1991). The mutant CC-400 is known to retain a rudimentary cell wall. Of the seven wall layers (W1-W7), it lacks the inner triplet of W2-W4-W6 and the accompanying intracellular space layers W3-W5. The innermost layer, W1, is present, as is the external loose fibrous network of W7 (Harris, 2009, pgs 38-39). For CC-3395, a breakdown of residual wall components is unavailable; it may however be suggested that any wall remnants present are more rudimentary than that of CC-400. When examined with Fourier Transform Infrared spectromicroscopy (FTIR) resonant Mie-type scattering – a spectral artefact whose intensity has been linked to the presence of cell walls in *C. reinhardtii* (Svensen et al., 2007) – was least pronounced for CC-3395 cells, despite their high degree of roundness (Goff, unpublished data). In addition, visual observation of both mutant lines indicated much greater variation in the size and shape of CC-400 cells (Figure 2.2, Figure 4.8). Cells of CC-3395 cultures were overwhelmingly round, while cultures of CC-400 included round, ovaloid, oblong, sickle-shaped, and irregularly-shaped cells. This suggests that the presence of cell wall remnants W1 and W7 may help retain and induce such variation in size and shape, much as the full cell compliment retains the ovaloid shape of wild type cells.

1.5. Methodologies

1.5.1. Algal Growth

Axenic algal cultures were grown heterotrophically in liquid Tris-Acetate-Phosphate (TAP) media supplemented with arginine (Gorman and Levine, 1965; Harris, 1989) under uniform light conditions of 100 μmol photons m^{-2} s^{-1}. Experiments were carried out in sterilized Erlenmeyer flasks on a mechanical shaker. For growth and exposure trials, a known

concentration of algal cells was added to fresh media, or fresh media spiked with NAFCs. Initial cell concentration was determined by flow cytometry. For growth trials, subsequent culture densities were measured using spectrophotometric absorbance at 750 nanometres (nm).

1.5.2 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) allowed the visualization of modification of many biophysical parameters of living cells and biofilms (Neu et al., 2001; Neu et al., 2004). Here, it was used to investigate NAFC-induced modification in living cells of *C. reinhardtii*. Fluorescently labelled probes were used to investigate distribution and modification of polysaccharides (sugars, glycoproteins, and exopolymer matrix), nucleic acids (nucleus and mitochondria), neutral lipids, proteins, and cellular membranes (Lawrence et al., 2007; Lawrence et al., 2003). Chlorophyll autofluorescence was used to image the chloroplast. Images were collected in three channels (RGB) consisting of a chlorophyll autofluorescence (false-coloured blue), and one to two channels of fluorescently labelled dyes.

1.5.3 Analytical Lipid Analysis

Further analysis of lipid modification was done by thin layer chromatography. This allowed quantification of neutral lipid and phospholipid classes and amounts, and therefore analysis of changes induced in general lipid pools and to the cell membranes by exposure to NAFCs. Exposure to a variety of toxicants and stressors, including copper and cadmium (Visviki and Rachlin, 1994), osmotic stress (Guschina and Harwood, 2006), CO2 exposure (Thompson, 1996), and nutrient deprivation (Dean et al., 2008; Grossman, 2000; Heraud et al., 2005) can induce changes in fatty acid amounts and compositions; such changes in lipid metabolism in response to the environment has been said to be key to adaptation to acute and chronic stress (Thompson, 1996).

1.5.4 Mass Spectrometry

Due to the complex nature of OSPW, NAs, and NAFCs, identification of biologically-mediated NAFC removal or modification is a challenging proposition. Clear analysis of initial NAFC composition is an essential prerequisite to this process, leading to difficulties overlapping those of baseline NAFC characterization, though the same precision is not required as for NAFC fingerprinting. Mass spectrometry (MS) has emerged as an important tool in the study of the fate and characterization of NAFCs in an environmental context (Quagraine et al., 2005).

Soft ionization methods such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are of interest, as they do not require sample derivitization and yield spectra with rich information on the molecular ions (Headley et al., 2009b). Both yield good sensitivity, (Headley et al., 2009b; Hsu et al., 2000). In addition, adduct formation observed under APCI conditions was not noted with ESI techniques (Headley et al., 2002). As such, ESI MS emerged as the technique of choice for analysis of environmental samples (Headley et al., 2009b). However, given increased awareness of the degree of complexity of the NAFC mixture, a trend has developed toward the use of complimentary ionization techniques to obtain a more complete picture of the mixture (pers. com. J. Headley, 2012).

Low resolution ESI MS (ESI-LRMS) was used to examine the concentration of NAFCs in algal growth media after five days exposure. A cleanup (Armstrong et al., 2008) was used to reduce ion suppression caused by high salt content in algal growth media. Paired studies of high and low resolution mass spectrometry (HRMS and LRMS) have yielded similar results in studies of commercial NA mixtures; however, measurement of complex combinations of oil sands process water showed substantial differences, with LRMS resulting in numerous misclassifications and false-positives for NAs in mixture (Martin et al., 2008). No significant changes were noted using LRMS, but results were complicated by algal organic acids excreted to

the media that fell within the NA envelope measured by ESI-LRMS, time-dependent concentration of NAFCs in exposure and control samples, and the possibility of interconversion or biomodification of NAFC compounds rather than their complete removal. As such, complimentary HRMS was carried out to further characterize the mixture composition of algal-exposed NAFC.

1.5.5 Fourier Transform Infrared Spectromicroscopy

Optical microscopy has proven to be an essential approach for *in vivo* studies of single cells, allowing subcellular imaging; when coupled with appropriate molecular biology or staining techniques it can provide spatio-temporal resolution of individual biomolecules (Johnsson, 2009; Xie et al., 2008). These techniques, however, are limited to compounds and molecules for which labelling is available (Levi and Gratton, 2007), and studies of low molecular weight compounds which are difficult to label, or metabolism, tend to rely on bulk samples or sample preparation techniques which kill cells (Borland et al., 2008; Turner et al., 2008). Vibrational spectromicroscopy techniques offer a variety of advantages, and amongst these techniques Fourier-Transform Infrared (FTIR) spectromicroscopy is of particular interest due to its sensitivity, broad applicability, and ease of implementation (Levin and Bhargava, 2005). The replacement of the traditional globar light source with synchrotron infrared radiation eases some of the limitations of the technique due to increased brightness and increased throughput at diffraction limited spatial resolution, such as the size scale of individual cells; this has been used to obtain high signal-to-noise (s/n) measurements of individual biomolecules with subcellular resolution (Dumas et al., 2000; Holman and Martin, 2006).

Molecular vibrations absorb IR light at specific wavelengths, allowing investigation of biomolecules via the resultant spectra; previous studies have identified classes of biomolecules,

such as proteins and lipids, as well as mapped their intracellular distribution (Heraud et al., 2005; Jamin et al., 1998) and followed conformational changes over time (Birarda et al., 2010; Chen et al., 2001; Holman et al., 2000) or accumulation/depletion of metabolic products (Goff et al., 2010; Goff et al., 2009; Heraud et al., 2005).

Previous work (Dean et al., 2007; Goff et al., 2009; Palmucci et al., 2011) has established FTIR as a tool to study *in vivo* algal metabolism, as well as fundamental differences in physical structure. Here it was used to study NAFC-induced changes in gross morphological structure and cellular-level macromolecule pools of individual living cells of *C. reinhardtii*.

1.6 Research Objectives

The overall objective of this research was to investigate the toxicity of NAFCs to *C. reinhardtii* and determine if there was evidence for their biodegradation. It was hypothesized that toxicity of NAFCs would increase in a dose-dependent manner, that the cell wall would be involved in mediation of toxicity, and that any cell wall/NAFC interactions could help remove toxic compounds from the mixture. To this end, the following objectives were identified:

1. Determination of NAFC toxicity to wild-type *C. reinhardtii*
2. Determination of NAFC toxicity to cell wall mutants of *C. reinhardtii*
3. Investigation of the physiological response of wild-type *C. reinhardtii* and two cell wall mutants to NAFC exposure
4. Assessment of possible biotransformation, bioaccumulation, or biological removal of NAFCs by wild-type or cell wall mutants of *C. reinhardtii*.

CHAPTER 2
2.0 ASSESSMENT OF THE EFFECTS OF OIL SANDS NAPHTHENIC ACIDS ON THE GROWTH AND MORPHOLOGY OF *CHLAMYDOMONAS REINHARDTII* USING MICROSCOPIC AND SPECTROMICROSCOPIC TECHNIQUES

2.1 Introduction

Canada's Athabasca oil sands currently provide more than half of all Canadian crude oil (Canadian Association of Petroleum Producers, 2011), results in the production of huge volumes oil sands process water (OSPW) contaminated with residual bitumen, fine sediments, ions, and a variety of organic and inorganic compounds; the totality of which has been found to be acutely and chronically toxic, as summarized by Allen (2008). Of particular concern are salinity levels, pH, and dissolved organics (Energy Resources Conservation Board, 2009), with oil sands naphthenic acids (NAs) having been identified as a contaminant of concern (Clemente and Fedorak, 2005; Han et al., 2009; Headley and McMartin, 2004) and suggested as primary agents of toxicity, with toxic effects on various organisms including algae, protozoa, bacteria, invertebrates, fish, birds, and mammals (Frank et al., 2008; Quagraine et al., 2005). These compounds form a poorly understood complex mixture of alkyl-substituted mono- and poly-cyclic carboxylic acids that have the general chemical formula $C_nH_{2n+Z}O_2$. In addition, there is growing evidence that the acid extractable fraction referred to as oil sands naphthenic acids contains a variety of non-classical naphthenic acids in addition to other compounds (Headley et al., 2011b; Headley et al., 2011c); the complex mixture extracted from OSPW may be more properly referred to as naphthenic acid fraction components (NAFCs).

The amphipathic nature of classical NAs suggests the possibility of surfactant interactions with cell membranes as a possible basis for some acute toxic responses (Clemente

and Fedorak, 2005; Lewis, 1991; Quagraine et al., 2005), but the mechanisms of NAFC toxicity are poorly understood. It is important that we develop a better understanding of the toxic effects of the naphthenic acids found in tailings ponds and the mechanisms by which they interact with cells.

A number of algal and bacterial species have been identified as being tolerant to NAs (Leung et al., 2001, 2003; Quesnel et al., 2011). Amongst these is *Chlamydomonas frigida*, a green alga which was found to grow when exposed to NAFCs at up to 10-20 mgL^{-1} (Leung et al., 2003). *Chlamydomonas reinhardtii* is a commonly used model laboratory organism. Its genome has been sequenced, its metabolism is extremely well characterized, various mutants are available, and it is known to be tolerant to wide variations in pH, metal stress, and salinity, in addition to being a degrader of a variety of cyclic and cyclic-substituted compounds (Ellis, 1977; Gutenkauf et al., 1998; Lei et al., 2002). In this study, we use *C. reinhardtii* as a model organism to study the effects of oil sands naphthenic acids extracted from Athabasca region tailing ponds, through examination of growth rate, changes in growth form, and composition through imaging with visual, confocal laser scanning microscopy (CLSM), and Fourier-Transform infrared (FTIR) spectromicroscopic methods.

2.2 Methods and Materials

2.2.1 Algal Exposures

Wild type *C. reinhardtii* (WT [$1B^{-}$]) is a back crossed wild type strain (descended from lines obtained from Jean-David Rochaix, Department of Molecular Biology, University of Geneva). The cell wall mutants CC-400 (*cw15* mt+) and CC-3395 (*arg7-8 cwd* mt-) were obtained from the Chlamydomonas Resource Center (University of Minnesota). Both lack fully

assembled cell walls and are known to excrete unassembled wall glycoproteins into the media (Voigt et al., 1997; Voigt et al., 1991). Cultures were grown heterotrophically in liquid Tris-Acetate-Phosphate nutrient medium (TAP) supplemented with arginine (Harris, 1989). Experiments were carried out in sterilized Erlenmeyer flasks on a mechanical shaker under uniform light conditions of 100 μmol photons m^{-2} s^{-1}. For growth trials, $10x10^{10}$ cells were added to TAP spiked with NAFCs at 0, 10, 20, 50, and 100 mgL^{-1} in a total volume of 100 mL. The compounds referred to hereafter as NAFCs are the acid-extractable fraction of tailings pond water as per Armstrong et al. (2008); NA concentration of the extract was obtained through negative ion electrospray mass spectrometry. Cells for visual, CLSM, and FTIR microscopy underwent 24 hour exposure to 100 mgL^{-1} NAFCs at a cell concentration of $20x10^{10}$ L^{-1}, with controls consisting of an equal number of cells transferred to an equal volume of fresh media and examined at the same time point.

2.2.2 Growth Trials

Culture density was measured every 8 hours for 4 days via spectrophotometric absorbance at 750 nm (A750) using a Genesys*20 spectrophotometer (Fisher Scientific Company, Ottawa, Canada). Rate of growth during exponential phase was obtained from the slope of the linear portion of the graph of $\ln(A750^{T}_{0}-A750^{T}_{x})$ versus time. Three sets of exposures were carried out for all algae. Each set consisted of a full exposure series (0, 10, 20, 50, 100 mgL^{-1} NAFC) conducted in duplicate.

2.2.3 Statistical Analysis

Statistical analysis was done in SPSS, with a Shapiro–Wilk normality test for normality. For each algae, exposure conditions were compared using a one-way ANOVA, with a Dunnett post hoc to test for differences between control (0 mgL^{-1} NAFC) and exposure cultures (10, 20,

50, and 100 mgL^{-1} NAFC), and a Tukey HSD post hoc to compare between exposure treatments. Comparison of growth rates under control conditions was done using a one-way ANOVA and Tukey HSD post hoc. All α were set at 0.05.

Estimates of cell size were obtained measuring the largest diameter of cells in visual images of control cultures and those exposed to 100 mgL^{-1} NAFC. A bounding box was placed in the middle of a visual image and cells were measured going left to right, top to bottom, of the bounding box, until a count of 70 cells was reached. Comparison of cell size was done using an independent-samples two-tailed t-test (equal variance assumed).

2.2.4 Visual and Confocal Laser Scanning Microscopy Imaging

Visual imaging was performed using a Zeiss Axioplan fluorescence and AxioVision 4.7 imaging software (Carl Zeiss, Germany). For fluorescence CLSM, images were obtained using a Zeiss Biorad MRC 1024 confocal laser scanning microscope mounted on a Microphot SA epifluorescence microscope with a 60x numerical aperture 1.4 oil immersion plan apochromatic objective lens (Nikon, Tokyo, Japan). Cells were separately incubated with ConcanavalinA lectin (ConA) extracted from Jack-bean (*Canavalia ensiformis*) and SYPRO Orange (Sigma-Aldrich, Oakville, Canada) as per Neu et al. (2001). ConA was conjugated to the fluor fluorescein isothiocyanate which exhibits green fluorescence with an excitation wavelength of 488 nm; an emission bandpass filter of 520± 20 nm was used. SYPRO Orange has an excitation wavelength of 470 nm and an emission bandpass filter of 570± 20 nm was used. Images were analyzed using National Institute of Health's ImageJ (rsbweb.nih.gov/ij).

2.2.5 Fourier Transform Infrared Spectromicroscopy

Cultures were prepared as per Goff et al. (2009), with cells resuspended in an aqueous solution of heavy water (D_2O) and 1% agarose. D_2O was used to shift solvent absorption from

the amide region, while agarose was used to immobilize cells during measurements. Higher glycerol concentrations have been used in the past to immobilize Chlamydomonas cells for FTIR spectromicroscopy with minimal effect on cell viability and metabolism (Goff et al., 2009).

FITR spectromicroscopy measurements were carried out at the Canadian Light Source on beamline 01B1-01 (MidIR) using synchrotron light, with a Bruker IFS 66v/S interferometer coupled to a Hyperion 2000 IR confocal microscope in transmission mode. Cells in solution were loaded into a holder designed for work with living cells, and compressed between two 1 mm BaF_2 optical windows with a 15 μm spacer. To ensure selectivity of isolated single cells, confocal apertures were closed to 2-5 μm greater than the diameter of individual cells (variation in cell size is discussed in Section 3.1; apertures were primarily set to 12 to 20 μm). Confocal apertures of this size allow a light spot close to the diffraction limit and an acceptable signal-to-noise ratio, as well as allowing for any residual sample drift. Data collection and processing was done using OPUS spectroscopic data analysis software (Bruker Optics, Ettlingen, Germany). As is standard for FTIR measurements, differences in peaks were visually assessed. Presented spectra are an average of 10 individual cells, selected on the basis of absorbance intensity and lack of measurement artifacts (such as atmospheric compensation and baseline variation), baseline-corrected and normalized to the Amide 1 band.

2.3 Results

2.3.1 Cell Growth in the Presence of Oil Sands Naphthenic Acids

The addition of NAFCs to the WT *C. reinhardtii* cell cultures resulted in a dose-dependent decrease in the rate of growth ($F(4,10)=223.128$, $p=0.000$). However, exponential rates of growth were recorded even at the highest exposure concentration of 100 mgL^{-1} of NAFCs (Figure 2.1). NAFCs occur in tailing ponds at average concentrations of 40 – 120 mgL^{-}

[1], so high exposure levels above 100 mgL^{-1} of NAFCs are not environmentally relevant (Holowenko et al., 2002). The rate of growth of the control treatment differed significantly from all exposures, and there was a pattern of significant decrease in growth rate with increasing levels of NAFCs.

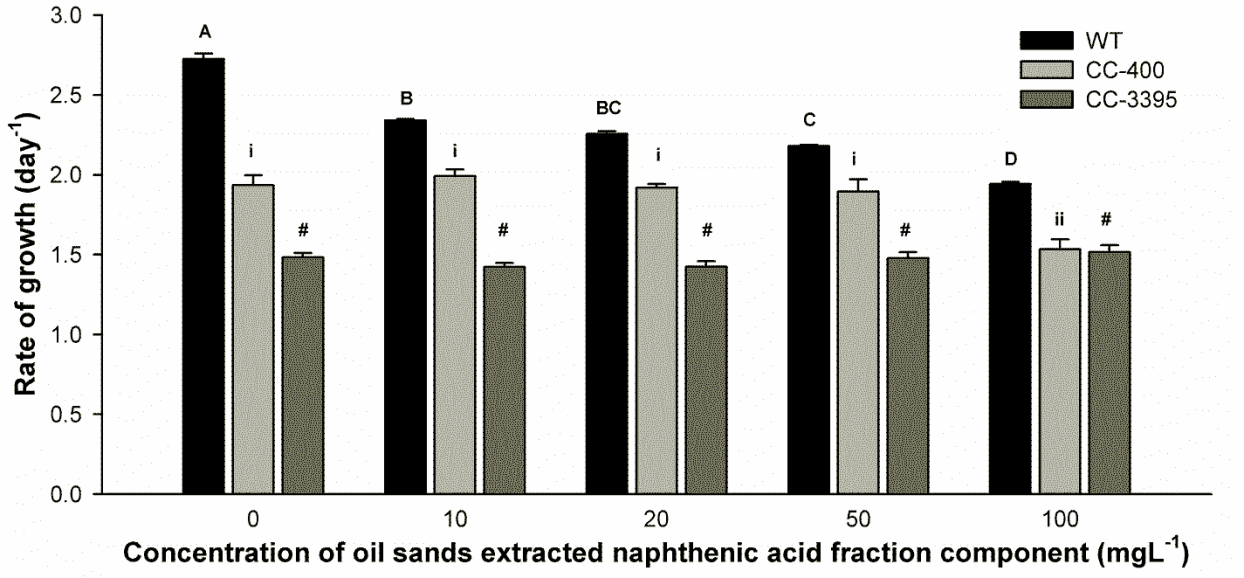

Figure 2.1: Impact of oil sands extracted naphthenic acid fraction components (NAFCs) on the rate of growth of *C. reinhardtii* wild type cells (WT) as well as the wall-less mutants CC-400 and CC-3395. The WT cultures exhibited a dose-depended in growth rate with exposure to increasing concentrations of NAFCs. CC-400's growth is unaffected until exposure to 100 mgL^{-1} of NAFC. CC-3395's growth rate is unimpacted by NAFC exposure at any level tested. Error bars are standard error. Homogeneous subsets from Tukey's test ($p < 0.05$) are indicated by the same letter/symbol. Full ANOVA tables in Tables 7.1-7.4.

Under control conditions, all three algal lines had rates of growth that differed significantly ($F(2,6)=200.521$, $p=0.000$). The first cell wall mutant, CC-400 had a rate of growth significantly lower than the wild type under control conditions, with NAFCs impacting its growth only after exposure to 100 mgL^{-1} ($F(4,10)=10.797$, $p=0.001$); the second cell wall mutant, CC-3395, had a control rate of growth significantly lower than that of CC-400 and WT, and was unaffected by NAFCs at any tested exposure concentration ($F(4,10)=1.405$, $p=0.301$).

Naphthenic acid exposure resulted in altered cell morphology. The cells of all three lines exhibited a trend to increasing roundness and diameter with exposures (Figure 2.2). Cell diameter for ovoid, unexposed, wild type cells averaged 9.6 ± 1.0 µm on the long axis, and round exposed cells average 12.6 ± 1.4 µm in diameter (Figure 2.2 G-H, M-N). In addition, the formation of palmelloids – four to sixteen cells remaining in the remnants of the mother cell wall – was noted in wild type cells (Figure 2.2 N).

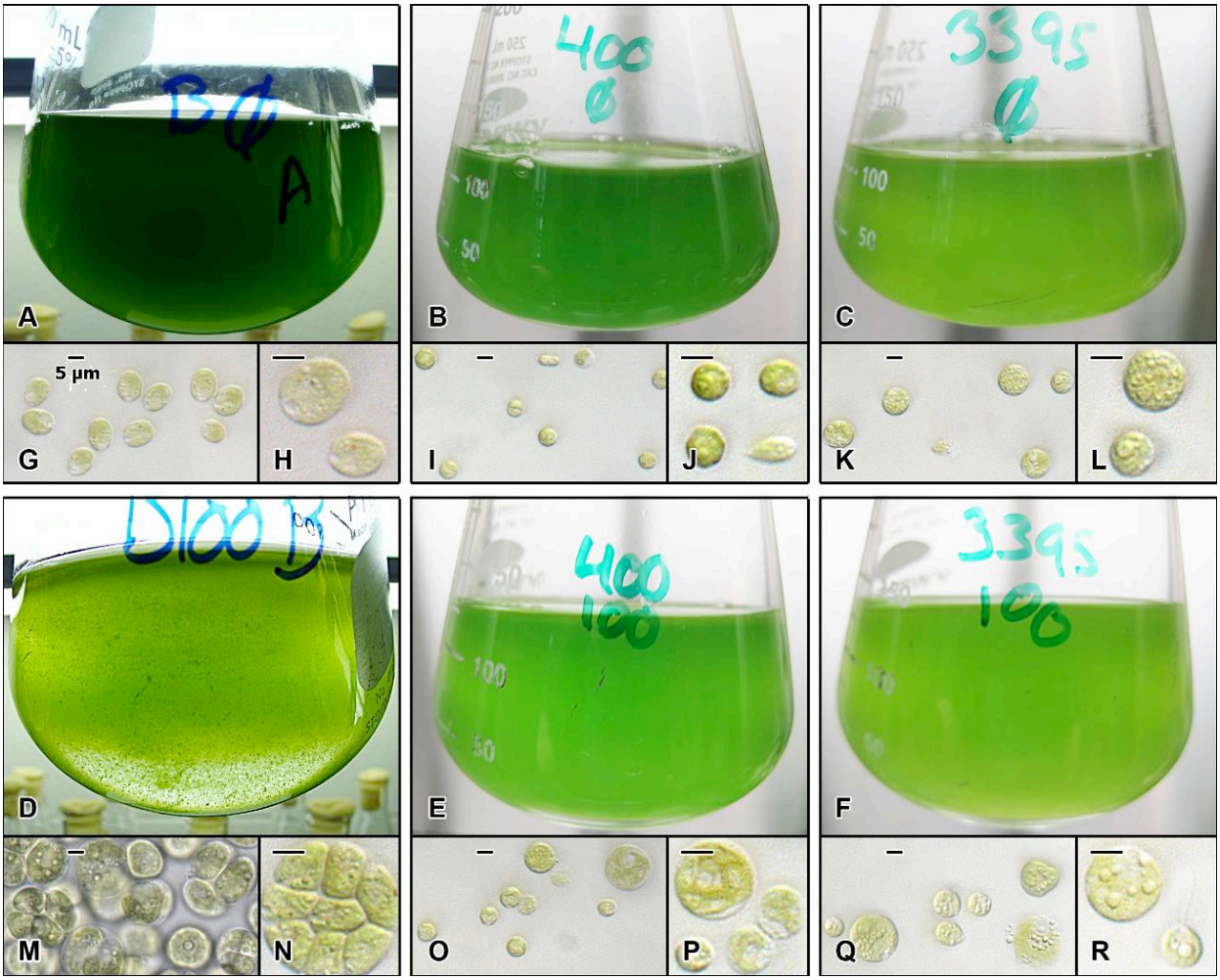

Figure 2.2: Changes in growth form and shape of C. reinhardtii after exposure to 100 mgL^{-1} of oil sands naphthenic acid fraction components (NAFCs). Cell cultures grown in control flasks (A, B, C) and with exposure to 100 mgL^{-1}NAFCs (D, E, F). At the cellular level, exposure to 100 mgL^{-1} NAFCs led to clumping of the wild type cells (M) and formation of palmelloid structures (N) compared to untreated cells (G, H). Clumping does not occur at either the visual or

microscopic level in either the CC-400 cells (E, O, P) or the CC-3395 cells (F, Q, R). Images A – F were taken after 72 hours exposure; G – R after 24 hours. All scale bars are 5µm.

The cell wall-deficient mutant CC-400 exhibited the greatest variation in cell size and shape under both exposed and unexposed conditions, with cells ranging from 4 - 13 µm (average 7.5 ± 1.7 µm) without exposure and 5-22 µm (10.5 ± 3.2 µm) after (Figure 2.2 I-J, O-P). Cell wall mutant CC-3395 were found to be round, with the diameter of control cells ranging from 8 – 12 µm (average 9.6 ± 1.2 µm) and exposed 9 – 15 µm (average 11.9 ± 1.7 µm) (Figure 2.2 K-L, Q-R). Independent two-tailed t-tests indicated differences between control and exposed population diameters at a significance level of $p \leq 0.01$ (n=70; WT: $t(138)=-14.7$, $p=0.00$; CC-400: $t(138)=-6.88$, $p=0.00$; CC-3395: $t(138)=-8.92$, $p=0.00$). Increased vacuolization was seen in both cell wall mutants (Figure 2.2 I-L, O-R).

Exposure to NAFCs altered the growth form of wild type cells. As shown in Figure 2.2, increased NAFC concentrations caused a shift from isolated cells evenly distributed through the media to macroscopic clumps containing palmelloids, individual cells, and exopolymer matrix. The change was dose dependent with clumping increasing along the concentration gradient (photographic assessment of 0-10-20-50-100 mgL^{-1} exposure series, not shown). This change was most marked when the wild type cells in the untreated flask (Figure 2.2 A) were visually compared to those in the 100 mgL^{-1} treatment flask (Figure 2.2 D). Similar clumping was not observed in the CC-400 or CC-3395 control flasks (Figure 2.2 B, C) or following treatment with 100 mgL^{-1} NAFCs (Figure 2.2 E, F). The clumping visible at the macroscopic level was also observed in the wild type cell cultures under microscopic observation (Figure 2.2 M, N). The clumping appeared to be a result of both cells in close association with each other (Figure 2.2 M) and the formation of palmelloids (Figure 2.2 N). No such effects were observed in either the CC-400 or CC-3395 cultures (Figure 2.2 O-R).

2.3.2 Confocal Laser Scanning Microscopy

Staining of the exopolymer matrix with ConA-FITC allowed additional changes in cell structure to be visualized (Figure 2.3). In wild type cells unexposed to NAFCs, ConA staining was faintly present along the external wall, with some cells exhibiting increased fluorescence localized to the flagellar region (Figure 2.3 A). After 24 hours of exposure to 100 mgL^{-1} NAFC, this pattern had changed, with more intense fluorescence indicating increased ConA binding surrounding entire cells and palmeloids; in addition, strong ConA fluorescence was found in the space between closely grouped cells, suggesting the presence of an extended glycoprotein exopolymer (Figure 2.3 B).

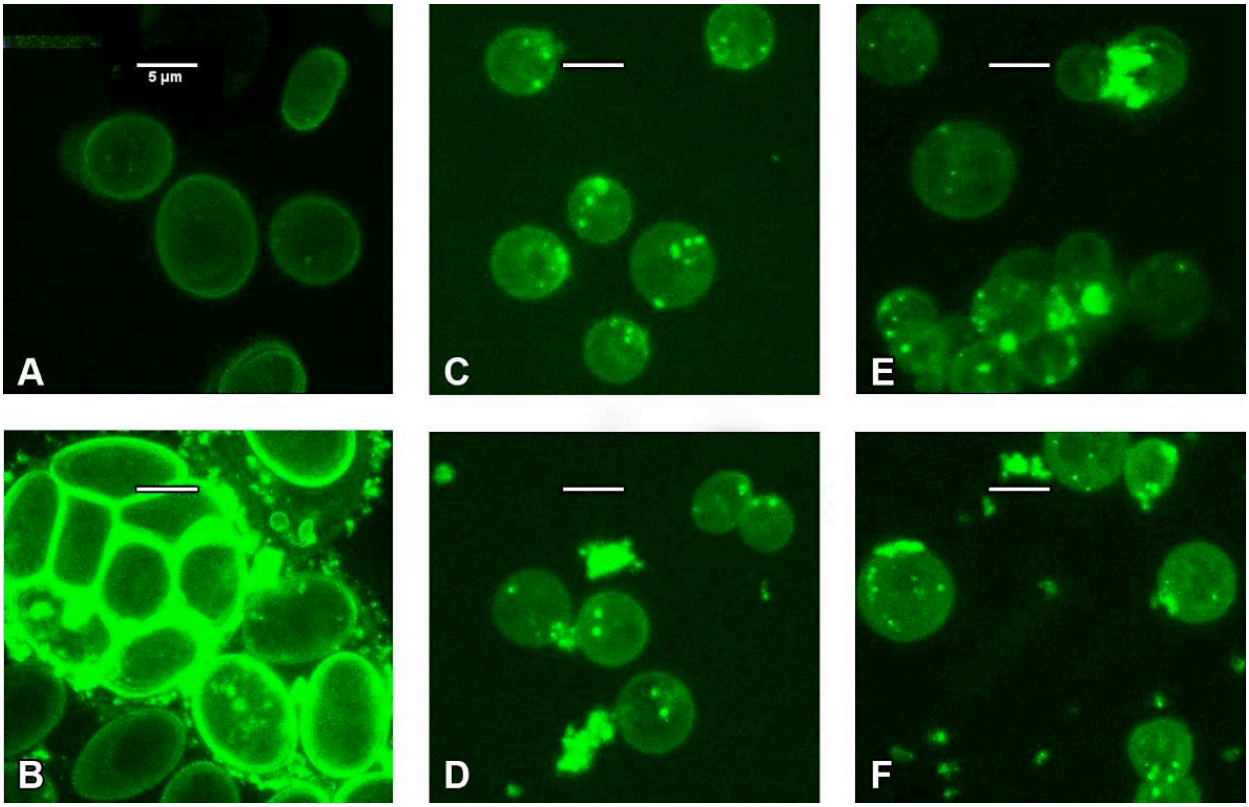

Figure 2.3: ConcanavalinA binding to *C. reinhardtii* under normal conditions and after 24 hours exposure to 100 mgL^{-1} of oil sands naphthenic acids. Wild type cells control (A) and exposed (B); CC-400 control (C) and exposed (D); CC-3395 control (E) and exposed (F). All scale bars are 5 µm.

The cell wall mutants exhibited extremely low baseline ConA binding, likely due to decreased presence of glycoprotein cell walls (Figure 2.3 C-F). There is, however, a visible increase in stainable material in the background after exposure to NAFCs (Figure 2.3 E, F).

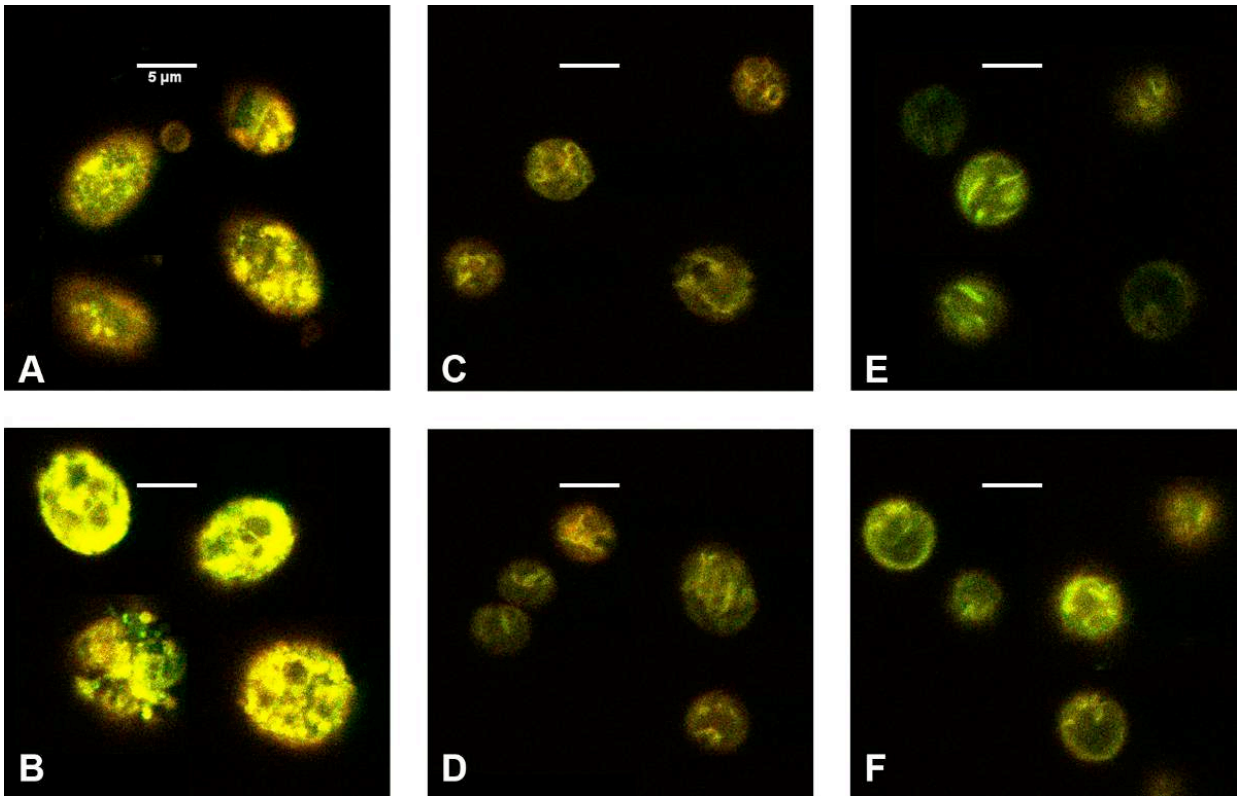

Figure 2.4: SYPRO Orange binding to *C. reinhardtii* under normal conditions and after 24 hours exposure to 100 mgL^{-1} of oil sands naphthenic acids. Wild type cells control (A) and exposed (B); CC-400 control (C) and exposed (D); CC-3395 control (E) and exposed (F). All scale bars are 5 µm.

Visualization of cell surface proteins with SYPRO Orange (Figure 2.4) also indicated modification of the WT cell wall after 24 hours exposure to 100 mgL^{-1} NAFCs (Figure 2.4 A, B). Protein-staining bodies increased in size and relative coverage of cell surface. Protein staining was again lower for the cell wall mutants; minor modification was apparent in CC-400 exposed to NAFCs in the form of smoothing of surface features (Figure 2.4 C, D) whereas no changes were observed in cells of CC-3395 (Figure 2.4 E, F).

2.3.3 Fourier Transform Infrared Spectromicroscopy

Numerous changes were observed in the infrared spectra obtained from cells exposed to NAFCs compared to untreated cells (Figure 2.5). In particular, changes in the protein bands Amide I (AI, ~1650 cm^{-1}) and Amide II (AII, ~1545 cm^{-1}) were seen in the wild type and CC-400 cells treated with NAFCs (Figure 2.5 A, B) but not observed in the CC-3395 cells (Figure 2.5 C).

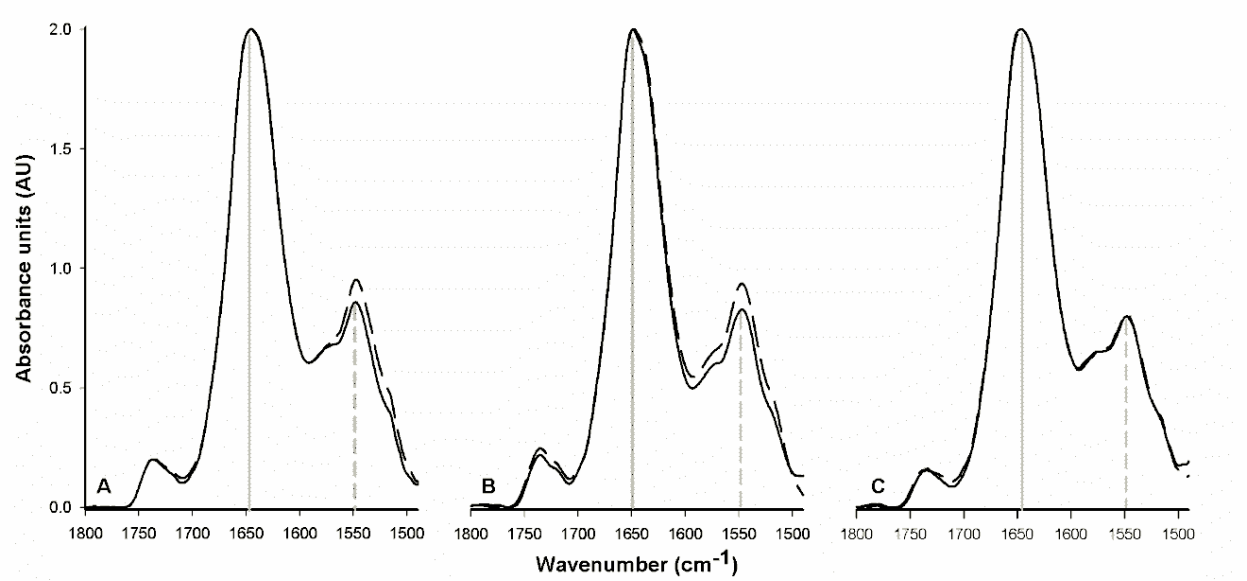

Figure 2.5: Fourier transform infrared spectra of cells of *C. reinhardtii*. Wild-type (A), CC-400 (B), and CC-3395 (C). Control cells (solid line) were transferred to fresh media 24 hours before measurement; exposure cells (broken line) were transferred to media containing 100 mgL^{-1} of oil sands naphthenic acids 24 hours before measurement. Spectra are an average of 10 cells, baseline-corrected and normalized to the Amide I peak. Amide I peak (solid line) and Amide II peak (broken line).

In Figure 2.5, it can be observed that there is an increase in the ratio of the AI and AII bands after NAFC exposure in wild type cells and in the cell wall mutant CC-400, again change was not observed in CC-3395 cells. Though the precise ratio varied due to innate biological culture variation, as well as synchrotron beam strength and position, these patterns of change were consistently found.

2.4 Discussion

In this study we examined the tolerance of the green alga *C. reinhardtii* to naphthenic acids extracted from oil sands process water. In our tests, *C. reinhardtii* exhibited exponential growth at NAFC concentrations of 100 mgL^{-1}, a tolerance significantly higher than predicted based on *C. frigida*'s environmental tolerance to NAFC concentrations up to 10 – 20 mgL^{-1} (Leung et al., 2003). While it is possible that this difference is species-specific, it seems unlikely that a laboratory strain would exhibit greater tolerance than an algal strain exposed to NAFCs in its evolutionary environment. This information suggests that naphthenic acids extracted from oil sands process water may not act directly as primary agents of toxicity; or may act as such only with exposure to synergistic agents of toxicity under field conditions. This is in agreement with recent work by Quesnel et al. (2011) who recently reported algal tolerances to Sigma-Aldrich model NA compounds up to 300 mgL^{-1} in *Dunaliella tertiolecta*. This is in keeping with the notion that in the natural environment NAFCs act synergistically with other OSPW toxicants, such as salinity, pH, heavy metals, and polycyclic aromatic hydrocarbons (PAHs).

Of particular interest, the cell wall mutants CC-400 and CC-3395 exhibited an increased tolerance to NAFC exposure in comparison to the wild type. Previous studies have compared the tolerance of *C. reinhardtii* strains that lack cell walls to metals and heavy metals (Macfie et al., 1994; Macfie and Welbourn, 2000; Prasad et al., 1998) hydrodynamic stress (Barbosa et al., 2003), and cancer drugs (Maucourt et al., 2002). Contrary to these studies our findings demonstrated that cell wall-deficient mutants are less affected by exposure to NAFCs than the wild type strain. The growth rate of CC-400 was significantly affected only when exposed to 100 mgL^{-1}, while the growth rate of the CC-3395 mutant was unaffected upon treatment with 100

mgL^{-1} of NAFC (Figure 2.1). It is worth noting that *D. tertiolecta*, found by Quesnel et al. (2011) to be unaffected by 300 mgL^{-1} of most model NAs at 14 days of exposure, has a naked membrane and lacks a cell wall. Given these observations, it is reasonable to presume that there may be some interaction between NAs/NAFCs and cell walls. If there is NAFC-induced disruption of the cell wall, it may increase sensitivity to heavy metals and other stressors such as alkalinity, pH or heavy metals not present under laboratory conditions, contributing to the observed toxicity variation between environmental and laboratory conditions.

The cell wall of *C. reinhardtii* is composed of seven layers (W1-W7) of hydroxiproline-rich glycoproteins, and is known to regulate intracellular transport and uptake (Azencott et al., 2007). Glycoproteins contain a wide variety of charged residues that are known to bind a number of compounds such as metals (Macfie et al., 1994) and therefore influence environmental interactions. The cell wall mutant CC-400 is known to have a rudimentary cell wall, retaining the innermost and outermost layers, W1 and W7 (Harris, 2009); the remaining layers are synthesized and excreted into the media without assembly (Voigt et al., 1991). Specific breakdown of missing wall layers in CC-3395 is not available; it can however be suggested that it its wall is less substantial than that of CC-400. Cells of CC-3395 exhibit lower levels of resonant Mie-type scattering in the FTIR spectra, the intensity of this phenomena having been tied to the presence of cell walls in *C. reinhardtii* (Svensen et al., 2007). Perhaps most importantly, cells of CC-400 cultures exhibited a greater variance in cell shape and size than those of CC-3395; CC-3395 cells were overwhelmingly round, while CC-400 contained round, ovaloid, and even oblong or sickle-shaped cells, suggesting the presence of cell wall remnants W1 and W7 may help induce and retain such variations in shape. If CC-400 does retain a greater number of wall layers than CC-3395, and NAFC-cell wall interactions are

involved in toxicity, it could explain why the growth of CC-400 is somewhat decreased at 100 mgL^{-1} of NAFC while CC-3395 remains unaffected.

The limited effect of NAFCs on the growth of cell wall-deficient mutants (CC-400 and CC-3395) combined with our Concanavalin A, SYPRO Orange, and FTIR data strongly suggests that oil sands NAFCs are capable of interacting with the glycoprotein cell walls. Concanavalin A, a sugar binding lectin that exhibits a high degree of specificity for glycoproteins containing α-D-mannose and α-D-glucose, was used to image cell wall glycoproteins and exopolymer matrixes. ConA binding visualized using CLSM (Figure 2.3) was greater in WT cells exposed to NAFCs. The binding pattern changed as well, becoming more evenly distributed along the cell walls, as well as exhibiting increased extracellular exopolymer staining. While the specific reason is unknown, this increased binding capacity indicates a change in the surface structure/chemistry of these cells, especially when combined with the surface protein modification visualized with SYPRO Orange. There are a number of possible mechanisms by which NAFCs could induce this difference, including modification of oligosaccharide side chains, exposure of binding sites, or a build up of glycosylated peptides along the outside of palmelloids or individual cell walls. Natural variation in the degree of binding of fluorescent anti-bodies to cell wall polypeptides and polycolonal antibodies has been found to fluctuate with cell reproductive stage, with strong variation in the degree of reaction to mother cell walls (Harris, 2009). This appears to indicate changes in the exposed surface proteins as mother cell walls prepare for lysis. Artificial modification of external cell wall proteins (signalled by changes in the pattern and intensity of ConA and SYPRO Orange binding) may thus interfere with the release of daughter cells by interfering with or interrupting native changes, leading to palmelloid formation.

Induction of palmelloid formation and changes in algal growth form were present only in wild type cells. Microscopic imaging indicated a change in the shape of the exposed cells with a shift from oval to round (Figure 2.2 A, D). Many of the "clumps" of cells observed in the wild type cultures exposed to 100 mgL^{-1} of NAFC appear to contain palmelloids. Normally, mitotic division in *C. reinhardtii* involves the formation of two daughter cells within the cell wall remnants of the mother cell, followed by breakdown of the wall remnants and release of the daughter cells into the media. The high frequency of mother cells containing upwards of 8 daughter cells indicates a change in the ability of the daughter cells to degrade the surrounding wall. This suggests a role for NAFCs in blocking the degradation of *C. reinhardtii* cell walls following mitosis. This has been observed previously, in *C. reinhardtii* and *C. eugametos* cells treated with a variety of organic acids (Iwasa and Murakami, 1968; Nakamura et al., 1975), and in cultures severely deprived of phosphorous (Olsen et al., 1983) or under heavy rotifer predation (Lurling and Beekman, 2006); normal eyespots and flagella were observed within palmelloids, suggesting their formation is due to cell wall abnormalities rather than loss or alteration of flagella. Palmelloid formation has also been observed in a *C. reinhardtii* mutant that had a defect in O-glycosylation (Vallon and Wollman, 1995). The changes induced were too subtle to be seen with microscopic methods, yet resulted in palmelloid formation in asexually reproducing cells and prevented sexual reproduction. It can therefore be extrapolated that even minor cell wall alterations can have significant impacts, resulting in defects in both sexual and asexual reproduction in Chlamydomonas cells. The formation of palmelloids in wild type cultures of *C. reinhardtii* is therefore strongly indicative of NAFC-cell wall interactions, through general modification or alteration of crucial oligosaccharide side chains, especially when taken in concert with the modified ConA fluorescence.

Information from FTIR further supports the suggestion that NAFCs are altering the cell wall structure. After NAFC exposure, there was a distinctive change in the ratio of the AI/AII bands in cells of the wild type and cell wall mutant CC-400, which retains cell wall layers W1 and W7, but not in those of CC-3395 (Figure 2.5). This ratio is a useful indicator of protein secondary structure and cell health (Holman et al., 2000). As this change is equally present in CC-400 (low growth stress at 100 mgL^{-1} NAFC) and WT (high growth stress at 100 mgL^{-1} NAFC) it is unlikely to be a general stress indicator, such as the formation of heat shock proteins (Lindquist and Craig, 1988), and more likely to be a physiological response of the cell wall protein pool to NAFC exposure.

2.5 Conclusions

Though the complex mixture that makes up NAFCs is poorly understood, one class of compounds grouped within this umbrella is known to be classical NAs, which exhibit anionic surfactant properties. Though surfactant toxicity shows extreme variation between individual species and surfactant compounds, they are generally known to bind to and denature cell wall proteins and alter membrane permeability (Lewis, 1990). Our data suggests NAFCs are capable of modifying the algal cell wall, leading to palmelloid formation and reduced growth rates. This is further supported by imaging data which demonstrate the failure of wild type cells exposed to NAFCs to disintegrate the remnants of the mother cell wall as during standard mitotic division, resulting in palmelloid formation. If the main interaction of NAFCs is with the cell wall, it may help explain why a cell wall-less halophile *D. tertiolecta* was extremely tolerant of specific NAs (Quesnel et al., 2011), as well as the increased tolerance to NAFCs found in cell wall-less mutants of *C. reinhardtii*.

The real toxicity of NAFCs to green algae may be indirect. Under environmental conditions, NAFC-induced formation of palmelloids and macroscopic clumps may contribute to mechanical removal of algae from the water column; they may unable to remain suspended and effectively be removed from tailings ponds with NAFC concentrations above a threshold level. In addition, if NAFC exposure results in disruption or modification of cell wall proteins and altered membrane fluidity, it may increase sensitivity to heavy metals and other stressors known to have a greater effect on cell wall mutants. It is thus possible that interactions between cell walls and NAFCs are potentiating the toxicity of other tailings ponds components such as metals, salinity, pH, and PAHs.

A number of questions remain regarding the interaction of NAFCs with algae such as *C. reinhardtii*, and the direct mechanisms of toxicity for its constituent compounds. However, the combination of growth data, changes in morphology, surface chemistry, and changes to the protein bands (AI, AII) all align with expected surfactant effects, and offer strong evidence that NAFCs are capable of interacting with glycoprotein cell walls in a manner important in their toxicity.

CHAPTER 3
3.0 EVALUATION OF BIOLOGICALLY MEDIATED CHANGES IN OIL SANDS NAPHTHENIC ACID COMPOSITION BY *CHLAMYDOMONAS REINHARDTII* USING NEGATIVE-ION ELECTROSPRAY ORBITRAP MASS SPECTROMETRY

3.1 Introduction

Oil sands extraction and development in Fort McMurray, Alberta, Canada, results in the production of large volumes of oil sands process water (OSPW), a complex and toxic mixture of compounds that includes naphthenic acids (NAs) (Allen, 2008). In addition, there is growing evidence that the acid extractable fraction referred to as oil sands naphthenic acids contains a variety of non-classical naphthenic acids in addition to classical (O_2) NAs and other compounds (Headley et al., 2011b; Headley et al., 2011c), including classes with higher degrees of oxidation (O_3 or O_4) and those containing nitrogen and/or sulphur (such as O_2S). This mixture is perhaps more correctly referred to as oil sands naphthenic acid fraction components (NAFCs) to distinguish it from purely classical or commercially available NAs (Headley et al., 2011a).

Natural biodegradation of NAFCs has been reported, but the rate of toxicity reduction is slow, with relatively rapid dissipation of z=0 and z=-2 classical NAs, and the remaining recalcitrant fraction exhibiting half-lives on the order of 12.8-13.6 years (Han et al., 2009; Holowenko et al., 2002). A number of species of algae, bacteria, and some emergent macrophytes have been found to contribute to the biotransformation and dispersal of some NAFCs (Armstrong et al., 2008; Headley et al., 2009a; Quesnel et al., 2011).

The broader NAFC mixture derived from OSPW has been found to be harder to degrade than commercial NAs (Scott et al., 2005). Furthermore, there are differences in the susceptibility of NAFC compounds to degradation; those which exhibit higher toxicity (low MW, low proportion of multi-ring structures, lower carboxylic acid content) are more easily degraded, while those recalcitrant to degradation (higher MW, higher proportion of multi-ring structures, increased branching, methyl substitutions) are more likely to exhibit lower toxicity (Herman et al., 1994; Lo et al., 2006). It is possible that this is due to the positively correlated relationships between bioavailability and biodegradation and between bioavailability and toxicity. For example, increased polycyclic aromatic hydrocarbon (PAH) bioavailability is known to speed rates of biodegradation (Tiehm et al., 1997). The evidence for algal degradation of NAs and NAFCs has been mixed, with the possibility of degradation by *Selenastrum* sp. (Headley et al., 2008) and definite degradation by *Dunaliella tertiolecta* (Quesnel et al., 2011). While a number of other species tested have shown no evidence of degradation (summarized in Headley et al., 2008; Quagraine et al., 2005), the majority of previous studies have used low resolution mass spectrometry, which would have resulted in bulk measurements of NAFCs and potentially obscured or masked the removal or modification of mixture components.

Previous work (Chapter 2) showed that NAFC exposure in the unicellular green algae *Chlamydomonas reinhardtii* resulted lower levels of growth suppression than suggested by the environmentally relevant sister species, *C. frigida*. In addition, the presence of a cell wall or cell wall remnants was found to potentiate NAFC toxicity (Figure 2.1). Furthermore, spectromicroscopy techniques indicated interaction with cell wall and cell surface proteins, similar to those predicted by surfactant interaction. The presence of NAFC-protein interactions capable of inducing toxicity (Figure 2.1), altering protein secondary structure (Figure 2.5), and

altering cell surface protein structure (Figures 2.3 and 2.4) suggest the possibility of physical interactions strong enough to physically remove bound NAFC from media.

In this study, Orbitrap high resolution mass spectrometry (150,000 resolving power at m/z 200) was used to investigate changes in the composition of an NAFC aqueous mixture mediated by the unicellular green algae *Chlamydomonas reinhardtii*, and two of its cell wall deficient mutant strains (CC-400 and CC-3395).

3.2 Materials and Methods

3.2.1 Materials

An NAFC extract was prepared from OSPW collected from an oil sands extraction operation (Fort McMurray, AB, Canada). The standard, composed of the acid-extractable fraction which includes NAFCs, was prepared as per Rogers et al. (2002a) and Janfada et al. (2006). Wild type *C. reinhardtii* (WT [1B^{-}]) is a back crossed wild type strain (descended from lines obtained from Jean-David Rochaix, Department of Molecular Biology, University of Geneva). The cell wall mutants CC-400 (*cw15* mt+) and CC-3395 (*arg7-8 cwd* mt-) were obtained from the Chlamydomonas Resource Center (University of Minnesota).

3.2.2 Algal Exposures

Algae were grown axenically in sterilized Erlenmeyer flasks containing liquid, arginine-supplemented, Tris-Acetate-Phosphate (TAP) nutrient media (Harris, 1989). For exposure studies, $10x10^{10}$ cells were added to 100 mL of TAP (control) or 100 mL TAP spiked with 100 mgL^{-1} of NAFCs. Cultures were grown for four days on mechanical gyratory shakers under uniform light conditions of 100 μmol photons m^{-2} s^{-1}. Homogenized media samples were taken immediately after spiking and at four days. The 10 mL samples were centrifuged at 3,900 RCF

for four minutes to pellet out algal cells, and the top 8.5 mL removed and frozen at -20 °C. Media blanks, with no algal cells added, were used to control for non-biologically mediated processes. Exposures on all three algal strains and controls (TAP only and TAP+100 mgL^{-1} NAFC) were performed once in triplicate.

3.2.3 Mass Spectrometry Analysis

Algal growth media contains high concentrations of buffers, salts, and other compounds; preliminary investigation (data not shown) found that direct injection of the TAP media for analysis resulted in ion suppression. To minimize these matrix effects, sampled supernatant underwent a cleanup process prior to MS analysis using ENV+ solid phase extraction (SPE) columns (Biotage, Chartlottesville, VA, USA). Samples were acidified to pH < 2 with formic acid, then run through the SPE column, rinsed with deionised water acidified with formic acid, and eluted in methanol. The methanol extract was evaporated under an N_2 gas stream, and the remaining sample reconstituted in 1 ml of 50:50 acetonitrile/water containing 0.1% NH_4OH_3. The prepared samples were run on a linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher Scientific) using the procedure and data processing techniques described by Headley et al. (2011c).

3.2.4 Statistical Analysis

Statistical analysis of exported and processed data was done using SPSS. Paired t-tests (two-tailed) were used to compare individual components of the mixture immediately after spiking and after four days incubation with algae. Comparisons of the concentration of O_3 compounds at day four were done using a one-way ANOVA with a Dunnett post hoc to test for differences between control media (non-biological) and media containing algae, and a Tukey HSD post hoc to compare between algal treatments.

3.3 Results

Information about NAs and NAFCs can be reported in terms of speciation (O_2, O_2S, O_3, etc.), and in terms double bond equivalents (DBEs), which represents the number of rings present in a compound plus the number of double bonds to carbon atoms (Bae et al., 2011). Statistically significant changes in NAFC mixture composition were found after four days exposure to the three algal strains (Figure 3.1, Figure 3.2, Figure 3.3). These changes were specific to chemical species and algal strain, and their observation required the full NAFC speciation provided by high resolution mass spectrometry. With the exception of a decrease in the relative abundance of O_3 species, no changes were found in the NAFC composition of the algae-free control. HRMS reports NAFC amounts as relative percent abundance of the overall NAFC concentration; the Y-axis of all graphs is scaled as a percent of total overall NAFC content.

Classical naphthenic acids, represented here by O_2 species, made up the majority of the NAFC consortia, on the order of 90% of the total relative abundance of each sample. There was no change in the overall relative abundance of the O_2 species for any of the algal strains (WT Figure 3.1 A, CC-400 Figure 3.1 B, CC-3395 not pictured). The DBE distribution of the O_2 species, however, showed a different trend. As seen in Figure 3.1, there are changes in the O_2 DBE distribution over time in NAFC media from WT and CC-400 cultures. The analysis of WT media showed an increased relative abundance of O_2 compounds with a DBE of 1 ($t(2)= -20.2$, $p=0.02$) and the removal of those with DBE of 8 ($t(2)=18.7$, $p=0.03$). The media of CC-400 showed a minor, though significant, decrease in the relative abundance of O_2 compounds with a

DBE of 7 ($t(2)=2.92$, $p=0.09$), and the removal of those with a DBE of 8 ($t(2)=32.3$, $p=0.01$). No such changes were present in the non-algal control media or that of CC-3395.

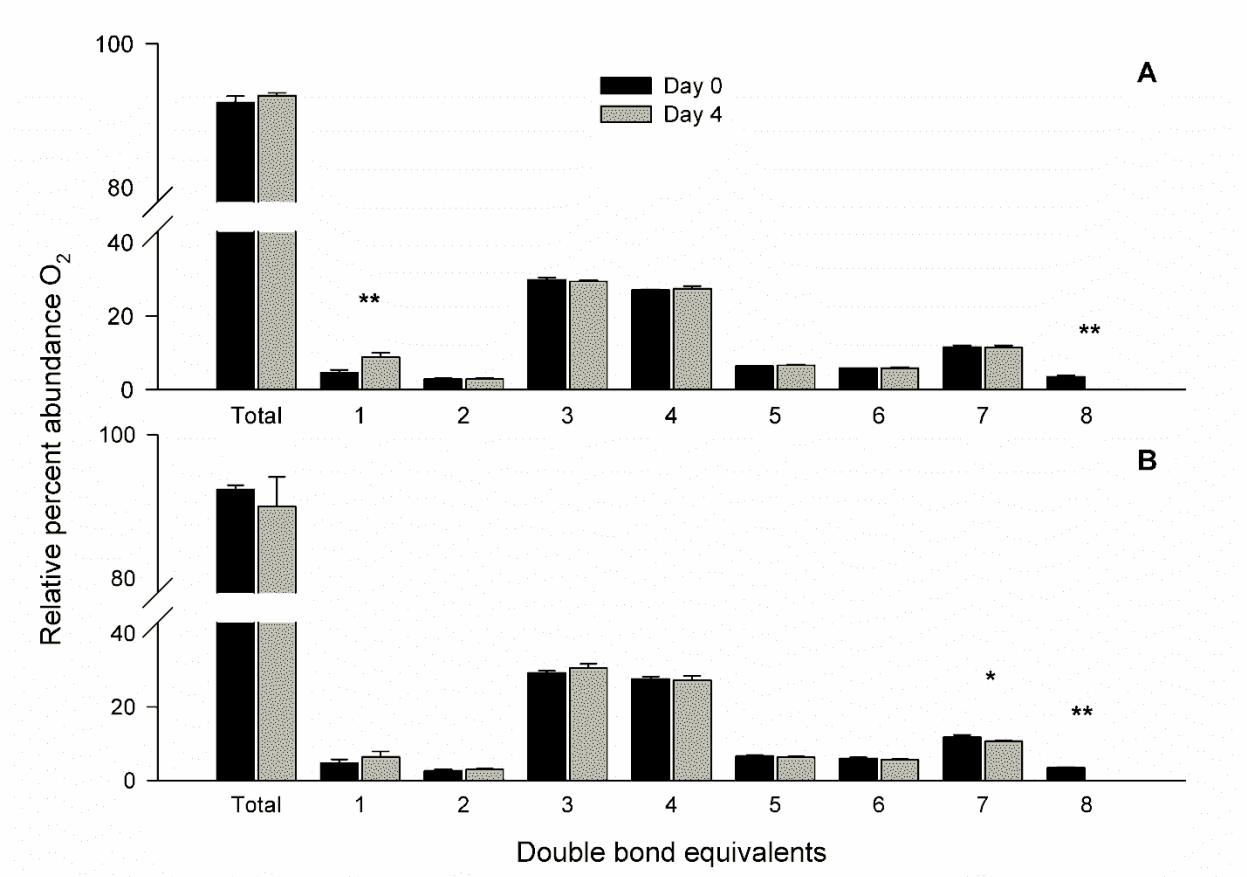

Figure 3.1: Relative abundances of O_2 species in naphthenic acids fraction component mixtures at time zero and after four days incubation with wild type *Chlamydomonas reinhardtii* (A) and the *C. reinhardtii* cell wall deficient mutant CC-400 (B). Significant differences in the average of the mean indicated by * for $p < 0.10$ and ** for $p < 0.05$. n=3.

As illustrated in Figure 3.2, changes were observed in the relative abundance of O_2S species. For NAFC mixtures exposed to all algal strains, there was a significant decrease in the overall relative abundance of all O_2S compounds. The O_2S species made up approximately 4.5% of the total NAFC mixture at time zero, and were composed primarily of compounds with a DBE of 4 or 5. Minor amounts of O_2S species with a DBE of 6 were also present, though not subjected to statistical analysis because of low relative abundances. This process was less

compound and algae specific than changes in O_2 composition (Figure 3.2 WT, CC-400, CC-3395). For WT and CC-3395 there was a significant decrease in O_2S species with a DBE of 4 and 5, as well as an overall decrease in the relative abundance of total O_2S (WT: DBE 4 $t(2)=3.51$, $p=0.07$; DBE 5 $t(2)=5.17$, $p=0.03$; Total $t(2)=6.26$, $p=0.03$; CC-3395: DBE 4 $t(2)=4.37$, $p=0.04$; DBE 5 $t(2)=3.88$, $p=0.06$; Total $t(2)=4.30$, $p=0.05$). A similar pattern was found in CC-400 with the exception of a non-significant decrease in O_2S DBE 4 (DBE 4 $t(2)=2.55$, $p=0.13$; DBE 5 $t(2)=5.12$, $p=0.04$; Total $t(2)=3.16$, $p=0.09$).

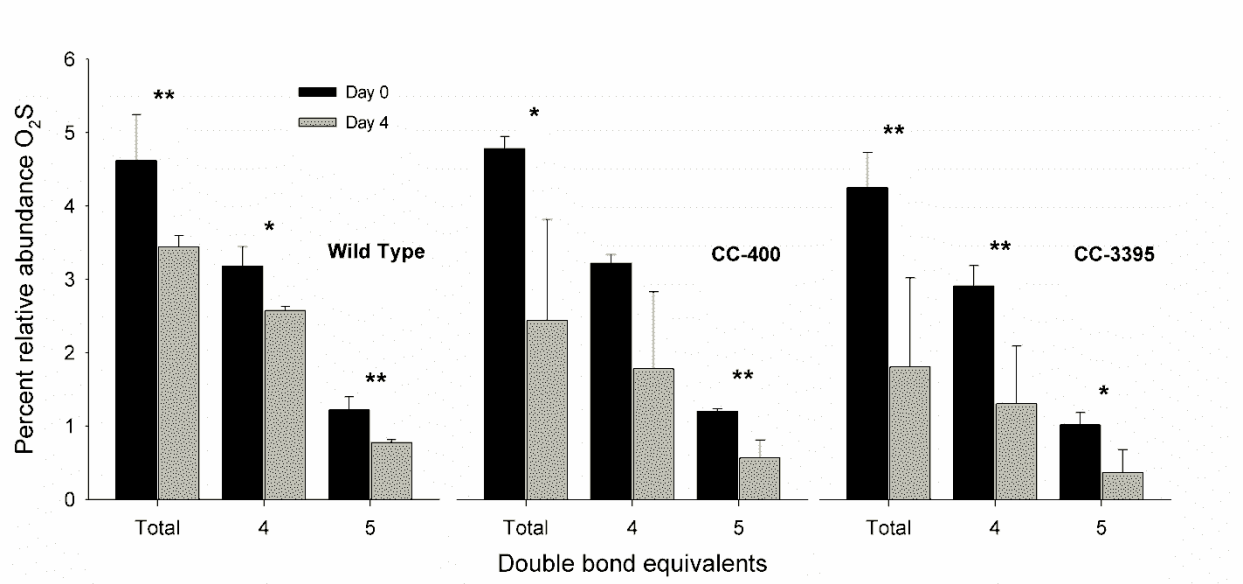

Figure 3.2: Relative abundance of O_2S species of the naphthenic acid fraction component mixture in aqueous media at time zero and at termination after four days of algal exposure. For all algae, the relative abundance of O_2S in media decreased over time. Error bars are standard deviations. Significant differences in the average of the mean indicated by * for $p < 0.10$ and ** for $p < 0.05$. O_2S TOTAL represents the sum of all double bond equivalents for O_2S species, including those not analysed individually due to low overall abundance.

Changes were also observed in the total relative abundance of the more oxidized O_3 species following incubation with algal cultures (Figure 3.3, 3.4). The O_3 class of NAFCs occurred at even lower abundance than the O_2S compounds, at approximately 1% of the total mixture used in this study. The O_3 class included compounds with DBEs of 2 – 7; however due to the low overall relative abundance, no statistical analysis could be reliably calculated on the

individual DBE species of the O_3 group. The non-biological control blank (TAP media plus NAFCs) exhibited an overall decrease in the relative abundance of O_3 species ($t(2)=3.39$, $p=0.08$), a pattern not present in media incubated with algal cultures (Figure 3.3).

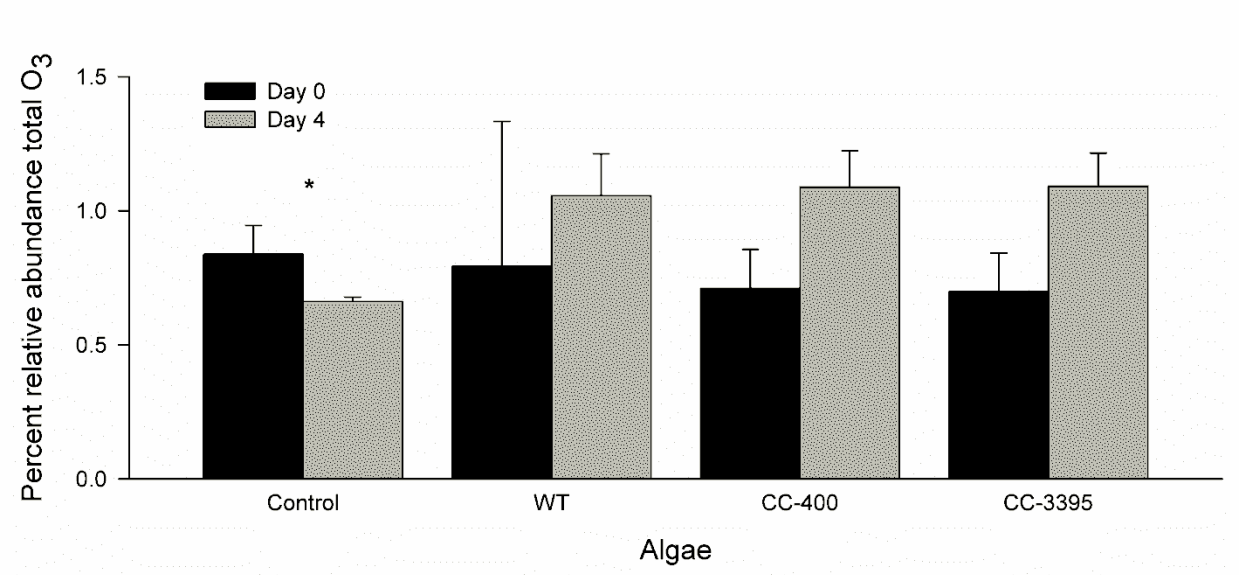

Figure 3.3: Overall relative abundance of O_3 species in naphthenic acid fraction component mixture in algal growth media at time zero and at termination after four days of algal exposure. Measure includes all double bond equivalent groups of the O_3 species. Significant differences in the average of the mean indicated by * for $p < 0.10$. Error bars are standard deviation.

When overall relative abundances of O_3 species were compared at termination time at day four (Figure 3.4), the concentration in control media was found to differ from that in algal media ($F(3,8)=8.562$, $p=0.007$). This appeared likely due to both the decreased relative abundance in control media and the absolute (though non-significant) increase in the relative abundance of O_3 compounds in algal media. Even with HRMS, it was not possible to discern if these were algal organic acids which read within the NAFC envelope, or if they were secondary NAFCs resulting from algal-mediated biotransformation.

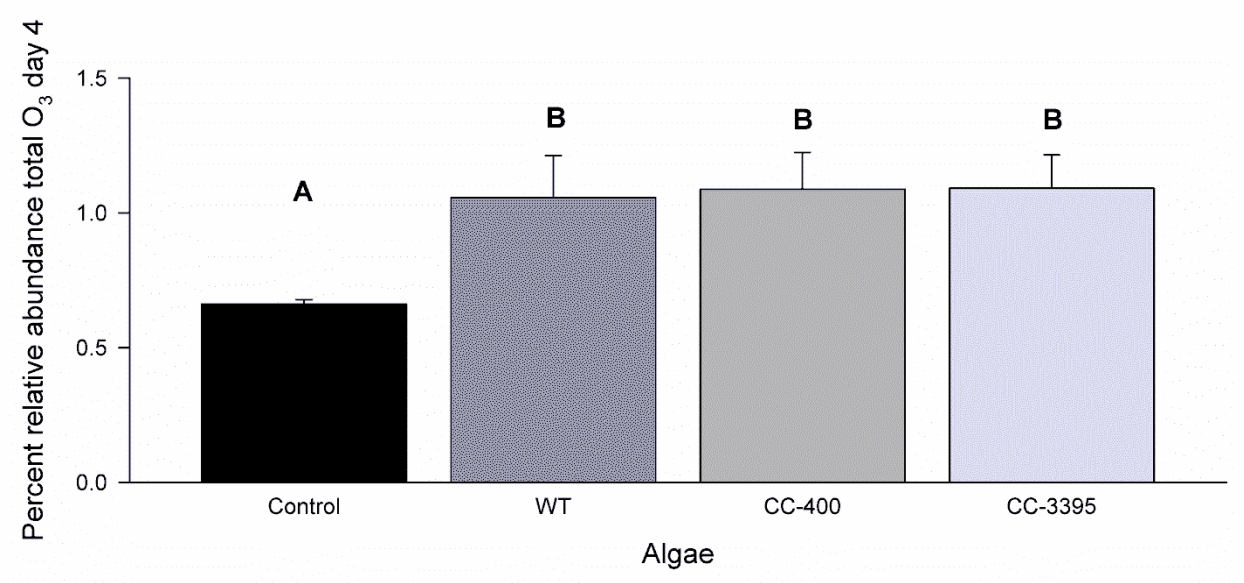

Figure 3.4: Overall relative abundance of O_3 species in naphthenic acid fraction component mixture in algal growth media at termination after four days of algal exposure. Measure includes all double bond equivalent groups of the O_3 species. Error bars are standard deviation. $p < 0.05$ for homogeneous subsets.

A wide variety of other non-classical NAFC classes were observed via HRMS, including various nitrogen and sulphur containing compounds and those with a higher degree of oxidation (N_2SO_4, O_3S, O_4-O_6, and many others); however they were relatively minor components in the mixtures investigated and no comment was possible on related changes.

3.4 Discussion

Based on our results, *C. reinhardtii* appeared capable of modifying the composition of oil sands extracted NAFCs, either by sorption to the cell and cell wall components, or via metabolism. This process was highly specific, and algae-compound interactions appeared to be based both on cell wall structure and the class and structure of the NAFC constituents. The amounts of classical NAs, represented by O_2 species, changed in a fashion specific to both algal strain and DBE. NAFCs incubated with wild type cells showed removal of DBE 8 and an increased relative amount of DBE 1 (Figure 3.1 A). Cell wall mutant CC-400, which retains a

partial cell wall, appeared to mediate reduction in DBE 7 and the removal of DBE 8 (Figure 3.1 B). Results for O_2S compounds (Figure 3.2) suggested that the relative abundance of all DBE classes of these compounds were diminished by algal exposure for all tested strains.

These differences between compound classes and cell lines were not surprising, and a more specific breakdown of NAFC mixture composition would be expected to yield more variation. In studies of *C. reinhardtii*, as with other algae, there can be markedly different responses to similar compounds: *C. reinhardtii* is capable of removing pyrene (Lei et al., 2002) but not benzo[a]pyrene (Warshawsky et al., 1995). Other studies examining the removal of a variety of chlorinated, nitrated, and sulfonated benzoic acids by *Chlamydomonas reinhardtii* (Gutenkauf et al., 1998) showed it to be able to discriminate very specifically between the individual carboxylic acid compounds. Differences in cell wall structure and exposed binding sites would also be expected to increase the variation in response. Toxicity assessments (Chapter 2) showed high levels of variation between these algal lines in their susceptibility to NAFC toxicity. The cell wall deficient mutants exhibited greater tolerance to NAFCs than the wild type (WT) with its full glycoprotein cell wall (Figure 2.1). Cultures of WT cells exhibited a dose-dependent decrease in growth rate when exposed to NAFCs, with exponential growth continuing with exposure to 100 mgL^{-1} NAFCs. The two cell wall mutants were less affected; CC-400's rate of growth was decreased only after exposure to 100 mgL^{-1} of NAFCs, while CC-3395's growth was non-impacted at this level. Additional evidence suggested that the NAFCs have a surfactant interaction with the cell wall proteins (Figures 2.3 – 2.5).

The removal of O_2 class compounds with a DBE of 8 by wild type, fully walled cells and the cell wall mutant CC-400 which retains a partial cell wall suggested the possibility of sorption and removal of NAFCs from the aqueous phase. The glycoprotein cell walls of *C. reinhardtii*

contain a number of charged residues capable of selectively binding compounds such as metal ions (Macfie et al., 1994). This process is known to be dynamic and responsive and may also be influenced by contaminant concentration; it may involve increased production of binding sites and excretion of specific proteins and exudates (Kola et al., 2004). The variation in cell wall structure and available binding sites between the different cell wall mutants and WT cells may help explain the differences in the degree of toxicity induced by NAFC exposure, as well as differences in the removal of specific compounds. Though the body of research on algal tolerance to and degradation of NAFCs is still growing, a number of the algae found in tailings ponds by Leung et al. (2001, 2003) were naked or exhibited abnormal cell wall morphology. One marine algae, *Dunaliella tertiolecta*, that has been found to exhibit high tolerance to five model NAs and degrade four (Quesnel et al., 2011) also lacks a cell wall.

The only change observed in the non-algal control was a decrease in the relative abundance of O_3 compound classes over time (Figure 3.3). At termination, the algae increased relative abundances of O_3 compared to control as well as a non-significant absolute increase; there was no statistical change in the WT cells, but in comparison to the decrease in the control media, this suggests there may have been O_3 species added to the media by algal growth. The nutrient media used for culture growth contained organic acids, which *C. reinhardtii* is known to metabolize. In addition, *C. reinhardtii* excretes a variety of organic acids as part of its metabolism. While decreased relative abundances of compounds could likely be attributed to specific biomodification or physical removal by *C. reinhardtii*, increased levels of compounds reported as NAFCs must be more carefully interpreted. Changes could have been due to the formation of secondary NAFCs after metabolism of primary compounds, or attributable to biological secretion of endogenously metabolized organic acids, potentially increased due to the

toxic exposure. Control algal cultures in blank TAP media showed a decrease in the relative abundance of O_3 present in the media, which suggested that this production is not a product of a metabolic pathway under non-stressed conditions. Exposure to 100 mgL^{-1} of NAFCs induced increased toxicological effects in WT cells as compared to CC-400 and the unaffected CC-3395 (Figure 2.1) whereas the relative abundance of O_3 at day 4 was relatively equivalent between all three lines.

3.5 Conclusions

Overall, this research showed that *C. reinhardtii* was capable of modifying the relative composition of NAFCs in solution, in a way that was dependent upon chemical compound classes and cell wall structure. Though the mechanisms of removal were not clear, it appeared that for some compounds sorption by cellular components played an important role. These results would not have been discernible with unit mass resolution MS analysis of classical NAs, as the changes in the distribution of NAFCs that were observed are not resolved with low mass resolution typically employed in earlier investigations.

CHAPTER 4
4.0 INDUCTION OF PHYSICAL AND METABOLIC CHANGES IN *CHLAMYDOMONAS REINHARDTII* BY EXPOSURE TO OIL SANDS NAPHTHENIC ACIDS

4.1 Introduction

There is a large and growing interest in the toxicological impacts of byproducts of industrial oil sands production. This oil sands process water (OSPW) is highly saline and alkaline, and contains residual bitumen, fine sediments, ions, and a variety of organic and inorganic compounds; this mixture has been found to be acutely and chronically toxic across a variety of taxa (summarized in Allen, 2008), with special interest paid to the role of pH, salinity, and dissolved organics (Energy Resources Conservation Board, 2009). Of the dissolved organics, naphthenic acids (NAs) have been identified as a contaminant of concern and possible agent of primary toxicity (Clemente and Fedorak, 2005; Han et al., 2009; Headley and McMartin, 2004), with toxic effects observed in a broad array of organisms (Frank et al., 2008; Quagraine et al., 2005). Oil sands NAs are a highly complex and poorly understood mixture of alkyl-substituted mono- and poly-cyclic carboxylic acids, and there is growing evidence that the acid-extractable fraction previously referred to as oil sands NAs contains a variety of non-classical naphthenic acids and other organic compounds (Headley et al., 2011b; Headley et al., 2011c). This mixture is herein referred to as naphthenic acid fraction components (NAFC) to differentiate it from purely classical or model NAs.

Though the toxicity of OSPW and NAFCs is well established, the mechanisms of toxicity are not. Model NAs and NAFCs have been shown to exhibit different levels or pathways of

toxicity (Armstrong et al., 2008; Garcia-Garcia et al., 2011; Peters et al., 2007), and various constituents of NAFC mixtures have been found to elicit different degrees of toxic response (Jones et al., 2011). Previous work (Chapter 2) has already established the importance of the cell wall of *Chlamydomonas reinhardtii* in susceptibility to NAFC toxicity: the fully walled wild type (WT) exhibits the greatest sensitivity to NAFC exposure, and the cell wall mutants CC-400 (retaining the innermost and outermost layers) and CC-3395 (believed to be completely naked) are relatively tolerant (Figure 2.1). Confocal laser scanning microscopy (CLSM) imaging of cell surface glycoproteins (Figure 2.3) and proteins (Figure 2.4) appear to indicate cell wall modification in WT cells exposed to 100 mgL^{-1} NAFCs, and Fourier-transform infrared spectromicroscopy (FTIR) spectra (Figure 2.5) indicated changes in protein confirmation for 100 mgL^{-1} NAFC-exposed cultures of WT and CC-400. As previously discussed, this selective toxicity and evidence of interaction with cell wall proteins is consistent with the general predicted impacts of surfactant toxicity. The highly selective nature of the modification of NAFC composition, based on algae strain, chemical species, and chemical structure, is also consistent with the highly specific nature of surfactant-cell interactions. As such, it was important to conduct further investigation of the potential of the importance of surfactant effects in NAFC toxicity.

Classical NAs are anionic surfactants lacking the functional groups that would be expected to target a specific reactor; as such, if the amphipathic properties of these compounds are important in toxicity the expected acute mode of toxicity would be narcosis (Frank et al., 2009) from NA/NAFC-induced disruption of the cell membrane, increased membrane permeability leading to metabolite leakage, or the disruption of surface protein confirmations interfering with membrane function and transport (Van Hamme et al., 2006). Metabolic leakage

is often characterized by the presence of nucleic acid in the media, cellular swelling and loss of shape, followed by loss of integrity and finally lyses; this effect has been found to be more pronounced in algal strains lacking a cell wall (Sun et al., 2004). Microbial work by Glover et al. (1999) examined the effect of four surfactants on a number of species and found that while surfactant exposure increased membrane leakage and fluidity, this was not correlated with the degree of toxicity observed, suggesting that other factors such as protein binding and loss of tertiary structure (Goncalves et al., 2003) might play an important role. Furthermore, charged surfactants (both anionic and cationic) have been found to be more strongly denaturing than neutral ones (Nyberg, 1979).

It is important to note that it is difficult to generalize the environmental impacts of surfactants and anionic surfactants, as well as the possibility for synergistic toxicity, as the complex interactions vary widely based on the specific structure of the surfactant, multiple environmental factors, possible co-toxins, and the individual organism exposed. Species sensitivity to a given surfactant and the effect of similar surfactants on the same algae may vary by three to four orders of magnitude (Lewis, 1990). They may or may not act synergistically with metals (Lewis, 1992; Van Hamme et al., 2006), but there is a trend to increased surfactant toxicity when algae are co-exposed to surfactants and oil or surfactants and high levels of salinity (summarized in Lewis, 1992), both important factors given the high levels of salinity in OSPW and presence of residual bitumen.

In this work, further examination was made of physiological changes induced in *C. reinhardtii* and its two cell wall mutants, CC-400 and CC-3395, including predicted surfactant effects such as changes to phospholipids and lipid metabolism, DNA distribution, membrane visualization, protein distribution, and other morphological changes.

4.2 Materials and Methods

4.2.1 Algal Exposures

Three strains of *C. reinhardtii* were used in these experiments. The wild type (WT, 1B-) is a backcrossed wild type descended from lines obtained from Jean-David Rochaix (Department of Molecular Biology, University of Geneva). Two cell wall mutants lacking fully assembled cell walls, CC-400 (*cw15* mt+) and CC-3395 (*arg7-8 cwd* mt-) were obtained from the Chlamydomonas Resource Centre (University of Minnesota). Both produce a full complement of wall glycoproteins that they are unable to assemble, and are instead excreted into the media (Voigt et al., 1997; Voigt et al., 1991). Cultures were grown photoheterotrophically in liquid Tris-Acetate-Phosphate (TAP) media supplemented with arginine (Harris, 1989) in sterilized Erlenmeyer flasks. Light conditions were a uniform 100 μmol photons m^{-2} s^{-1}, and flasks were agitated on a mechanical shaker to prevent settling. Exposures were 24 hours in length and carried out three times; flasks consisted of fresh TAP spiked with $20x10^{10}$ cells L^{-1} and 100 mgL^{-1} NAFCs, or controls of fresh TAP spiked with $20x10^{10}$ L^{-1} cells.

4.2.2 Lipid Analysis

Thin-layer chromatography (TLC) was performed to isolate and quantify neutral/polar lipid composition in algae grown under control conditions and NAFC exposure (Miquel and Browse 1992, Lightner et al. 1994, Christie 2003). Determination of fatty acid/oil composition was carried out using Lee et al.'s (1998) solvent system and analyzed via gas chromatography. Cells from control and exposed cultures were harvested by flash-freezing pellets formed by centrifugation at 3900 and 20,800 RCF. Prior to lipid extraction, samples were lyophilized and dry weights were determined, with sample weights compared to GC results to determine overall

lipid contend and amounts of specific fatty acids. Samples were places in bead beater tubes with 1 mL of 2:1 chloroform:methanol, along with 100-200 µL of 0.5 mm glass beads, and agitated in a beat-beater for three times for 60s at 4°C. The tube contents, as well as the rinse liquid (3 x 1 mL rinses with 2:1 chloroform:methanol) were transferred to glass tubes and evaporated under N_2. Samples were rehydrated using 100 µL chloroform and 30 µL was spotted on Silica Gel 60 F_{254} TLC plates alongside standards for separation, using a mobile phase of 70:30:1 hexane:ethyl ether:glacial acetic acid.

Plates for lipid visualization were developed in 0.05% primulin in acetone (w/v). TLC spot scrapings were transferred to glass tubes with an internal standard of 100 µg 15:0 fatty acid and 2 mL of 3 N methanolic HCl and incubated at 80°C for two hours. Following incubation, 2 mL 0.9% NaCl was added and extracted twice with 2 mL hexane. Following a final N_2 evaporation, samples were resuspended in 15 µL hexane and injected into an Agilent 6890N network gas chromatography (GC) system using a DB-23 column with Chemstation software. Samples were held at an initial temperature of 160°C for 1 minute, then ramped to 240°C at a rate of $4°C min^{-1}$ and held there for 10 minutes (Browse 1986).

4.2.3 Fourier-Transform Infrared Spectromicroscopy

Samples were prepared after Goff et al. (2009), with cells suspended in an aqueous D_2O solution containing 1% agarose as an immobilizing agent in order to shift solvent absorption away from the amide region and prevent cellular movement during measurements. Fourier-transform infrared spectromicroscopy (FTIR) experiments were carried out in transmission mode at beamline 01B-01 (MidIR) at the Canadian Light Source using a Bruker QFS 66v/S interferometer coupled to a Hyperion 2000 IR confocal microscope. Samples were loaded in a holder designed for work with living cells consisting of two 1mm BaF_2 optical windows and a 15

μm spacer. Confocal apertures 2-6 μm in diameter greater than individual cells (cells were 6-20 μm on the short axis depending on cell line and exposure conditions; for detailed discussion on variation on cell size and shape see section 2.3.1.) were used to ensure selective measurement of individual cells. Apertures had a minimum diameter of 12 μm, allowing a light spot close to the diffraction limit and an acceptable signal-to-noise ratio. Increased presence of the solvent system in the measurement of smaller cells was accounted for via background measurement. Data was collected and processed using OPUS spectroscopic data analysis software (Bruker Optics, Ettlingen, Germany). Presented spectra are an average of 10 individual cells, selected on the basis of absorbance intensity and lack of measurement artifacts (such as atmospheric compensation and baseline variation), baseline-corrected and normalized to the Amide 1 band.

4.2.4 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was carried out using a Microphot SA epifluorescence microscope coupled with a Zeiss Biorad MRC 1024 confocal laser scanning microscope and a 60x NA 1.4 oil immersion plan apochromatic objective lens (Nikon, Tokyo, Japan). Cells were incubated with Nile Red and ConcanavalinA (ConA) extracted from *Canavalia ensiformis* and conjugated to the fluor fluorescein isothiocyanate, or with one of SYTO 9 (S9), SYPRO Orange (SO), or FM1-43 (all chemicals obtained from Sigma-Aldrich, Oakville, Canada), as per Neu et al. (2001). Excitation wavelengths/emission wavelengths were 568 nm/605 nm, 488 nm/522 nm, 288 nm/522 nm, 488 nm/598 nm, and 488 nm/598 nm respectively. All emissions used a bandpass filter of ± 20 nm.

4.2.5 Statistical Analysis

Statistical analysis was done in SPSS. Differences in algal lipid composition in cultures with and without exposure to 100 mgL^{-1} NAFCs were done using independent samples t-test

(two-tailed, equal variance assumed) to compare the average of the means. *df* for all tests was 4, n=3. All α were set at 0.05.

4.3 Results

4.3.1 Lipid Analysis

4.3.1.1 Phospholipid Breakdown

Under control conditions, the three algal strains had similar diversities in the types of phospholipids present, though there were differences in the relative abundances of different classes. WT and CC-400 cultures were found to be more similar to each other than to CC-3395; for these first two, ~70% of phospholipids were represented by three classes (18:3n-3, 16:0, and 16:3), other classes represented less than 10% of total phospholipids. Phospholipid composition of the cell wall mutant CC-3395 was less concentrated, with four phospholipids that each contributed more than 10% of phospholipid totals (18:3n-3, 16:0, 16:3, 18:1n-9). Table 4.1 summarizes the phospholipid diversity for each line under control and exposure conditions, as well as the abundance of each phospholipid species relative to the others present.

Table 4.1: Ranked abundances of classes of phospholipids for wild type cells and the cell wall mutants CC-400 and CC-3395 under control conditions and after exposure to 100 mgL^{-1} naphthenic acid fraction components. Bolded ranks indicate a class that makes up at least 10% of the total phospholipid mass.

	Wild Type		CC-400		CC-3395	
	Control	Exposed	Control	Exposed	Control	Exposed
14:0	--	--	11	11	--	11
16:0	**2**	**2**	**2**	**3**	**2**	**2**
16:1n-5	7	--	6	5	8	8
16:1n-7	9	--	10	10	7	7
16:2	10	--	9	7	10	10
16:3	**3**	**3**	**3**	**2**	**3**	**3**
18:0	8	--	8	9	9	9
18:1n-9	5	5	7	8	**4**	**4**
18:1n-7	6	6	5	6	6	6
18:2n-6	4	4	4	4	5	5
18:3n-3	**1**	**1**	**1**	**1**	**1**	**1**

Exposure to 100 mgL^{-1} NAFCs resulted in changes in the phospholipid composition of WT cells, most obvious in a loss of diversity, with the disappearance of the four classes of phospholipids initially present at the lowest concentration: 16:1n-5, 16:1n-7, 16:2, and 18:0. This was apparent when measured as absolute amounts of individual phospholipid classes, as well as when calculating each class as a percentage of total phospholipids present (Table 4.2). In addition, when examined as a proportion of total phospholipids, there was an increase in the relative abundance of 16:3 and 18:2n-6. At a lower significance level ($p<0.10$), we saw the concurrent increase in 16:0 and 18:3n-3 that accompanied this concentration of phospholipids into the classes initially present at highest abundance.

Table 4.2: Phospholipid composition of wild type cells with and without exposure to 100 mgL^{-1} naphthenic acid fraction components. Absolute amount of phospholipid indicates mg of the given lipid per gram of dried sample. % total phospholipids indicates what proportion of total phospholipids are made up by the given class. Data in bold indicates a significant difference in the average of the means in control and exposed cultures ($p<0.05$) as per two-tailed, independent sample t-test. n=3, df=4, *t* found in Table 7.5.

	Absolute amount phospholipid					As a % of total phospholipids				
	Control (SD)		/	Exposed (SD)		Control (SD)		/	Exposed (SD)	
14:0	--	--	/	--	--	--	--	/	--	--
16:0	14	(2.5)	/	25	(11)	21%	(0.43%)	/	27%	(4.1%)
16:1n-5	**2.5**	**(0.55)**	**/**	**--**	**--**	**3.7%**	**(0.37%)**	**/**	**--**	**--**
16:1n-7	**1.4**	**(0.26)**	**/**	**--**	**--**	**2.1%**	**(0.18%)**	**/**	**--**	**--**
16:2	**0.85**	**(0.11)**	**/**	**--**	**--**	**1.3%**	**(0.07%)**	**/**	**--**	**--**
16:3	13	(2.2)	/	22	(7.9)	**19%**	**(0.66%)**	**/**	**23%**	**(2.1%)**
18:0	**1.7**	**(0.28)**	**/**	**--**	**--**	**2.5%**	**(0.12%)**	**/**	**--**	**--**
18:1n-9	5.1	(1.0)	/	5.1	(4.6)	7.4%	(0.27%)	/	4.3%	(3.9%)
18:1n-7	2.7	(0.52)	/	2.1	(3.7)	3.9%	(0.24%)	/	1.7%	(3.0%)
18:2n-6	5.9	(1.1)	/	9.3	(3.7)	**8.7%**	**(0.16%)**	**/**	**9.7%**	**(0.45%)**
18:3n-3	20	(3.6)	/	33	(13)	30%	(1.06%)	/	34%	(3.0%)

Though there was an increase in the total amount of phospholipid present in cultures exposed to NAFCs, this difference was non-significant owing to increased variation in exposed cultures. Exposed cultures also saw an increase in the peroxidation index (Table 4.3), which is a unitless representation of the lipid stability. A higher lipid peroxidation index indicates increased susceptibility to degradation and oxidative damage.

Table 4.3: Selected analytical measures of phospholipid composition in cultures with and without exposure to 100 mgL^{-1} naphthenic acid fraction components. Data in bold indicates a significant difference in the average of the means (as per two-tailed, independent sample t-test) in control and exposed cultures ($p<0.05$) in italics ($p<0.10$). n=3, df=4, t found in Table 7.5.

	Wild Type		CC-400		CC-3395	
	Control (SD)	Exposed (SD)	Control (SD)	Exposed (SD)	Control (SD)	Exposed (SD)
mg phospholipid/g dry sample weight	68 (12)	96 (40)	**69 (5.1)**	**41 (5.0)**	60 (0.48)	63 (3.2)
Peroxidation index	**181 (1.2)**	**194 (12)**	*177 (14)*	*195 (4.6)*	162 (12)	165 (11)
Double bonds/mg saturated phospholipid	12 (2.2)	9.1 (5.1)	**9.3 (1.9)**	**20 (3.3)**	12 (1.7)	11 (1.4)
Double bonds/mg unsaturated phospholipid	3.6 (0.71)	3.3 (2.0)	**3.6 (0.26)**	**6.3 (0.70)**	3.5 (0.15)	3.4 (0.22)

Cultures of CC-400 cells also exhibited changes in phospholipid composition with NAFC exposure, though these changes were not as pronounced (Table 4.4). While there was no change in diversity, the overall amount of phospholipid decreased significantly (Table 4.3). In cultures exposed to NAFCs, there were significantly lower masses of 16:0, 18:0, 18:1n-9, and 18:2n-6; however, the relative composition of the phospholipid pool experienced fewer changes, exhibiting only a decreased relative abundance of 16:0 and an increased relative abundance of 18:3n-3.

Table 4.4: Phospholipid composition of CC-400 cells with and without exposure to 100 mgL^{-1} naphthenic acid fraction components. Absolute amount of phospholipid indicates mg of the given lipid per gram of dried sample. % total phospholipids indicates what proportion of total phospholipids are made up by the given class. Data in bold indicates a significant difference in the average of the means (as per two-tailed, independent sample t-test) in control and exposed cultures (p<0.05) in italics (p<0.10). n=3, df=4, t found in Table 7.5.

	Absolute amount phospholipid					As a % of total phospholipids				
	Control (SD)		/	Exposed (SD)		Control (SD)		/	Exposed (SD)	
14:0	0.10	(0.17)	/	0.042	(0.073)	0.13%	(0.2%)	/	0.11%	(0.20%)
16:0	**17**	**(1.9)**	**/**	**9.3**	**(1.7)**	**25%**	**(3.0%)**	**/**	**20%**	**(0.90%)**
16:1n-5	2.5	(0.14)	/	1.9	(0.49)	3.6%	(0.43%)	/	3.9%	(0.14%)
16:1n-7	0.22	(0.38)	/	0.087	(0.15)	0.30%	(0.51%)	/	0.24%	(0.41%)
16:2	1.6	(0.25)	/	1.3	(0.34)	2.3%	(0.23%)	/	2.6%	(0.10%)
16:3	12.4	(2.5)	/	11	(3.24)	18%	(3.0%)	/	23%	(1.6%)
18:0	**2.1**	**(0.35)**	**/**	**1.1**	**(0.35)**	3.1%	(0.67%)	/	2.2%	(0.28%)
18:1n-9	**2.1**	**(0.25)**	**/**	**1.1**	**(0.11)**	3.1%	(0.60%)	/	2.4%	(0.34%)
18:1n-7	2.9	(0.98)	/	1.6	(0.40)	4.2%	(1.1%)	/	3.3%	(0.09%)
18:2n-6	**5.9**	**(0.13)**	**/**	**3.7**	**(0.67)**	8.6%	(0.56%)	/	8.0%	(0.41%)
18:3n-3	21.7	(2.3)	/	16	(3.8)	**32%**	**(1.7%)**	**/**	**35%**	**(0.26%)**

After loss of approximately 40% of total phospholipids, the remaining phospholipids showed higher preponderance of double bonds, as measured by the number of double bonds present per mg of saturated phospholipid, and per mg of unsaturated phospholipid (Table 4.3). The value of the peroxidation index also increased in a similar manner and value to WT cells, but this change was only significant at the level of $p < 0.10$.

In comparison, cultures of CC-3395 did not show these signs of altered phospholipid composition in response to NAFC exposure. The only significant difference between control and exposed cultures was the appearance of minor amounts of 14:0 phospholipid in response to NAFC exposure.

Table 4.5: Phospholipid composition of CC-3395 cells with and without exposure to 100 mgL^{-1} naphthenic acid fraction components. Absolute amount of phospholipid indicates mg of the given lipid per gram of dried sample. % total phospholipids indicates what proportion of total phospholipids are made up by the given class. Data in bold indicates a significant difference in the average of the means (as per two-tailed, independent sample t-test) in control and exposed cultures (p<0.05) in italics (p<0.10). n=3, df=4, t found in Table 7.5.

	Absolute amount phospholipid					As a % of total phospholipids				
	Control (SD)		/	Exposed (SD)		Control (SD)		/	Exposed (SD)	
14:0	**--**	**--**	**/**	**0.23**	**(0.021)**	**--**	**--**	**/**	**0.37%**	**(0.014%)**
16:0	12	(0.93)	/	13	(0.83)	21%	(1.7%)	/	20%	(1.4%)
16:1n-5	2.1	(0.12)	/	2.3	(0.060)	3.5%	(0.22%)	/	3.7%	(0.11%)
16:1n-7	3.2	(0.61)	/	3.0	(0.75)	5.3%	(1.1%)	/	4.8%	(1.1%)
16:2	1.0	(0.17)	/	1.1	(0.13)	1.6%	(0.27%)	/	1.7%	(0.17%)
16:3	9.3	(1.8)	/	10	(1.8)	15%	(2.9%)	/	16%	(2.5%)
18:0	1.6	(0.11)	/	1.7	(0.012)	2.7%	(0.20%)	/	2.8%	(0.15%)
18:1n-9	8.0	(1.4)	/	7.8	(1.6)	13%	(2.4%)	/	12%	(2.5%)
18:1n-7	3.2	(0.11)	/	3.3	(0.086)	5.3%	(0.22%)	/	5.3%	(0.14%)
18:2n-6	4.6	(0.53)	/	4.9	(0.27)	7.6%	(0.82%)	/	7.8%	(0.32%)
18:3n-3	15	(1.1)	/	16	(1.5)	25%	(1.6%)	/	25%	(2.0%)

4.3.1.2 Other Lipids

Exposure to NAFCs induced changes in other lipid classes in addition to phospholipids. As seen in Table 4.6, there were a variety of effects on the composition of free fatty acids (FFAs), triacylglycerols (TAGs), cholesterol esters (CEs), and 1-2 and 1-3 diacylglyercols (1-2 DAGs and 1-3 DAGs). There were baseline differences under control conditions between WT, CC-400, and CC-3395; these differences are not discussed here, rather analysis focused on the changes within each line induced by NAFC exposure.

Table 4.6: Summary of changes in 1-2 diacyglyerols (1-2 DAGs), 1-3 diacyglycerols (1-3 DAGs), triacylglycerols (TAGs), free fatty acids (FFAs), and cholesterol esters (CEs) after exposure to 100 mgL^{-1} naphthenic acid fraction components for wild type (WT) cultures, and cell wall mutants CC-400 and CC-3395. Amounts are mg lipid/gram dried sample. Bolded values indicate $p<0.05$; italicized values are $p<0.10$. n=3, df=4, t in Table 7.6.

		Wild Type						CC-400						CC-3395					
		Control (SD)		/	Exposed (SD)			Control (SD)		/	Exposed (SD)			Control (SD)		/	Exposed (SD)		
1-2 DAG	Total	0.18	(0.060)	/	0.27	(0.090)		0.32	(0.089)	/	0.41	(0.11)		0.26	(0.070)	/	0.18	(0.049)	
	16:0	0.18	(0.060)	/	0.27	(0.090)		0.23	(0.074)	/	0.20	(0.052)		0.17	(0.053)	/	0.13	(0.042)	
	18:0	–	–	/	–	–		**0.10**	**(0.033)**	/	**0.22**	**(0.06)**		0.092	(0.031)	/	0.045	(0.015)	
1-3 DAG	Total	**0.30**	**(0.10)**	/	**2.9**	**(0.79)**		1.4	(0.46)	/	0.96	(0.33)		0.40	(0.11)	/	0.37	(0.11)	
	16:0	**0.14**	**(0.049)**	/	**0.71**	**(0.20)**		**0.48**	**(0.16)**	/	**–**	**–**		0.24	(0.063)	/	0.17	(0.052)	
	18:0	**0.16**	**(0.054)**	/	**0.60**	**(0.19)**		0.80	(0.27)	/	0.96	(0.33)		0.16	(0.04 4)	/	0.20	(0.058)	
	18:1n -9	**–**	**–**	/	**0.44**	**(0.15)**		**0.085**	**(0.029)**	/	**–**	**–**		–	–	/	–	–	
	18:2n -6	**–**	**–**	/	**0.69**	**(0.23)**		–	–	/	–	–		–	–	/	–	–	
	18:3n -3	**–**	**–**	/	**0.46**	**(0.16)**		–	–	/	–	–		–	–	/	–	–	
TAG	Total	**0.25**	**(0.085)**	/	**0.75**	**(0.20)**		0.81	(0.5 1)	/	0.97	(0.26)		0.16	(0.056)	/	0.31	(0.26)	
	16:0	*0.25*	*(0.085)*	/	*0.51*	*(0.17)*		0.52	(0.47)	/	0.58	(0.17)		0.16	(0.056)	/	0.13	(0.21)	
	18:0	**–**	**–**	/	**0.24**	**(0.080)**		*0.19*	*(0.061)*	/	*0.39*	*(0.12)*		**–**	**–**	/	**0.086**	**(0.028)**	
	18:1n -9	–	–	/	–	–		**0.093**	**(0.032)**	/	**–**	**–**		–	–	/	–	–	
	18:2n -6	–	–	/	–	–		–	–	/	–	–		**–**	**–**	/	**0.091**	**(0.029)**	
FFA	Total	0.62	(0.39)	/	1.6	(1.0)		1.3	**(0.34)**	/	**3.3**	**(0.97)**		0.60	(0.26)	/	0.38	(0.12)	
	16:0	0.29	(0.31)	/	0.94	(0.87)		0.52	(0.16)	/	0.95	(0.31)		0.36	(0.20)	/	0.21	(0.070)	
	16:1n -5	–	–	/	–	–		**0.34**	**(0.12)**	/	**–**	**–**		–	–	/	–	–	
	16:3	–	–	/	–	–		**–**	**–**	/	**0.14**	**(0.047)**		–	–	/	–	–	
	18:0	*0.33*	*(0.10)*	/	*0.63*	*(0.20)*		0.40	(0.12)	/	0.46	(0.20)		0.25	(0.073)	/	0.17	(0.054)	
	18:1n -7	–	–	/	–	–		**–**	**–**	/	**0.08**	**(0.027)**		–	–	/	–	–	
	18:2n -6	–	–	/	–	–		**–**	**–**	/	**0.22**	**(0.066)**		–	–	/	–	–	
	18:3n -3	–	–	/	–	–		**–**	**–**	/	**1.4**	**(0.42)**		–	–	/	–	–	
CE	Total	**0.40**	**(0.11)**	/	**1.0**	**(0.35)**		*0.79*	*(0.42)*	/	*0.18*	*(0.18)*		0.30	(0.10)	/	0.21	(0.17)	
	16:0	*0.23*	*(0.08)*	/	*0.53*	*(0.18)*		0.32	(0.24)	/	0.18	(0.18)		0.12	(0.042)	/	0.078	(0.10)	
	16:1n -7	–	–	/	–	–		**0.11**	**(0.036)**	/	**–**	**–**		0.080	(0.027)	/	0.067	(0.023)	
	18:0	**0.17**	**(0.06)**	/	**0.51**	**(0.17)**		**0.36**	**(0.19)**	/	**–**	**–**		0.10	(0.032)	/	0.065	(0.081)	

In WT cells exposed to NAFCs, there was a significant increase in the overall abundance and diversity of TAGs and 1-3 DAGs, an increase in the presence of CEs, and a weakly significant ($p < 0.10$) increase in 18:0 FFAs. The cell wall mutant CC-400 had a significant increase in the overall abundance and diversity of FFAs, an increase in 18:0 1-2 DAG, and a loss of diversity in TAGs, 1-3 DAGs, and CEs. The fewest changes were observed in CC-3395, which exhibited an increased diversity of TAGs with NAFC exposure.

4.3.1.3 Lipid FTIR

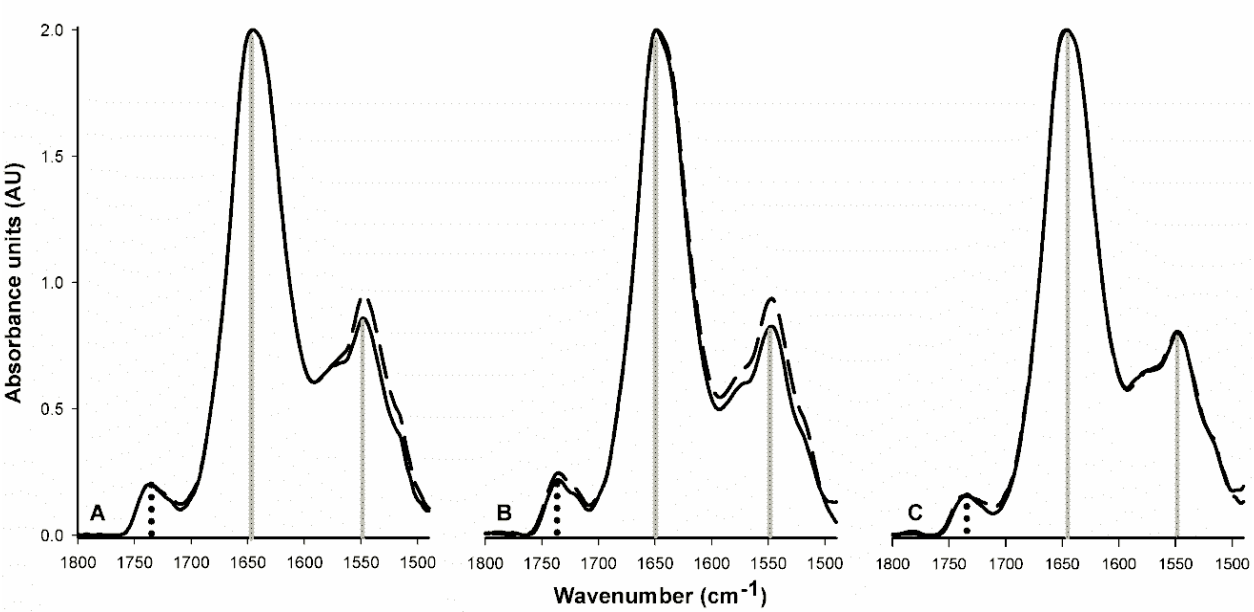

Figure 4.1: Fourier transform infrared spectromicroscopy of single living cells. Spectra are the average of ten individual cells, baseline corrected and normalized to the Amide I band. Wild type (A), CC-400 (B), CC-3395 (C). Solid black lines represent control cells, dashed black lines represents cells exposed to 100 mgL^{-1} oil sands naphthenic acid components for 24 hours. Solid grey lines are the Amide I (1650 cm^{-1}) and Amide II (1550 cm^{-1}) peaks. Lipid peak (1740 cm^{-1}) is marked by the black dotted line.

When examined with Fourier-transform infrared spectromicroscopy, there were moderate to no changes in the overall relative abundance of cellular lipids. The spectra in Figure 4.1 represent the average spectra of individual algal cells. The ratio of the lipid peak (1740 cm^{-1}) to

the Amide I protein peak (1650 cm^{-1}) can be used as a measure of lipid accumulation or depletion relative to the protein pool (Dean et al., 2010), though this was somewhat complicated by the change in the ratio of Amide I to Amide II (1550 cm^{-1}) peaks in exposed WT and CC-400 cultures. This change is often indicative of changes to protein confirmation and secondary structure (Holman et al., 2000). When compared to Amide I, there was no change in amount of lipids present for WT or CC-3395 cells, while CC-400 exhibited a slight increase. When the increased level of Amide II was taken into account, WT and CC-400 cells exposed to NAFCs appeared to show a minor decrease in the presence of lipids. Despite these variations, it was clear that by these measures, there were none to minor changes in the presence of lipids in comparison to protein.

4.3.1.4 Lipid Confocal Using Nile Red

Contrary to TLC and FTIR analysis of WT cells, visualization of neutral stainable lipids with Nile Red led to a sharp decrease in apparent lipid levels after exposure to 100 mgL^{-1} NAFCs (Figure 4.2 A, B). Control cells exhibited a relatively even distribution of neutral stainable lipids across the cells, with some concentration into lipid droplets. In exposed cells, not only was the overall lipid fluorescence decreased, but also the distribution. Lipids were concentrated along the cell membrane, and droplets were absent.

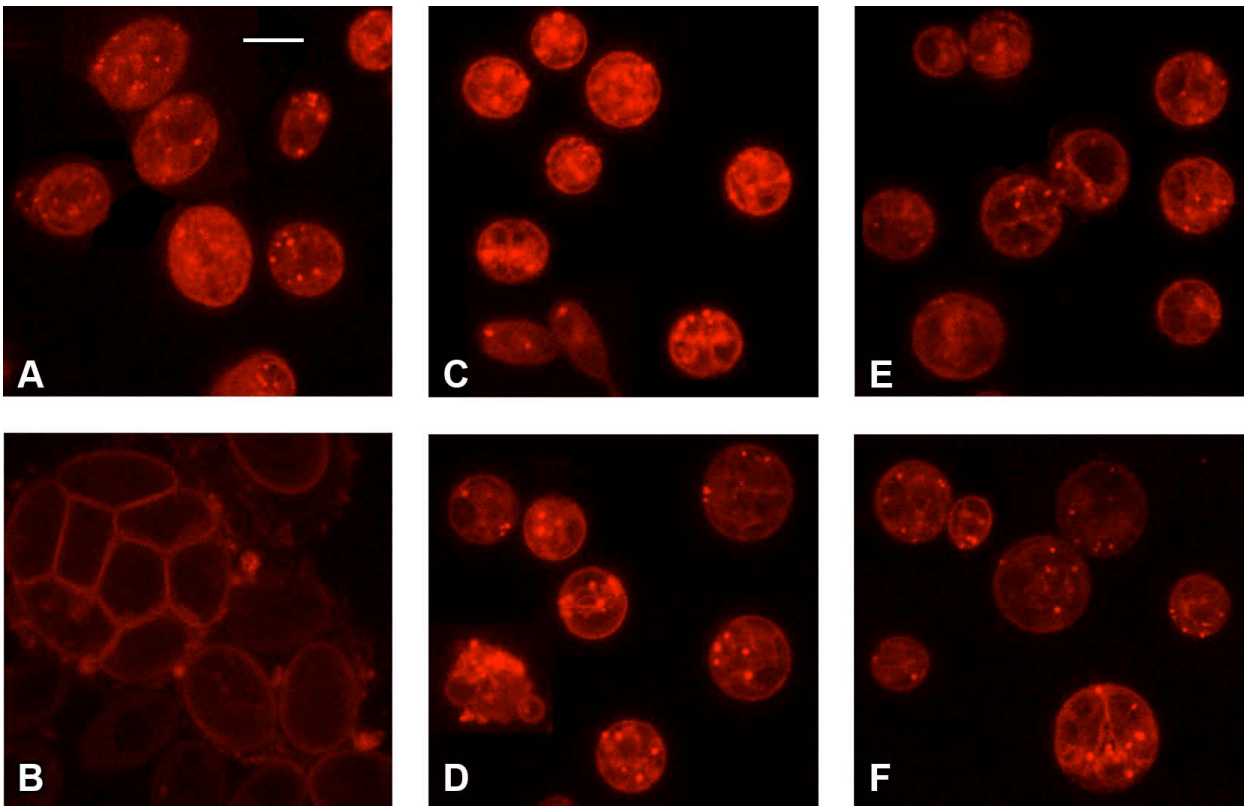

Figure 4.2: Nile red fluorescence enabling the visualization of neutral lipids. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions. Cells of CC-3395 under control (E) and exposed (F) conditions. Scale bare is 5 µm.

The cell wall mutants CC-400 and CC-3395 both exhibited differences from WT cells under control conditions (Figure 4.2 A, C, E). Due to a lower baseline fluorescence, images for both mutants were taken at a higher laser intensity than WT cells. This corroborates the similar ratios of lipid:Amide I seen with FTIR (Figure 4.1) in all lines, as these mutants lacked some to all cell wall glycoproteins, and would be expected to exhibit lower absolute levels of lipids to maintain the same lipid:protein ratio. Control cells of CC-400 exhibited lipid distribution throughout the cell, with some concentration along the cell membrane and the presence of large lipid droplets. After 24 hours exposure to 100 mgL^{-1} NAFCs (the lowest concentration found to have any impact on culture growth rate) there was an overall decrease in the neutral stainable lipids, though this change was not as pronounced as in exposed WT cells. There remained lipid

distributed throughout the cell, with some concentration along the outer membrane. Lipid droplets were also present, though they appear to be smaller in size than in unexposed cells. Cultures of CC-3395 appeared to have lower baseline lipid fluorescence than those of CC-400, and lipid droplets present were fewer and smaller. They again exhibited lipid distribution throughout the cell, with minor concentration along the outer membrane. After 24 hours exposure to 100 mgL^{-1} NAFCs (a concentration found to have no effect on the culture growth rate) few physiological changes were detected, with the exception of what appeared to be the concentration of lipid droplets.

4.3.2 Other Physiological Changes

4.3.2.1. Membrane Confocal Using FM1-43

Normally staining with FM1-43 allows only visualization of outer cell membranes; it is likely the use of DMSO as a carrier for FM1-43 allowed penetration of the dye into the cell and visualization of internal membranes (Figure 4.3). In WT cells exposed to NAFCs (Figure 4.3 B), much of the internal detail was lost when compared to unexposed control cells (Figure 4.3 A). Autofluorescence and visual imaging confirmed the presence of the chloroplast and other structures, so this change was not likely due to loss of internal structures, but more likely related to decreased dye penetration.

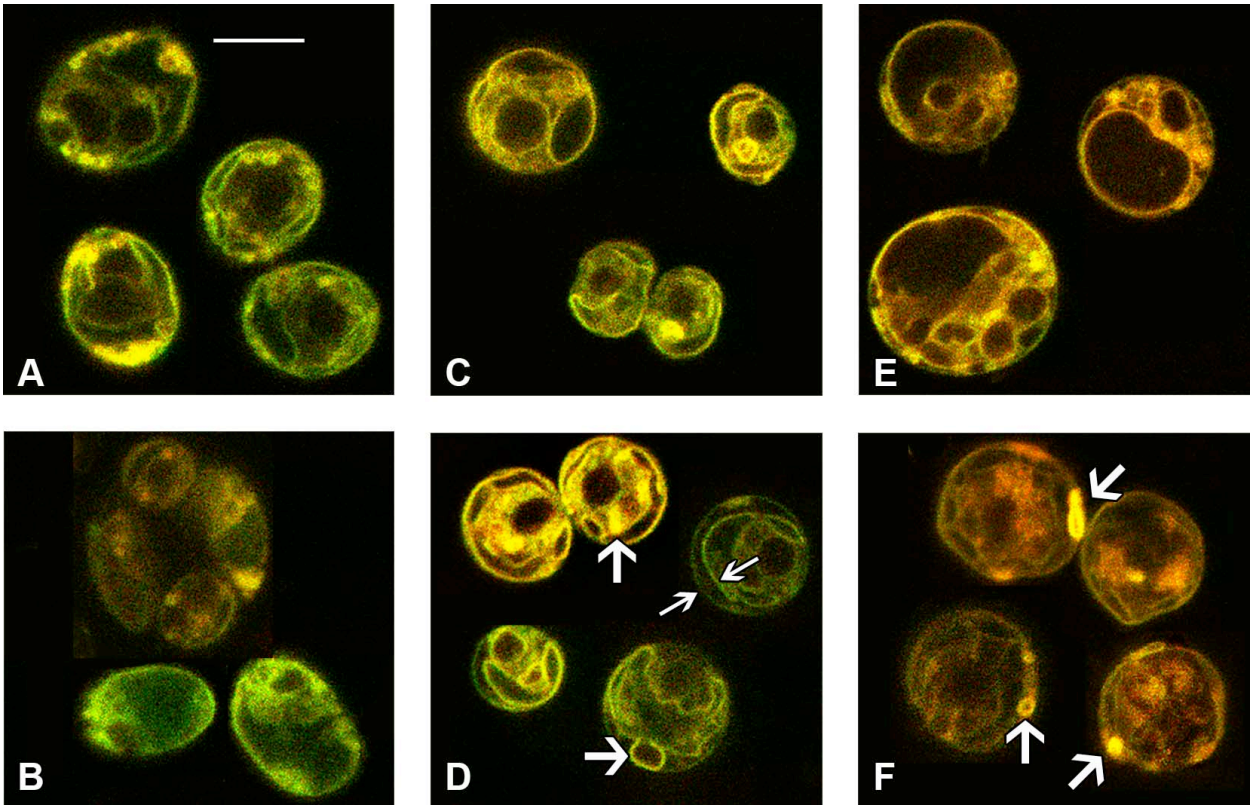

Figure 4.3: FM1-43 visualization of membrane lipids. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (B). Cells of CC-400 under control (C) and exposed (D) conditions; arrows indicate brightly staining bodies and vacuoles, small arrows indicate increased space between the chloroplast and cell membranes. Cells of CC-3395 under control (E) and exposed (F) conditions; arrows indicate actively excreted vesicles and free dye in the intracellular space. Scale bar is 5 µm.

For the cell wall mutant CC-400, exposure to NAFCs appeared to increase the variability of staining. Control cells were fairly uniform in brightness, with well-defined fluorescence of many internal membranes, including the chloroplast and vacuoles. Cells exposed to NAFCs exhibited large variation in overall fluorescence intensity, and appeared to have more well defined structures, including vacuoles. There appeared to be more space between the chloroplasts and the external membranes, and there were a number of small, brightly staining bodies present. The other cell wall mutant, CC-3395, exhibited similar fluorescence patters under control conditions. In cells exposed to 100 mg L^{-1} of NAFCs for 24 hours, there were some changes in the pattern of staining. Overall brightness was reduced, and there appeared to

be concentration of free dye into intracellular spaces. There were a number of small, globular areas of extremely concentrated fluorescence; during imaging the transport of these small vesicles to the cell surface was observed, as well as their subsequent excretion into the media. What appeared to be similar excretory vesicles were also noted in CC-400 cells exposed to NAFCs; however these were not observed to be actively excreted.

4.3.2.2 Protein Confocal Using SYPRO Orange

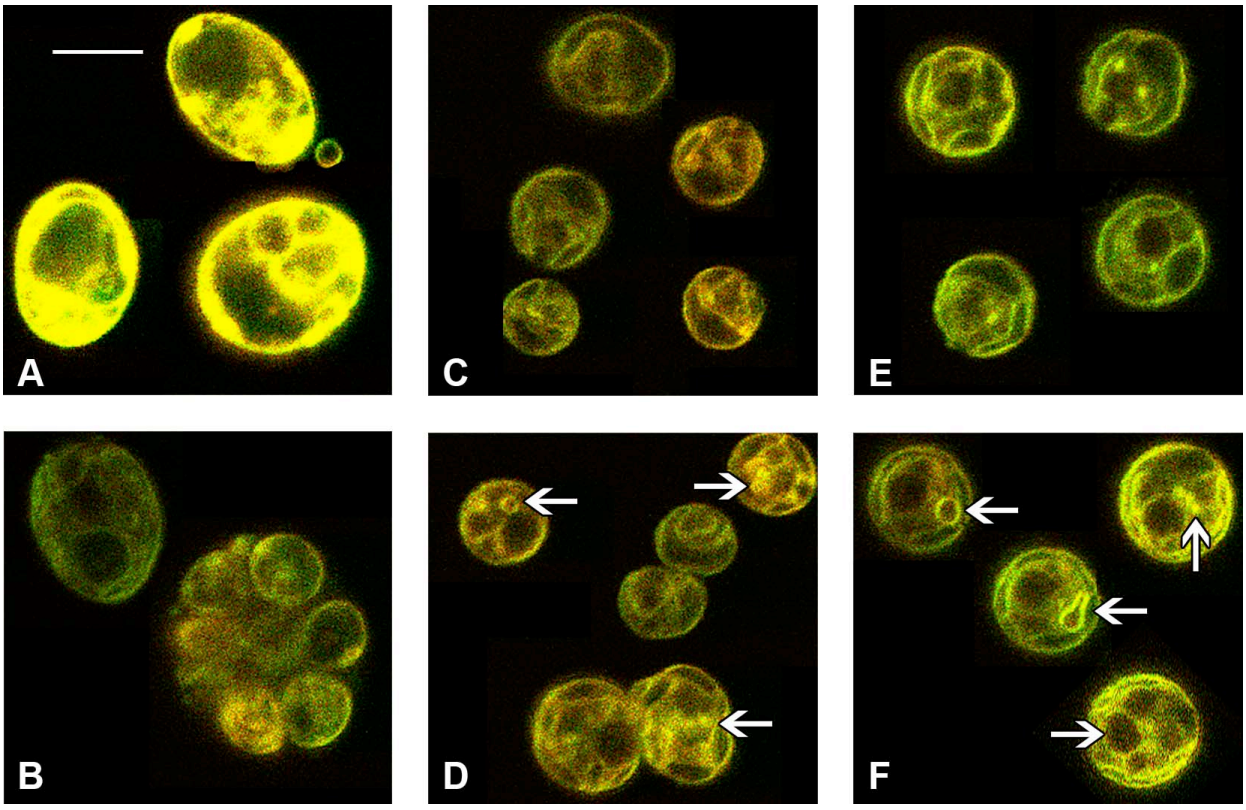

Figure 4.4: SYPRO Orange visualization of internal proteins visualized via a z-series slice through the centre of the cell. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions. Cells of CC-3395 under control (E) and exposed (F) conditions. Arrows indicate vesicles and granular structures. Scale bar is 5 µm.

As in the case of FM1-43, WT cells exposed to NAFCs and stained with SYPRO Orange appeared to have a decreased uptake of fluorescent dyes. The internal detail of control cells

(Figure 4.4 A) was much greater than that of cells exposed to NAFCs (Figure 4.4 B). No such changes were present in CC-400 cells (Figure 4.4 C, D), or CC-3395 (Figure 4.4 E, F), though exposed cells of CC-3395 appeared to have increased presence of small bodies that were likely to be granules or vesicles similar to those seen previously with FM1-43 (Figure 4.3).

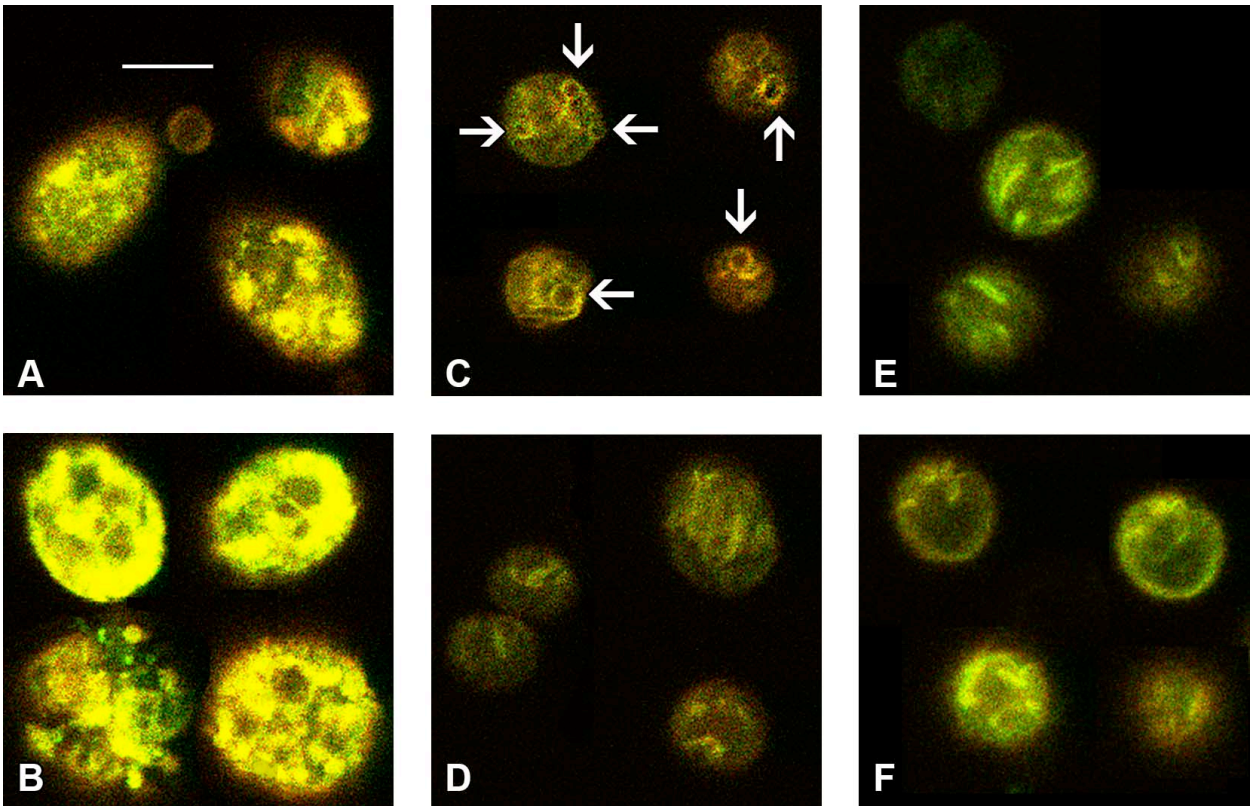

Figure 4.5: SYPRO Orange visualization of surface proteins, obtained via a z-series slice encompassing the top of the cell. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions; arrows highlight surface features lost with NAFC exposure. Cells of CC-3395 under control (E) and exposed (F) conditions. Images are from the cell surface. Scale bar is 5 µm.

In comparison to the decreased visualization of internal proteins in WT cells, cell surface protein staining with SYPRO Orange was increased (Figure 4.5 A, B). For the cell wall mutant CC-400, NAFC exposure appeared to decrease the presence of some surface features (Figure 4.5 C, D). No appreciable changes were noted in CC-3395 (Figure 4.5 E, F).

4.3.2.3 DNA Confocal Using SYTO 9

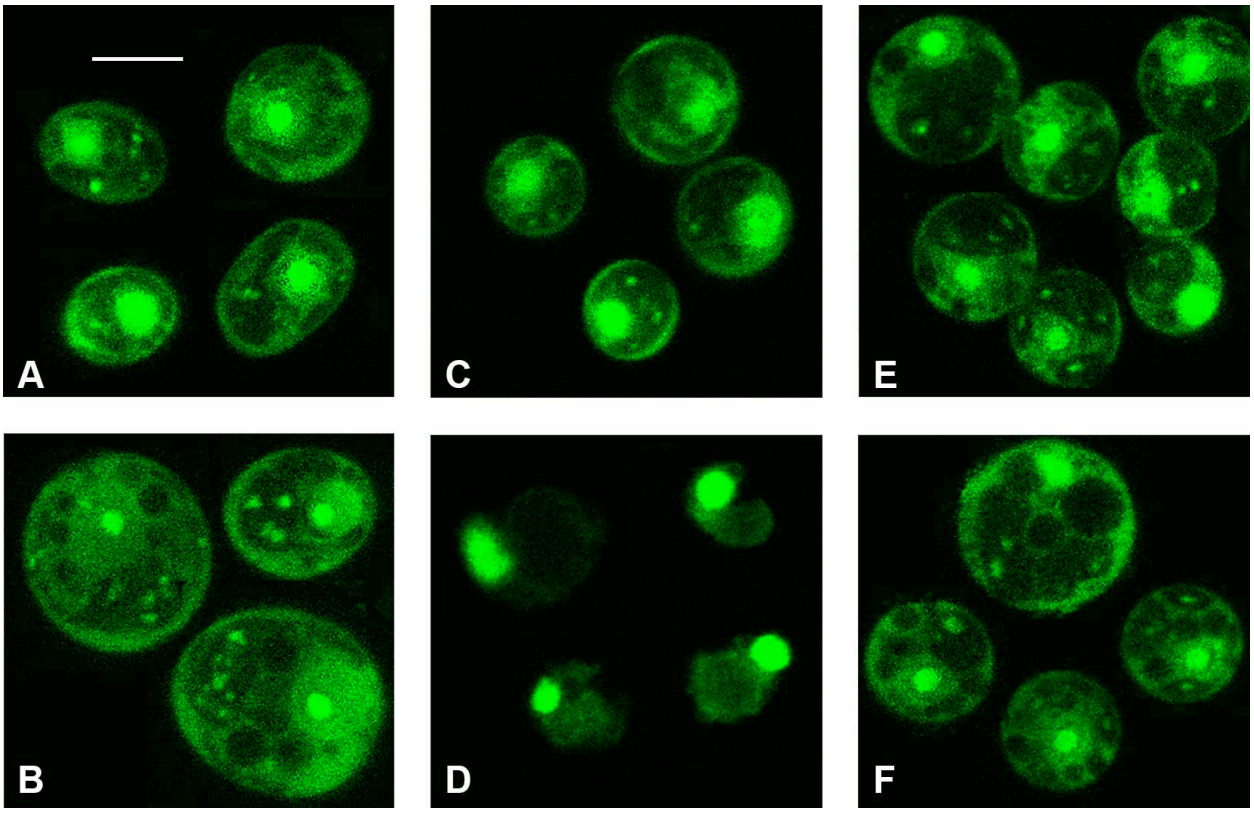

Figure 4.6: Visualisation of cytoplasm and DNA using SYTO 9. Smaller dots are mitochondria. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions. Cells of CC-3395 under control (E) and exposed (F) conditions. Scale bar is 5 µm.

Exposure to NAFCs for 24 hours also appeared to impact morphology of the nucleus and cytoplasm distribution (Figure 4.6). For all algal strains, there appeared to be a contraction of the size of the nucleus, though this trend was most pronounced in WT cells. For WT and CC-3395 cells, there was also an increased amount of cytoplasm present between the chloroplast and outer membrane, and an increase in vacuolization (Figure 4.6 A, B, E, F). It was unclear if this was due to swelling of the cell or retraction of the chloroplast. In comparison, cells of CC-400 exposed to NAFCs appeared to lose cytoplasmic definition; staining was less even across the cell as a whole and mitochondria were not visible.

4.3.2.4 Eyespots Visualization

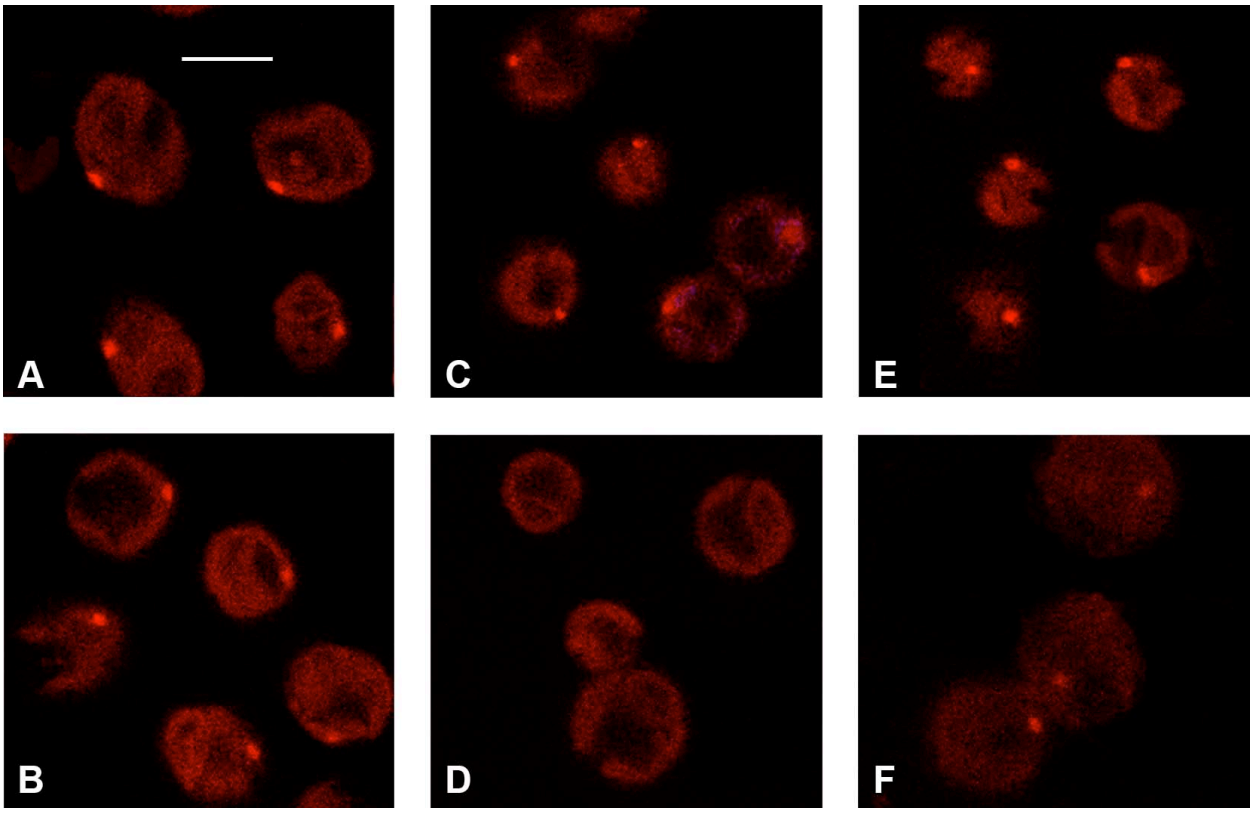

Figure 4.7: Representative cells showing autofluorescence of the eyespot (bright dot) and bleed through from chlorophyll autofluorescence (diffuse cup-shaped or circular fluorescence), indicating photosynthetic viability. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions. Cells of CC-3395 under control (E) and exposed (F) conditions. Scale bar is 5 μm.

Exposure to 100 mgL^{-1} of NAFCs had little effect on chlorophyll autofluorescence (Figure 4.7). There was, however, a loss of the structured eyespot in the cell wall mutant CC-400 (Figure 4.7 D).

4.3.2.5 Visible Transmitted Light Imaging

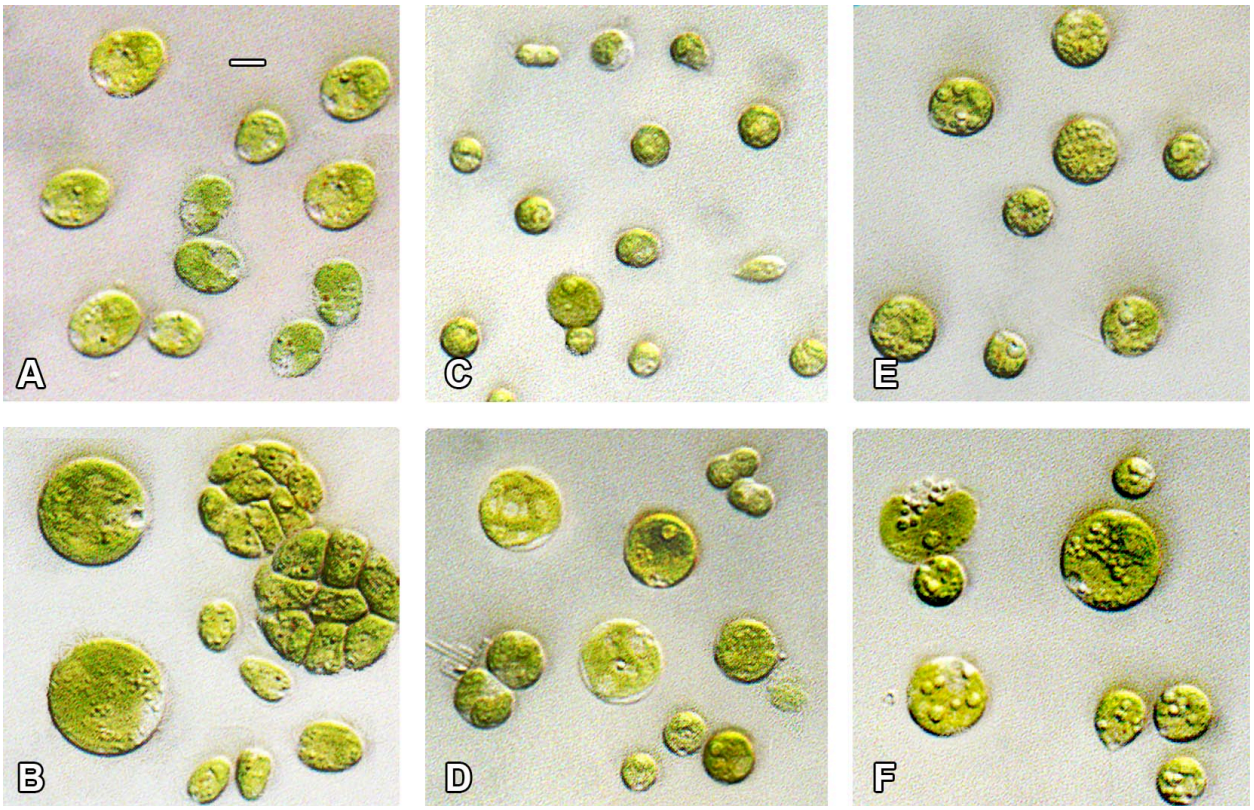

Figure 4.8: Representative cells under visible light. Wild type cells under control conditions (A) and after exposure to 100 mgL^{-1} naphthenic acid fraction components (NAFCs, B). Cells of CC-400 under control (C) and exposed (D) conditions. Cells of CC-3395 under control (E) and exposed (F) conditions. Scale bar is 5 µm.

Some of the changes induced by NAFC exposure were evident with visible transmitted light imaging (Figure 4.8). In contrast to other methods of visualization, many of these changes were similar across cell lines. In all cultures, exposure resulted in increased cell size and roundness, in addition to increased texture of the cell body. While it could not be positively ascertained what structures were responsible, it appeared likely to be a mix of the staining bodies revealed with SYPRO Orange (Figure 4.4) and FM1-43 (Figure 4.3), as well as the excretory vesicles imaged with FM1-43 (Figure 4.3) and vacuolization evident in S9 staining (Figure 4.6). Under visible light, these features were most pronounced in CC-3395, in keeping with the CLSM observations. The small bodies present in WT cells exposed to NAFCs were present at higher

concentration than would be expected from the corresponding CLSM images; this again suggested decreased uptake of fluorescent dye by exposed cells which would have been in keeping with alteration of membrane fluidity to exclude toxic substances or stabilize the membrane.

4.4 Discussion

As a whole, this work indicates that exposure to NAFCs mediated a number of physiological and metabolic responses in *C. reinhardtii*. Wild type cells and the cells of the two cell wall mutants, CC-400 and CC-3395, exhibited different reactions to NAFC exposure; as such it may be hypothesized that the cell wall and its structure contributes to the mediation and potentiation of NAFC toxicity.

After exposure to NAFCs, WT cells exhibited a sharp decrease in Nile Red fluorescence binding (Figure 4.2), which would be expected to correlate to a similar decrease in the presence of neutral stainable lipids, something that was not borne out by TLC analysis (Table 4.6) or the FTIR ratio of overall lipids to proteins (Figure 4.1). Taken together with the loss of detailed visualization of internal proteins (Figure 4.4) and membranes (Figure 4.3), this suggested that NAFC-exposed cells uptake lower levels of these fluorescently labelled dyes. As this response was unlikely to be specific to these three fluorescent dyes, it suggested that NAFC exposure resulted in altered interaction with and uptake of macromolecules from the environment. Two main physiological changes were observed that might have contributed to this modification of environmental interaction, the first being that the observed changes in phospholipid and lipid composition (Table 4.1, Table 4.2, Table 4.3) altered membrane permeability. The observed loss in phospholipid diversity was primarily attributable to the loss of C_{16} mono- and polyunsaturated

fatty acids. Combined with the increase in cholesterol esters (Table 4.6) this would be expected to have decreased membrane fluidity, as the presence of unsaturated phospholipids and cholesterol esters are known to respectively increase and decrease membrane fluidity (Cooper, 2000). This response might have been expected for a number of reasons, including: stabilization of the membrane in direct response to surfactant insertion into the bilayer, increased membrane stability in response to modification of cell wall protein confirmation, or reduction of cross-membrane diffusion and leakage. The second change that might have contributed to decreased dye uptake was the modification and accumulation of cell surface proteins (Figure 4.1, Figure 4.5) and an extensive exopolymer matrix (Figure 2.3). Any of these combinations of factors would have been expected to result in the altered interaction with environmental macromolecules, either as a result of NAFC-altered physiology or cellular attempts to limit uptakes of substances such as NAFCs.

Exposure to surfactants is known to alter protein confirmations and physically induce changes to membrane permeability via insertion (Lewis, 1990, 1991), both of which result in altered environmental interaction, and align perfectly with the changes seen in WT cells upon NAFC exposure. The increase in vacuolization seen with S9 (Figure 4.6) was not obvious with the other dyes, but also suggested differences in intracellular transport or osmotic balance, as did the increased layer of cytoplasm between the chloroplast and cell membrane, and the reduced nuclear volume (Finan et al., 2011). However, it was not clear if this was due to cellular swelling, chloroplast shrinkage, or a combination of the two, though the increased cell roundness (Figure 4.8) and similar chloroplast volumes/autofluorescence (Figure 4.7) suggested this was attributable to swelling. Both effects would have been characteristic of metabolic leakage

induced by increased membrane permeability (Sun et al., 2004) or pore formation (Carrillo et al., 2003).

The cell wall mutant CC-400 also exhibited a variety of physiological changes in response to NAFC exposure, though these were less pronounced than those in the fully-walled WT. There was a similar decrease in Nile Red fluorescence (Figure 4.2), though at a much less pronounced level. Exposed cells had a sharp decrease in the amount of phospholipids present, though there was no decrease in phospholipid diversity (Table 4.3, Table 4.4), and changes in other lipid classes – including neutral stainable lipids – were primarily related to changes in diversity, rather than overall amounts (Table 4.6), again consistent with changing permeability or lipid metabolism. Visualization of membranes (Figure 4.3) and internal proteins (Figure 4.4) retained the same degree detail as in control cells, though both showed increased presence of granules/vesicles similar those shown to be actively excreted by cells of CC-3395 exposed to NAFCs. Changes in the distribution and definition of the nucleus, chloroplast, and mitochondria were present in NAFC-exposed cells (Figure 4.6); given the exponential growth of these cells under the given exposure conditions (Figure 2.1), it is unlikely that this represented a loss of mitochondria and cytoplasmic integrity. However, the swelling, loss of cell shape, distribution of nuclear-staining material, as well as the loss of chloroplast integrity in some cells (Figure 4.8 D) were again similar effects to what would be expected given metabolic leakage (Sun et al., 2004). This has been reported not to be directly correlated to surfactant toxicity (Glover et al., 1999) and may be less important than protein interaction (Van Hamme et al., 2006). The loss of eyespot integrity (Figure 4.7 D) observed here has also been observed with organic solvent exposure (Soto et al., 1979) and may be consistent with stress and toxic effects. It is speculated that the arrangement of the eyespot of *Chlamydomonas* spp. is stabilized by the presence of a

variety of hydrophobic proteins, and a number of the eyespot mutants show defects in specific proteins (Harris, 2009, pgs 62-64), suggesting its structure is sensitive to changes in protein confirmation such as those than can be induced by surfactant exposure. The penetration of some surfactants into the cell itself is also known to potentially disrupt thylakoid organization (Cserhati et al., 2002; Popova and Kemp, 2007), similar to that seen in some cells of CC-400 (Figure 4.8), and the different wall and membrane structure would be expected to impact the ease of penetration of some of the complex NAFC mixture into the cell (Rosen et al., 2001; Vonlanthen et al., 2011).

Despite an overall relative tolerance to NAFC exposure, the 40% decrease in phospholipid amounts (Table 4.1), smoothing of cell surface protein features (Figure 4.5), and loss of structural cholesterol esters (Table 4.6) were suggestive of surfactant-induced solubilization of membrane lipids that may occur at certain surfactant/lipid ratios (Carrillo et al., 2003). CC-400 retains the innermost cell wall layer, W1, and the outermost layer, W7. While W1 is an insoluble fibrous frame, W7 is looser amorphous network of branching fibres and non-structural glycoproteins (Harris, 2009, pgs 29-33) and is likely responsible for the wide variation in the shape and size of unexposed cells that was observed (Figure 4.8 C, D). Given that surfactant:protein interactions are generally stronger than protein:membrane lipid interactions (Jones, 1992) and the loose binding of the W7 layer, it is not unreasonable to postulate its removal. This was possible in NAFC-exposed WT cells as well, with the SYPRO Orange visualized changes in surface protein and FTIR changes in protein confirmation due to modification of cell wall layers W1-W6. Though there was no loss of diversity in phospholipids as with exposed WT cells, the changes in the relative amounts of specific phospholipids, as well as the increased number of double bonds per mg of saturated and unsaturated phospholipid

(Table 4.3), suggested changes to membrane fluidity as would be expected with surfactant exposure (Lewis, 1990; Sun et al., 2004; Van Hamme et al., 2006). Despite these changes, there was not the same evidence of reduced fluorescent dye uptake as in WT cells. It is possible however, that the increased surface glycoproteins and extensive exopolymer matrix found in exposed WT cells were strong contributors to this effect. In addition, the effects of changes to the composition of phospholipid and lipid pools was different than that experienced by NAFC-exposed WT cells, where the changes were strongly indicative of decreased membrane fluidity. For exposed CC-400 cultures, presence of bodies seen to be excreted in CC-3395 cells suggested that this may also have been part of the cells' regulatory response to NAFCs.

Perhaps unsurprisingly, exposure to 100 mgL^{-1} of NAFCs resulted in few physiological changes for CC-3395. This mutant, believed to completely lack a cell wall, had a growth rate unaffected by exposure to NAFCs. Nile Red staining (Figure 4.2 E, F) revealed no changes in the overall concentration of neutral lipids; there was a possible concentration into lipid droplets but this was difficult to quantify. Exploration of surface (Figure 4.5) and cell (Figure 4.4) proteins revealed few changes, with the exception of the appearance of small bodies in the cell cross section believed to be granules or vesicles. Membrane visualization with FM1-43 (Figure 4.3) was perhaps the most interesting, as it revealed the presence of small vesicles actively secreting the uptaken dye. Imaging with S9 (Figure 4.6) was also suggestive of possible changes to the osmotic balance, given the visualization of a variety of vacuoles, minor decrease in nuclear volume, and an increased buffer of cytoplasm between the chloroplast and cell membrane, though as with exposed WT cells, it was unclear if this was due to cell swelling or to chloroplast contraction, and resembled physiological changes induced by increased metabolite leakage (Sun et al., 2004). Analysis of cellular phospholipids revealed virtually no changes in the amount or

composition of phospholipids: the only significant change was the appearance of minor levels of 14:0 phospholipids not found in the examination of unexposed, control cells. Similarly, total lipid pools and metabolism appeared to be relatively unaffected, given the lack of significant changes save for increased TAG diversity. While CC-3395 exhibited only minor physical changes in response to NAFCs, there appeared to be a physiological modification in increased production and export of excretory vesicles.

4.5 Conclusions

The investigated effects of NAFC exposure on *C. reinhardtii* appeared to vary depending upon cell wall presence and structure. Given the differences in cell wall structure and membrane composition, and the broad variation in surfactant response based on chemical and species (Lewis, 1992; Venhuis and Mehrvar, 2004) this was expected. Some broad similarities were found that agreed with general surfactant effects, including changes in phospholipid composition and fluidity, differences in total lipid metabolism, protein modification, and indicators of osmotic stress or metabolite leakage. However, the specific mode of these effects varied depending upon the algal line.

Exposed WT cells evidenced decreased uptake of fluorescent dyes, decreased membrane fluidity (loss of unsaturated phospholipids and increased cholesterol), in addition to modification of cell surface proteins, changes in confirmation of overall protein pools, and signs of osmotic stress or metabolic leakage including cell swelling, shrinkage of the nucleus, and increased vacuolization. Cells of CC-400 exhibited different patterns of response: there was some smoothing of protein features on the cell surface, as well a 40% loss of phospholipids without a change in class diversity, which made it more difficult to quantify the effects on membrane

fluidity. There was not the same evidence of reduced uptake of fluorescent dyes as in WT, but visualization of the cytoplasm was highly indicative of metabolic leakage. In addition, the total loss of eyespots and disruption of chloroplast integrity in some cells suggested the possibility of increased internal contact with agents able to disrupt protein:membrane interactions or protein confirmations. Unsurprisingly, few changes were observed in CC-3395 after NAFC exposure. Aside from the shared indicators of osmotic stress or metabolic leakage, the effects appeared limited to the appearance of 14:0 phospholipids, the formation in intracellular granules, and increased active excretion of secretory vesicles.

The sum of induced changes in all algae was again consistent with surfactant effects, including changes to membrane fluidity, protein confirmation, and osmotic stress or metabolic leakage. While increases were visible in vacuolization, cell size, and cell roundness, the degree varied. In WT cells, total effects were consistent with altered environmental interaction through a combination of decreased membrane fluidity, changes in protein confirmation, and increased production of extracellular matrix. Cells of CC-400 exhibited changes in lipid composition dwarfed by the total loss in phospholipid amounts and the greatest degree of indication of metabolic loss, which suggested solubilization and removal of parts of the plasma membrane and some cell wall proteins, and different NAFC-induced effects on membrane fluidity than were found in WT cells. Though speculative, the difference in internal structure – loss of eyespot and changes in chloroplast organization – suggested different internal physiological responses than WT or CC-3395 cells. Exposure in CC-3395 cells appeared to have minimal influence on membrane fluidity or environmental uptake, though there was evidence of increased vesicle activity, possibly linked to increased excretion of substances taken from the environment (such as NAFCs), osmotic stress, or repair of the cell membrane. Changes in TAGs, DAGs, and FFAs

were also less pronounced than WT or CC-400, suggesting that the changes in lipid metabolism were minor. Similar vesicles and brightly staining bodies were observed in exposed CC-400 cells, though they were not actively observed to be exported, suggesting that it responded to a similar stressor in a similar manner, and combined the responses of the WT and CC-3395.

Overall, the presence of a cell wall appeared to be consistent with increased physiological stress and change. The retention of cell wall layers W1 and W7 in CC-400 also appeared to potentiate toxicity, though in a different manner than the fully-walled WT cells. This effect could be attributed to different interactions between NAFC compounds and a full cell wall in WT cells, or possibly to increased interaction with the cellular envelope in CC-400, especially if the loss of globular surface features visualized with SYPRO Orange indicated removal of the loosely bound framework of the W7 layer. Each of the three cell lines also had different phospholipid compositions under control conditions, suggesting their initial membrane fluidity was different; this might also have been a factor in NAFC toxicity. Regardless of the root cause, the presence of a cell wall or cell remnants appeared to result in a variable but significant response to NAFC exposure. Amongst the stress responses induced, NAFCs appeared to have the ability to induce modification of phospholipid membranes, lipid metabolism, and surface proteins.

CHAPTER 5
5.0 GENERAL CONCLUSIONS AND DISCUSSION

5.1 Completion of Research Objectives

5.1.1 Objectives 1 and 2

Determination of NAFC toxicity to wild type, fully-walled cells of Chlamydomonas reinhardtii. Exposure to NAFCs resulted in a dose-dependent decrease in the rate of growth of growth of WT cultures. Exponential growth was found even after exposure to 100 mgL^{-1} of NAFCs. This was unexpected given that the environmentally relevant species, *C. frigida*, has been found to disappear from waters containing more than 10-20 mgL^{-1} NAFCs (Leung et al., 2003).

Determination of NAFC toxicity to the cell wall mutants CC-400 and CC-3395. Compared to WT cultures, both cell wall mutants were relatively tolerant to NAFC exposure. The growth rate of CC-400 was impacted only after exposure to the highest tested concentration of NAFCs, 100 mgL^{-1}. Even this exposure concentration failed to elicit a change in the growth rate of CC-3395.

5.1.2 Objective 3

Investigation of NAFC-induced physiological responses of wild type Chlamydomonas reinhardtii and its cell wall mutants CC-400 and CC-3395. Observed physical changes varied by cell line, with WT and CC-400 cells exhibiting the greatest similarity, and CC-3395 cultures displaying only limited changes after exposure. A pattern of change present in all lines is the

observed increase in cell size and degree of roundness, as well as an associated increase in the presence of vacuoles and granules in the cytoplasm and apparent cell swelling.

The fully walled WT also had a change in growth form, with palmelloids and multicelluar clumps forming, the former held together by the remnant of the mother cell wall and the latter by increased amounts of exopolymer matrix. Visualization of the cell surface with CLSM indicated a buildup of proteins, and examination with FTIR indicated a change in the protein confirmation of the total cellular protein pool. There was evidence of decreased uptake of fluorescently labelled dyes after NAFC exposure, given the decrease in detail of internal membranes and proteins, and the sharp decrease in Nile Red staining neutral lipids was not correlated to a change in total amount of lipids. There was, however, a decrease in the diversity of phospholipids present in cells exposed to NAFCs, primarily through loss of C_{16} unsaturated fatty acids, and an increased peroxidation index. Cells also exhibited increased amounts and diversities of TAGs and 1-2 DAGs, and increased amounts of CEs.

The cell wall mutant CC-400, retaining cell wall layers W1 and W7, exhibits some similar patterns of change. There is a minor smoothing of surface protein features, and the same FTIR-observed change in the confirmation of cellular proteins. There was no sharp change in the staining of internal cellular features with fluorescently labelled dyes, but there was an increase in the between-cell variation in uptake, and an increase in FM1-43 and SYPRO Orange staining bodies, though they were not observed to be actively excreted. Variation was also present in chloroplast response, with some cells appearing to experience chloroplast disorganization, and all cells losing a visible eyespot. The appearance of the cytoplasm changed markedly, with a loss of internal detail and failure to follow the shape of the cell. Cells of CC-400 exposed to NAFCs experienced a loss of almost half of their phospholipids. Though there

was no change in the overall diversity of phospholipids, there was a change in the relative amounts of different phospholipids, as well as an increase in the number of double bonds present, as well as in the peroxidation index. There was a loss of diversity in TAGs, CEs, and 1-3 DAGS, and an increase in 18:0 1-2 DAG and in the overall diversity and abundance of FFAs.

The cell wall mutant CC-3395, believed to be completely naked, exhibited only minor physiological changes in response to NAFC exposure. In addition to the increased roundness and size seen in all lines, the increased vacuolization/presence of granules was most marked here as compared to WT and CC-400. Visualization with the membrane stain FM1-43 allowed the observance of the production and active transport of secretory vesicles of some form. The only lipid changes detected were minor increases in diversity in the form of the appearance of small amounts of 14:0 phospholipid, and increased TAG diversity.

5.1.2 Objective 4

Assessment of potential biomodification of NAFCs. Changes in the NAFC mixture composition were observed after incubation with algal cultures. These changes were specific to the algal line and DBE of individual NAFC species. Changes to the composition of classical (O_2) NAFCs were mediated by WT and CC-400 cultures. Both removed all DBE 8 compounds from the media. There was also an increase in DBE 1 after culturing with WT cells and a decrease in DBE 7 after culturing with CC-400. Non-classical O_2S compounds were much less specific, with all algae mediating an overall decrease and the decrease of individual DBEs of the O_2S species. Changes were also found in the relative abundance of O_3: O_3, which also occurs in the nutrient media, had a decreased relative abundance in the control NAFC culture lacking algae, as well as in the non-NAFC algae-only control cultures. However, in the flasks containing both algae and NAFCs, there was an increase in their relative abundance. This change was

significant in CC-400 and CC-3395; there was a similar increase in WT media but the change was non-significant due to a high standard deviation at the start of the experiment.

5.2 Synthesis of Research Data

5.2.1 The Role of the Cell Wall in Toxicity and Dissipation

In *C. reinhardtii* the presence of the cell wall appeared to play an important role in susceptibility to NAFC toxicity. Though all lines tested exceeded the tolerance threshold of 10-20 mgL^{-1} predicted by the environmentally relevant species, the fully walled WT cells were most strongly affected by NAFC exposure, with a reduction in growth rate found at the lowest exposure level (10 mgL^{-1}), followed distantly by CC-400, which retains wall layers W1 and W7, and whose rate of growth was impacted at the highest level of exposure (100 mgL^{-1}). The second cell wall mutant, CC-3395, had a rate of growth unresponsive to NAFC exposure. Though the mechanisms behind this were unclear, various forms of evidence suggested that this difference in toxicity was linked to the presence of the cell wall. In WT and CC-400 cultures, changes to the cell surface protein confirmation after NAFC exposure were indicated by changes in the FTIR AI/AII ratio; in addition, investigation via CLSM with SYPRO Orange showed a buildup of surface proteins in exposed WT cultures, and loss of surface protein features in CC-400. In WT cultures, the observed appearance of palmelloid cell balls was also suggestive of changes to protein confirmation and the inability to degrade the original mother cell wall and release the daughter cells.

Furthermore, MS analysis of aqueous NAFCs indicated that only WT and CC-400 cells were able to remove classical (O_2) NAFCs from solution, or modify their composition. The high DBE of the compounds removed (DBE 8 for WT, DBE 7 and 8 for CC-400) also suggested the likelihood of sorption and physical removal. The mutant CC-3395, believed to be completely

naked, had rate of growth unaffected by exposure even to 100 mgL^{-1} NAFCs, and was unable to remove classical NAFCs. Changes to non-classic components of the mixture (O_2S, O_3) were much less specific, and all three algae appeared to reduce the presence of O_2S compounds of all DBEs, a pattern potentially indicative of biometabolism or sorption. An apparent increase in the relative abundance of O_3 compounds was found in all strains (though it was non-significant in WT cells). However, it was not possible to discern if this was a result of the transformation of NAFCs to secondary metabolites, or if it was due to metabolic algal O_3 organic acids indistinguishable from NAFC O_3 compounds.

5.2.2 Changes to Membranes and Lipid Metabolism

Exposure to NAFCs changed lipid and phospholipid composition in the two algal lines whose growth was affected by exposure. WT cells showed the most dramatic change in phospholipid composition, losing a number of C_{16} unsaturated and C_{18} saturated phospholipids, which occurred as relatively minor components. Combined with the increased diversity and amount of cholesterol esters present, this was suggestive of decreased membrane fluidity. The changes in CC-400 phospholipid composition after NAFC exposure were primarily related to an overall loss of ~40% of the amount of phospholipids present per gram of dehydrated sample. Phospholipid diversity in CC-400 remained unchanged, and though there were changes in the relative abundance of individual phospholipids, an increase in the peroxidation index, and an increase in the number of double bonds present per gram of saturated and unsaturated phospholipids. However, the effect upon membrane fluidity was more difficult to ascertain than for exposed WT cells, as there was not the same straight forward loss of phospholipid classes known to increase membrane fluidity, and increase of cholesterol esters which act to stabilize the membrane. The overall loss of phospholipids and cholesterol esters suggested losses from the

cell membrane, possibly due to solubilization of the membrane due to NAFC interactions. There were no changes in the phospholipid composition of exposed CC-3395 cells, save for the appearance of small amounts of 14:0.

All three lines exhibited changes in lipid metabolism; as expected, these were most minor in CC-3395, which only showed an increase in the diversity of TAGs. WT and CC-400 also showed changes across 1-2 DAGs, 1-3 DAGs, TAGs, FFAs, and CEs, the specific changes varied between the two lines, indicating that the induced responses differed.

5.2.3 Other Morphological Changes

Exposure to NAFCs induced a variety of morphological changes in all algae, though many of these differed between algal lines. Several changes were observed consistently across algae species, primarily indicators of osmotic stress or metabolic leakage. Cell sizes increased with NAFC exposure, as did the degree of roundness. A larger cytoplasmic space was observed between the chloroplast and cell membrane in exposed cells, as was an increase in the degree of vacuolization and the presence of granules. In WT cells, these latter two changes were obvious only in images taken with visible light, rather than visible light and CLSM of FM1-43 and internal SYPRO Orange: NAFC-exposed WT cells appeared to exhibit decreased uptake of fluorescent dyes, most exemplified by the apparent disappearance of neutral lipids stained with Nile Red, but not found with FTIR or TLC techniques. In addition to the formation of palmelloid cell balls, NAFC exposure also induced the production of large amounts of exopolymer matrix around individual cells and used to bind together large multi-cellular clumps.

In contrast, CC-400 exhibited signs of cytoplasm loss, increased staining variability, loss of the eyespot, and in some cells, loss of chloroplast integrity. Though combined with its sharp loss of phospholipid, these changes might have been expected to have a greater effect upon the

cell population than those changes observed in WT cells, this was not the case, as measured by decreased growth rate. The loss of chloroplast integrity in some exposed cells, and eyespots from the entire culture, were also potentially indicative of internal loss exposure to organic solvents or other agents capable of denaturing proteins. Exposed CC-400 cultures also shared some hallmarks of the one major change exhibited by CC-3395: increased active excretion.

NAFC-exposed cultures of CC-3395 were observed to have higher rates of formation and active excretion of secretory vesicles. It was unknown if these bodies were functioning in osmotic balance, removal of toxic compounds, or membrane repair. Some of these same bodies were seen in NAFC-exposed CC-400, but in that line they were not witnessed in active excretion.

5.2.4 Evidence Suggesting the Importance of Surfactant Interactions in Toxicity

Though the precise mode of toxic action of NAs and NAFCs remains unknown, this work offers a strong suggestion that in *C. reinhardtii* surfactant interactions are important determining factors in toxicity. Surfactant toxicities are incredibly hard to predict or to generalize; they depend upon the individual surfactant compound and species exposed. Similar compounds can have extremely different effects upon the same species, and very similar species can react to the same compound in disparate ways. As such, the response of these three algal strains to exposure to NAFCs would be expected to vary, along with their ability to remove NAFC compounds from solution; the different cell wall structures and membrane compositions would be expected to interact differently with the complex mix of surfactants and toxicants that make up the naphthenic acid fraction components. The two main hallmarks of surfactant toxicity, however, are modification of surface proteins and their confirmations, and changes in membrane fluidity.

Both of these have been observed in *C. reinhardtii* exposed to NAFCs, in addition to evidence of osmotic stress or metabolic leakage, both also frequently caused by surfactant exposure.

The linking of susceptibility to NAFC toxicity and cell wall presence would agree with the suggestion by Van Hamme et al. (2006) that protein binding may be more important in toxicity than membrane permeation. Fully walled WT cells were most affected by NAFC exposure, as defined by decreased growth and reproduction even with exposure to 10 mgL^{-1}, and exhibited high degrees of surface protein modification. They also exhibited controlled changes in phospholipid membrane composition (likely indicative of decreased membrane fluidity) and environmental uptake. Conversely, the cell wall mutant CC-400 exhibited changes indicative of minor modification to surface proteins, but lost ~40% of their phospholipids and exhibited the most severe signs of membrane permeabilization, organic solvent exposure, and metabolic leakage, but maintained their baseline rate of growth until exposure to 100 mgL^{-1}NAFCs. In contrast, the algal strain whose growth was not impacted by NAFC exposure, CC-3395, was believed to be completely naked, and exhibited none of these hallmarks of protein modification or modification of membrane structure.

These findings were in contrast to the majority of studies on the role of the cell wall in toxicity (Barbosa et al., 2003; Macfie et al., 1994; Macfie and Welbourn, 2000; Maucourt et al., 2002; Prasad et al., 1998), in which wall-less mutants were more susceptible to toxic insult, and were again suggestive of the importance of cell wall proteins in NAFC interaction and toxicity.

5.2.5 The Overall Role of NAFCs in Toxicity to *C. reinhardtii*

Under laboratory conditions, NAFCs were found to be much less toxic to *C. reinhardtii* than predicted by the NA tolerance of an environmentally relevant species, *C. frigida*, which evolved with thousands of years' exposure to low levels of NAFC components. Whereas *C.*

fridiga disappears from tailings ponds with NA contents greater than 10-20 mgL^{-1}, *C. reinhardtii* was found to exhibit exponential growth even after exposure to 100 mgL^{-1} NAFC, and its cell wall mutants CC-400 and CC-3395 were found to be relatively unresponsive. While possible that these differences were due to inter-species differences, this was more likely attributable to differences between laboratory and environmental exposure conditions. It is possible that NAFCs do not act as the primary agent of toxicity in green algae similar to *C. reinhardtii*, or that their full impact can only be seen synergistically in action with other OSPW components. Other compounds in OSPW may act synergistically with NAFC-induced stress: high salinity, pH stress, and oil products are known to increase the toxic impact of surfactants (Lewis, 1992). Separately, the observed changes in cell wall and membrane structure, as well as changes in environmental interaction, would be expected to have impacts on environmental viability. Finally, it is possible that the most ecologically relevant effects of NAFC exposure come after a certain threshold concentration, when the formation of palmelloid structures and multicellular clumps could simply result in physical removal of algae from the water column via settling.

5.3 Future Work

Though this work offers strong suggestions of the role of the cell wall and the importance of surfactant interactions in NAFC toxicity, there is still much to be done to elucidate the mechanisms of toxic action. Future work should include attempts to ascertain if the patterns of response to exposure hold true for algal species native to the Athabasca region and found in the oil sands, or even simply for other species of green algae. Ideally, a method for directly observing NAFC binding to proteins and membranes could be found that could be utilized at environmentally relevant concentrations.

One of the most important discoveries for mediation of toxicity of OSPW would be establishing which NAFC compounds exhibit the highest toxicity; this work indicates that there are very selective interactions between the class and structure of NAFCs and how they interact with algae. It is possible that the selective removal of certain fragments of OSPW could result in a marked reduction in toxicity. Similarly, further work is needed to figure out potential synergistic actions between NAFCs and salinity, pH, and metals. With the indication that NAFCs may not act alone as primary agents of toxicity, it is possible that simple mediation of other stressors could again reduce the overall toxicity of OSPW.

Finally, it is important that high resolution mass spectrometry be included in bioremediation strategies, when possible. *C. reinhardtii* was capable of mediating changes in the NAFC mixture that would not have been detected using standard low resolution mass spectrometry, and the use of this technique should be helpful in identifying species capable of mediating biological transformation of NAFC mixtures.

CHAPTER 6
6.0 REFERNCES

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

Amarak Zettler, L. A., Gomez, F., Zettler, E., Keenan, B. G., Amils, R., and Sogin, M. L. (2002). Eukaryotic diversity in Spain's River of Fire - This ancient and hostile ecosystem hosts a surprising variety of microbial organisms. *Nature* **417**, 137-137.

Armstrong, S. A. (2008). Dissipation and phytotoxicity of oil sands naphthenic acids in wetland plants, University of Saskatchewan, Saskatoon.

Armstrong, S. A., Headley, J. V., Peru, K. M., and Germida, J. J. (2008). Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering* **43**, 36-42.

Azencott, H. R., Peter, G. F., and Prausnitz, M. R. (2007). Influence of the cell wall on intracellular delivery to algal cells by electroporation and sonication. *Ultrasound in Medicine and Biology* **33**, 1805-1817.

Bae, E., Yeo, I. J., Jeong, B., Shin, Y., Shin, K. H., and Kim, S. (2011). Study of Double Bond Equivalents and the Numbers of Carbon and Oxygen Atom Distribution of Dissolved Organic flatter with Negative-Mode FT-ICR MS. *Analytical Chemistry* **83**, 4193-4199.

Barbosa, M. J., Albrecht, M., and Wijffels, R. H. (2003). Hydrodynamic stress and lethal events in sparged microalgae cultures. *Biotechnology and Bioengineering* **83**, 112-120.

Birarda, G., Grenci, G., Businaro, L., Marmiroli, B., Pacor, S., Piccirilli, F., and Vaccari, L. (2010). Infrared microspectroscopy of biochemical response of living cells in microfabricated devices. *Vibrational Spectroscopy* **53**, 6-11.

Boerger, H., Mackinnon, M., and Aleksiuk, M. (1986). Use of bioassay techniques to evaluate the effectiveness of natural and chemical detoxification of tar sands tailings water. *Canadian Technical Report of Fisheries and Aquatic Sciences*, 131-146.

Bollig, K., Lamshoeft, M., Schweirner, K., Marner, F.-J., Budzikiewicz, H., and Waffenschmidt, S. (2007). Structural analysis of linear hydroxyproline-bound O-glycans of Chlamydomonas reinhardtii-conservation of the inner core in Chlamydomonas and land plants. *Carbohydrate Research* **342**, 2557-2566.

Borland, L. M., Kottegoda, S., Phillips, K. S., and Allbritton, N. L. (2008). Chemical analysis of single cells. *Annual Review of Analytical Chemistry* **1**, 191-227.

Canadian Association of Petroleum Producers (2011). Crude oil. Forecasts, markets, and pipelines. Calgary, Alberta.

Carrillo, C., Teruel, J. A., Aranda, F. J., and Ortiz, A. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica Et Biophysica Acta-Biomembranes* **1611**, 91-97.

Chalaturnyk, R. J., Scott, J. D., and Ozum, B. (2002). Management of oil sands tailings. *Petroleum Science and Technology* **20**, 1025-1046.

Chen, X., Shao, Z. Z., Marinkovic, N. S., Miller, L. M., Zhou, P., and Chance, M. R. (2001). Conformation transition kinetics of regenerated *Bombyx mori* silk fibroin membrane monitored by time-resolved FTIR spectroscopy. *Biophysical Chemistry* **89**, 25-34.

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

Conrad Environmental Aquatics Technical Advisory Group (CEATAG) (1998). Naphthenic acids background information discussion report. (A. D. o. Energy, ed.), Edmonton, Alberta, Canada.

Cooper, G. M. (2000). "The Cell: A molecular approach," 2nd/Ed. Sinauer Associates, Sunderland, MA, USA.

Crowe, A. U., Plant, A. L., and Kermode, A. R. (2002). Effects of an industrial effluent on plant colonization and on the germination and post-germinative growth of seeds of terrestrial and aquatic plant species. *Environmental Pollution* **117**, 179-189.

Cserhati, T., Forgacs, E., and Oros, G. (2002). Biological activity and environmental impact of anionic surfactants. *Environment International* **28**, 337-348.

Das, B. K., Roy, A., Singh, S., and Bhattacharya, J. (2009). Eukaryotes in acidic mine drainage environments: potential applications in bioremediation. *Reviews in Environmental Science and Bio/Technology* **8**, 257-274.

Dean, A. P., Martin, M. C., and Sigee, D. C. (2007). Resolution of codominant phytoplankton species in a eutrophic lake using synchrotron-based Fourier transform infrared spectroscopy. *Phycologia* **46**, 151-159.

Dean, A. P., Nicholson, J. M., and Sigee, D. C. (2008). Impact of phosphorus quota and growth phase on carbon allocation in *Chlamydomonas reinhardtii*: an FTIR microspectroscopy study. *European Journal of Phycology* **43**, 345-354.

Dean, A. P., Sigee, D. C., Estrada, B., and Pittman, J. K. (2010). Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresource Technology* **101**, 4499-4507.

Dokholyan, B. K., and Magomedov, A. K. (1983). The effect of sodium naphthenate on the viability and physiological and biochemical indices of fish. *Voprosy Ikhtiologii* **23**, 1013-1019.

Dumas, P., Carr, G. L., and Williams, G. P. (2000). Enhancing the lateral resolution in infrared microspectrometry by using synchrotron radiation: applications and perspectives. *Analusis* **28**, 68-74.

Ellis, B. E. (1977). Degradation of phenolic compounds by freshwater algae. *Plant Science Letters* **8**, 213-216.

Energy Resources Conservation Board (2009). Alberta's energy reserves 2008 and supply/demand outlook 2009-2018. (E. R. C. Board, ed.), pp. 220. Government of Alberta, Calgary.

Fan, T. P. (1991). Characterization of naphthenic acids in petroleum by fast atom bombardment mass spectrometry. *Energy & Fuels* **5**, 371-375.

Finan, J. D., Leddy, H. A., and Guilak, F. (2011). Osmotic stress alters chromatin condensation and nucleocytoplasmic transport. *Biochemical and Biophysical Research Communications* **408**, 230-235.

Frank, R. A., Fischer, K., Kavanagh, R., Burnison, B. K., Arsenault, G., Headley, J. V., Peru, K. M., Van der Kraak, G., and Solomon, K. R. (2009). Effect of carboxylic acid content on

the acute toxicity of oil sands naphthenic acids. *Environmental Science & Technology* **43**, 266-271.

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

Garcia-Garcia, E., Pun, J., Perez-Estrada, L. A., Din, M. G. E., Smith, D. W., Martin, J. W., and Belosevic, M. (2011). Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. *Toxicology Letters* **203**, 62-73.

Gentes, M. L., McNabb, A., Waldner, C., and Smits, J. E. G. (2007). Increased thyroid hormone levels in tree swallows (*Tachycineta bicolor*) on reclaimed wetlands of the Athabasca oil sands. *Archives of Environmental Contamination and Toxicology* **53**, 287-292.

Glover, R. E., Smith, R. R., Jones, M. V., Jackson, S. K., and Rowlands, C. C. (1999). An EPR investigation of surfactant action on bacterial membranes. *Fems Microbiology Letters* **177**, 57-62.

Goff, K. L., Quaroni, L., Pederson, T., and Wilson, K. E. (2010). Measurement of ethanol formation in single living cells of *Chlamydomonas reinhardtii* using synchrotron Fourier Transform Infrared spectromicroscopy. *In* "WIRMS 2009 5th International Workshop on Infrared Microscopy and Spectroscopy with Accelerator Based Sources" (A. Predoi-Cross and B. E. Billinghurst, eds.), Vol. 1214, pp. 54-26. American Institute of Physics Conference Proceedings, Banff, Alberta, Canada.

Goff, K. L., Quaroni, L., and Wilson, K. E. (2009). Measurement of metabolite formation in single living cells of *Chlamydomonas reinhardtii* using synchrotron Fourier-Transform Infrared spectromicroscopy. *Analyst* **134**, 2216-2219.

Goncalves, A. M. D., Aires-Barros, M. R., and Cabral, J. M. S. (2003). Interaction of an anionic surfactant with a recombinant cutinase from Fusarium solani pisi: a spectroscopic study. *Enzyme and Microbial Technology* **32**, 868-879.

Gorman, D. S., and Levine, R. P. (1965). Cytochrome F and plastocyanin - their sequence in photosynthetic electron transport chain of *Chlamydomonas reinhardi*. *Proceedings of the National Academy of Sciences of the United States of America* **54**, 1665-9.

Government of Alberta (2009). Environmental management of Alberta's oil sands. (O. S. M. Division, ed.). Government of Alberta, AB, Canada.

Government of Alberta (2012). Alberta energy: Facts and Statistics. Vol. 2012.

Greene, D. L., Hopson, J. L., and Li, J. (2006). Have we run out of oil yet? Oil peaking analysis from an optimist's perspective. *Energy Policy* **34**, 515-531.

Grossman, A. (2000). Acclimation of *Chlamydomonas reinhardtii* to its nutrient environment. *Protist* **151**, 201-224.

Guschina, I. A., and Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research* **45**, 160-186.

Gutenkauf, A., Duker, A., and Fock, H. P. (1998). Fate of substituted benzoates in the freshwater green alga, Chlamydomonas reinhardtii 11-32b. *Biodegradation* **9**, 359-368.

Hadwin, A. K. M., Del Rio, L. F., Pinto, L. J., Painter, M., Routledge, R., and Moore, M. M. (2006). Microbial communities in wetlands of the Athabasca oil sands: genetic and metabolic characterization. *Fems Microbiology Ecology* **55**, 68-78.

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

Harris, E. H. (1989). The Chlamydomonas sourcebook. (E. H. Harris, ed.), pp. 780. Academic Press, San Diego.

Harris, E. H. (2001). Chlamydomonas as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 363-406.

Harris, E. H. (2009). "The Chlamydomonas Sourcebook: Introduction to Chlamydomonas and its laboratory use," 2nd/Ed. Academic Press, San Diego.

Headley, J. V., Barrow, M. P., Peru, K. M., and Derrick, P. J. (2011a). Salting-out effects on the characterization of naphthenic acids from Athabasca oil sands using electrospray ionization. *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* **46**, 844-854.

Headley, J. V., Barrow, M. P., Peru, K. M., Fahlman, B., Frank, R. A., Bickerton, G., McMaster, M. E., Parrott, J., and Hewitt, L. M. (2011b). Preliminary fingerprinting of Athabasca oil sands polar organics in environmental samples using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Communications in Mass Spectrometry* **25**, 1899-1909.

Headley, J. V., Du, J. L., Peru, K. M., Gurprasad, N., and McMartin, D. W. (2008). Evaluation of algal phytodegradation of petroleum naphthenic acids. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering* **43**, 227-232.

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

Headley, J. V., Peru, K. M., Armstrong, S. A., Han, X. M., Martin, J. W., Mapolelo, M. M., Smith, D. F., Rogers, R. P., and Marshall, A. G. (2009a). Aquatic plant-derived changes in oil sands naphthenic acid signatures determined by low-, high- and ultrahigh-resolution mass spectrometry. *Rapid Communications in Mass Spectrometry* **23**, 515-522.

Headley, J. V., Peru, K. M., and Barrow, M. P. (2009b). Mass spectrometric characterization of naphthenic acids in environmental samples: A review. *Mass Spectrometry Reviews* **28**, 121-134.

Headley, J. V., Peru, K. M., Barrow, M. P., and Derrick, P. J. (2007). Characterization of naphthenic acids from Athabasca oil sands using electrospray ionization: The significant influence of solvents. *Analytical Chemistry* **79**, 6222-6229.

Headley, J. V., Peru, K. M., Janfada, A., Fahlman, B., Gu, C., and Hassan, S. (2011c). Characterization of oil sands acids in plant tissue using Orbitrap ultra-high resolution mass spectrometry with electrospray ionization. *Rapid Communications in Mass Spectrometry* **25**, 459-462.

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

Heraud, P., Wood, B. R., Tobin, M. J., Beardall, J., and McNaughton, D. (2005). Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. *Fems Microbiology Letters* **249**, 219-225.

Herman, D. C., Fedorak, P. M., and Costerton, J. W. (1993). Biodegradation of cycloalkane carboxylic-acids in oil sand tailings. *Canadian Journal of Microbiology* **39**, 576-580.

Herman, D. C., Fedorak, P. M., Mackinnon, M. D., and Costerton, J. W. (1994). Biodegradation of naphthenic acids by microbrial populations indigenous to oil sands tailings. *Canadian Journal of Microbiology* **40**, 467-477.

Hirooka, T., Nagase, H., Hirata, K., and Miyamoto, K. (2006). Degradation of 2,4-dinitrophenol by a mixed culture of photoautotrophic microorganisms. *Biochemical Engineering Journal* **29**, 157-162.

Holman, H. N., Martin, M. C., Blakely, E. A., Bjornstad, K., and McKinney, W. R. (2000). IR spectroscopic characteristics of cell cycle and cell death probed by synchrotron radiation based fourier transform IR spectromicroscopy. *Biopolymers* **57**, 329-335.

Holman, H. Y. N., and Martin, M. C. (2006). Synchrotron radiation infrared spectromicroscopy: A noninvasive chemical probe for monitoring biogeochemical processes. *Advances in Agronomy, Vol 90* **90**, 79-127.

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

Hsu, C. S., Dechert, G. J., Robbins, W. K., and Fukuda, E. K. (2000). Naphthenic acids in crude oils characterized by mass spectrometry. *Energy & Fuels* **14**, 217-223.

Iwasa, K., and Murakami, S. (1968). Palmelloid formation of *Chlamydomonas* I: Palmelloid induction by organic acids. *Physiologia Plantarum* **21**, 1224-1233.

Jamin, N., Dumas, P., Moncuit, J., Fridman, W. H., Teillaud, J. L., Carr, G. L., and Williams, G. P. (1998). Highly resolved chemical imaging of living cells by using synchrotron infrared microspectrometry. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 4837-4840.

Janfada, A., Headley, J. V., Peru, K. M., and Barbour, S. L. (2006). A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils. *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* **41**, 985-997.

Johnsson, K. (2009). Visualizing biochemical activities in living cells. *Nature Chemical Biology* **5**, 63-65.

Jones, D., Scarlett, A. G., West, C. E., and Rowland, S. J. (2011). Toxicity of individual naphthenic acids to *Vibrio fischeri*. *Environmental Science & Technology* **45**, 9776-9782.

Jones, M. N. (1992). Surfactant interactions with biomembranes and proteins. *Chemical Society Reviews* **21**, 127-136.

Kalin, M., Wheeler, W. N., and Olaveson, M. M. (2006). Response of phytoplankton to ecological engineering remediation of a Canadian Shield Lake affected by acid mine drainage. *Ecological Engineering* **28**, 296-310.

Kavanagh, R. J., Frank, R. A., Oakes, K. D., Servos, M. R., Young, R. F., Fedorak, P. M., MacKinnon, M. D., Solomon, K. R., Dixon, D. G., and Van Der Kraak, G. (2011). Fathead minnow (Pimephales promelas) reproduction is impaired in aged oil sands process-affected waters. *Aquatic Toxicology* **101**, 214-220.

Kelly, E. N., Short, J. W., Schindler, D. W., Hodson, P. V., Ma, M. S., Kwan, A. K., and Fortin, B. L. (2009). Oil sands development contributes polycyclic aromatic compounds to the Athabasca River and its tributaries. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 22346-22351.

King, R. B., Long, G. M., and Sheldon, J. K. (1992). "Practical environmental bioremediation," 2nd/Ed. Lewis Publishers, Boca Raton.

Kirk, J. L., Klironomos, J. N., Lee, H., and Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environmental Pollution* **133**, 455-465.

Kola, H., Laglera, L. M., Parthasarathy, N., and Wilkinson, K. J. (2004). Cadmium Adsorption by Chlamydomonas reinhardtii and its Interaction with the Cell Wall Proteins. *Environmental Chemistry* **1**, 172-179.

Lai, J. W. S., Pinto, L. J., Kiehlmann, E., BendellYoung, L. I., and Moore, M. M. (1996). Factors that affect the degradation of naphthenic acids in oil sands wastewater by indigenous microbial communities. *Environmental Toxicology and Chemistry* **15**, 1482-1491.

Lawrence, J. R., Korber, D. R., and Neu, T. R. (2007). Analytical imaging techniques. *In* "Manual of Environmental Microbiology" (C. J. Hurst, R. L. Crawfork, J. L. Garland, D. A. Lipson, A. L. Mills and L. D. Stetzenbach, eds.), pp. 40-68. American Society for Microbiology Press, Washington, D.C.

Lawrence, J. R., Swerhone, G. D. W., Leppard, G. G., Araki, T., Zhang, X., West, M. M., and Hitchcock, A. P. (2003). Scanning transmission X-ray, laser scanning, and transmission electron microscopy mapping of the exopolymeric matrix of microbial biofilms. *Applied and Environmental Microbiology* **69**, 5543-5554.

Lei, A. P., Wong, Y. S., and Tam, N. F. Y. (2002). Removal of pyrene by different microalgal species. *Water Science and Technology* **46**, 195-201.

Leung, S. S., MacKinnon, M. D., and Smith, R. E. H. (2001). Aquatic reclamation in the Athabasca, Canada, oil sands: Naphthenate and salt effects on phytoplankton communities. *Environmental Toxicology and Chemistry* **20**, 1532-1543.

Leung, S. S., MacKinnon, M. D., and Smith, R. E. H. (2003). The ecological effects of naphthenic acids and salts on phytoplankton from the Athabasca oil sands region. *Aquatic Toxicology* **62**, 11-26.

Levi, V., and Gratton, E. (2007). Exploring dynamics in living cells by tracking single particles. *Cell Biochemistry and Biophysics* **48**, 1-15.

Levin, I. W., and Bhargava, R. (2005). Fourier transform infrared vibrational spectroscopic imaging: Integrating microscopy and molecular recognition. *Annual Review of Physical Chemistry* **56**, 429-474.

Lewis, M. A. (1990). Chronic toxicities of surfactants and detergent builders to algae - A review and risk assessment. *Ecotoxicology and Environmental Safety* **20**, 123-140.

Lewis, M. A. (1991). Chronic and sublethal toxicities of surfactants to aquatic animals - a review and risk assessment. *Water Research* **25**, 101-113.

Lewis, M. A. (1992). The effects of mixtures and other environmental modifying factors on the toxicities of surfactants to fresh-water and marine life. *Water Research* **26**, 1013-1023.

Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. *Annual Review of Genetics* **22**, 631-677.

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

Lurling, M., and Beekman, W. (2006). Palmelloids formation in Chlamydomonas reinhardtii: defence against rotifer predators? *Annales De Limnologie-International Journal of Limnology* **42**, 65-72.

Macfie, S. M., Tarmohamed, Y., and Welbourn, P. M. (1994). Effects of cadmium, cobalt, copper, and nickel on growth of the green alga Chlamydomonas *reinhardtii* - The influences of the cell wall and pH. *Archives of Environmental Contamination and Toxicology* **27**, 454-458.

Macfie, S. M., and Welbourn, P. M. (2000). The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). *Archives of Environmental Contamination and Toxicology* **39**, 413-419.

Macnaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y. J., and White, D. C. (1999). Microbial population changes during bioremediation of an experimental oil spill. *Applied and Environmental Microbiology* **65**, 3566-3574.

Martin, J. W., Han, X. M., Peru, K. M., and Headley, J. V. (2008). Comparison of high- and low-resolution electrospray ionization mass spectrometry for the analysis of naphthenic acid mixtures in oil sands process water. *Rapid Communications in Mass Spectrometry* **22**, 1919-1924.

Maucourt, K., Agarwal, M., Rene, B., and Fermandjian, S. (2002). Use of *Chlamydomonas reinhardtii* mutants for anticancer drug screening. *Biochemical Pharmacology* **64**, 1125-1131.

Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., Terry, A., Salamov, A., Fritz-Laylin, L. K., Marechal-Drouard, L., Marshall, W. F., Qu, L. H., Nelson, D. R., Sanderfoot, A. A., Spalding, M. H., Kapitonov, V. V., Ren, Q. H., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S. M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C. L., Cognat, V., Croft, M. T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Balle, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P. A., Lemaire, S. D., Lobanov, A. V., Lohr, M., Manuell, A., Meir, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J. V., Moseley, J., Napoli, C., Nedelcu, A. M., Niyogi, K., Novoselov, S. V., Paulsen, I. T., Pazour, G., Purton, S., Ral, J. P., Riano-Pachon, D. M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S. L., Allmer, J., Balk, J., Bisova, K., Chen, C. J., Elias, M., Gendler, K., Hauser, C., Lamb, M. R., Ledford, H., Long, J. C., Minagawa, J., Page, M. D., Pan, J. M., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A. M., Yang, P. F., Ball, S., Bowler, C., Dieckmann, C. L., Gladyshev, V. N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R. T., Brokstein, P., et al. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318**, 245-251.

Nakamura, K., Bray, D. F., and Wagenaar, E. B. (1975). Ultrastructure of *Chlamydomonas eugametos* palmelloids induced by chloroplatinic acid treatment. *Journal of Bacteriology* **121**, 338-343.

National Energy Board (2006). Canada's oil sands. Opportunities and challenges to 2015: An update. (N. E. Board, ed.), Calgary, Alberta.

Nero, V., Farwell, A., Lister, A., Van der Kraak, G., Lee, L. E. J., Van Meer, T., MacKinnon, M. D., and Dixon, D. G. (2006). Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to oil sands process-affected water. *Ecotoxicology and Environmental Safety* **63**, 365-377.

Neu, T. R., Swerhone, G. D. W., and Lawrence, J. R. (2001). Assessment of lectin-binding analysis for in situ detection of glycoconjugates in biofilm systems. *Microbiology* **147**, 299-313.

Neu, T. R., Woelfl, S., and Lawrence, J. R. (2004). Three-dimensional differentiation of photo-autotrophic biofilm constituents by multi-channel laser scanning microscopy (single-photon and two-photon excitation). *Journal of Microbiological Methods* **56**, 161-172.

Nyberg, H. (1979). Effects of some detergents on the phosphatases and phosphate accumulation of *Nitzschia actinastroides* (Bacillariophyceae). *Annales Botanici Fennici* **16**, 28-34.

O'Neill, M. A., and Roberts, K. (1981). Methylation analysis of cell wall glycoproteins and glycopeptides from *Chlamydomonas reinhardii*. *Phytochemistry* **20**, 25-28.

Olsen, Y., Knutsen, G., and Lien, T. (1983). Characteristics of phosphorus limitation in Chlamydomonas renihardtii (Chlorophyceae) and its palmelloids. *Journal of Phycology* **19**, 313-319.

Palmucci, M., Ratti, S., and Giordano, M. (2011). Ecological and evolutionary implications of carbon allocatiosn in marine phytoplankton as a function of nitrogen availability: A Fourier Transform infrared spectroscopy approach. *Journal of Phycology* **47**, 313-323.

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

Pollet, I., and Bendell-Young, L. I. (2000). Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. *Environmental Toxicology and Chemistry* **19**, 2589-2597.

Popova, A., and Kemp, R. (2007). Effects of surfactants on the ultrastructural organization of the phytoplankton, *Chlamydomonas reinhardtii* and *Anabaena cylindrica*. *Fundamental and Applied Limnology* **169**, 131-136.

Prasad, M. N. V., Drej, K., Skawinska, A., and Stralka, K. (1998). Toxicity of cadmium and copper in *Chlamydomonas reinhardtii* wild-type (WT 2137) and cell wall deficient mutant strain (CW 15). *Bulletin of Environmental Contamination and Toxicology* **60**, 306-311.

Quagraine, E. K., Peterson, H. G., and Headley, J. V. (2005). In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering* **40**, 685-722.

Quesnel, D. M., Bhaskar, I. M., Gieg, L. M., and Chua, G. (2011). Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*. *Chemosphere* **84**, 504-511.

Rogers, V. V., Liber, K., and MacKinnon, M. D. (2002a). Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. *Chemosphere* **48**, 519-527.

Rogers, V. V., Wickstrom, M., Liber, K., and MacKinnon, M. D. (2002b). Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings. *Toxicological Sciences* **66**, 347-355.

Rosen, M. J., Li, F., Morrall, S. W., and Versteeg, D. J. (2001). The relationship between the interfacial properties of surfactants and their toxicity to aquatic organisms. *Environmental Science & Technology* **35**, 954-959.

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

Sena, D. W., Kulacki, K. J., Chaloner, D. T., and Lamberti, G. A. (2010). The role of the cell wall in the toxicity of ionic liquids to the alga *Chlamydomonas reinhardtii*. *Green Chemistry* **12**, 1066-1071.

Smits, J. E., Wayland, M. E., Miller, M. J., Liber, K., and Trudeau, S. (2000). Reproductive, immune, and physiological end points in tree swallows on reclaimed oil sands mine sites. *Environmental Toxicology and Chemistry* **19**, 2951-2960.

Sogaard, D. H., Hansen, P. J., Rysgaard, S., and Glud, R. N. (2011). Growth limitation of three Arctic sea ice algal species: effects of salinity, pH, and inorganic carbon availability. *Polar Biology* **34**, 1157-1165.

Soto, C., Hellebust, J. A., Hutchinson, T. C., and Sheath, R. G. (1979). Effect of the hydrocarbon naphthalene on the morphology of the green flagellate *Chlamydomonas angulosa*. *Canadian Journal of Botany-Revue Canadienne De Botanique* **57**, 2729-2739.

Sun, X. X., Choi, J. K., and Kim, E. K. (2004). A preliminary study on the mechanism of harmful algal bloom mitigation by use of sophorolipid treatment. *Journal of Experimental Marine Biology and Ecology* **304**, 35-49.

Svensen, O., Frette, O., and Erga, S. R. (2007). Scattering properties of microalgae: the effect of cell size and cell wall. *Applied Optics* **46**, 5762-5769.

Thompson, G. A. (1996). Lipids and membrane function in green algae. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism* **1302**, 17-45.

Tiehm, A., Stieber, M., Werner, P., and Frimmel, F. H. (1997). Surfactant-enhanced mobilization and biodegradation of polycyclic aromatic hydrocarbons in manufactured gas plant soil. *Environmental Science & Technology* **31**, 2570-2576.

Turner, E. H., Cohen, D., Pugsley, H. R., Gomez, D. G., Whitmore, C. D., Zhu, C., and Dovichi, N. J. (2008). Chemical cytometry: the chemical analysis of single cells. *Analytical and Bioanalytical Chemistry* **390**, 223-226.

Vallon, O., and Wollman, F. A. (1995). Mutations affecting O-glycosylation in *Chlamydomonas reinhardtii* cause delayed cell wall degradation and sex-limited sterility. *Plant Physiology* **108**, 703-712.

van den Heuvel, M. R., Power, M., Richards, J., MacKinnon, M., and Dixon, D. G. (2000). Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. *Ecotoxicology and Environmental Safety* **46**, 334-341.

Van Hamme, J. D., Singh, A., and Ward, O. P. (2006). Physiological aspects - Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnology Advances* **24**, 604-620.

Venhuis, S. H., and Mehrvar, M. (2004). Health effects, environmental impacts, and photochemical degradation of selected surfactants in water. *International Journal of Photoenergy* **6**, 115-125.

Visviki, I., and Rachlin, J. W. (1994). Acute and chronic exposure of *Dunaliella salina* and *Chlamydomonas bullosa* to copper and cadmium - effects on ultrastructure. *Archives of Environmental Contamination and Toxicology* **26**, 154-162.

Voigt, J., Hinkelmann, B., and Harris, E. H. (1997). Production of cell wall polypeptides by different cell wall mutants of the unicellular green alga *Chlamydomonas reinhardtii*. *Microbiological Research* **152**, 189-198.

Voigt, J., Munzner, P., and Vogeler, H. P. (1991). The cell-wall glycoproteins of *Chlamydomonas reinhardtii* - analysis of the in vitro translation products. *Plant Science* **75**, 129-142.

Vonlanthen, S., Brown, M. T., and Turner, A. (2011). Toxicity of the amphoteric surfactant, cocamidopropyl betaine, to the marine macroalga, Ulva lactuca. *Ecotoxicology* **20**, 202-207.

Warshawsky, D., Cody, T., Radike, M., Reilman, R., Schumann, B., Ladow, K., and Schneider, J. (1995). Biotransformation of benzo[a]pyrene and other polycyclic aromatic hydrocarbons and heterocyclic analogs by several green algae and other algal species under gold and white light. *Chemico-Biological Interactions* **97**, 131-148.

Whyte, L. G., Greer, C. W., and Inniss, W. E. (1996). Assessment of the biodegradation potential of psychrotrophic microorganisms. *Canadian Journal of Microbiology* **42**, 99-106.

Xie, X. S., Choi, P. J., Li, G. W., Lee, N. K., and Lia, G. (2008). Single-molecule approach to molecular biology in living bacterial cells. *Annual Review of Biophysics* **37**, 417-444.

CHAPTER 7
7.0 APPENDIX A: SIGNIFICANCE TABLES

Table 7.1: One-way ANOVA for comparison of the rates of growth of wild type, CC-3395, and CC-400 under control conditions (0 mgL^{-1} naphthenic acid fraction components). n=3.

ANOVA

Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.376	2	1.188	200.521	.000
Within Groups	.036	6	.006		
Total	2.412	8			

Multiple Comparisons

Growth: Tukey HSD

Tukey HSD

(I) Algae	(J) Algae	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
WT	CC-3395	1.24343*	.06285	.000	1.0506	1.4363
	CC-400	.79076*	.06285	.000	.5979	.9836
CC-3395	WT	-1.24343*	.06285	.000	-1.436	-1.0506
	CC-400	-.45267*	.06285	.001	-.6455	-.2598
CC-400	WT	-.79076*	.06285	.000	-.9836	-.5979
	CC-3395	.45267*	.06285	.001	.2598	.6455

Table 7. 2: One-way ANOVA for comparison for growth rates of wild type *Chlamydomonas reinhardtii* after exposure to a dilution series of 0, 10, 20, 50, and 100 mgL^{-1} naphthenic acid fraction components. n=3.

ANOVA

Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.980	4	.245	223.128	.000
Within Groups	.011	10	.001		
Total	.991	14			

Multiple Comparisons

Dependent Variable:Growth

	(I) NAFC	(J) NAFC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0	10	.38557*	.02705	.000	.2965	.4746
		20	.46936*	.02705	.000	.3803	.5584
		50	.54677*	.02705	.000	.4577	.6358
		100	.78318*	.02705	.000	.6942	.8722
	10	0	-.38557*	.02705	.000	-.4746	-.2965
		20	.08379	.02705	.067	-.0052	.1728
		50	.16121*	.02705	.001	.0722	.2502
		100	.39761*	.02705	.000	.3086	.4866
	20	0	-.46936*	.02705	.000	-.5584	-.3803
		10	-.08379	.02705	.067	-.1728	.0052
		50	.07742	.02705	.097	-.0116	.1664
		100	.31382*	.02705	.000	.2248	.4028
	50	0	-.54677*	.02705	.000	-.6358	-.4577
		10	-.16121*	.02705	.001	-.2502	-.0722
		20	-.07742	.02705	.097	-.1664	.0116
		100	.23640*	.02705	.000	.1474	.3254
	100	0	-.78318*	.02705	.000	-.8722	-.6942
		10	-.39761*	.02705	.000	-.4866	-.3086
		20	-.31382*	.02705	.000	-.4028	-.2248
		50	-.23640*	.02705	.000	-.3254	-.1474
Dunnett t (2-sided)[a]	10	0	-.38557*	.02705	.000	-.4638	-.3074
	20	0	-.46936*	.02705	.000	-.5475	-.3912
	50	0	-.54677*	.02705	.000	-.6250	-.4686
	100	0	-.78318*	.02705	.000	-.8614	-.7050

Table 7.3: One-way ANOVA for comparison for growth rates of *Chlamydomonas reinhardtii* cell wall mutant CC-400 after exposure to a dilution series of 0, 10, 20, 50, and 100 mgL^{-1} naphthenic acid fraction components. n=3.

ANOVA

Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.405	4	.101	10.797	.001
Within Groups	.094	10	.009		
Total	.498	14			

Multiple Comparisons

Dependent Variable:Growth

	(I) NAFC	(J) NAFC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0	10	-.05833	.07903	.942	-.3184	.2018
		20	.01567	.07903	1.000	-.2444	.2758
		50	.03833	.07903	.987	-.2218	.2984
		100	.40167*	.07903	.003	.1416	.6618
	10	0	.05833	.07903	.942	-.2018	.3184
		20	.07400	.07903	.876	-.1861	.3341
		50	.09667	.07903	.739	-.1634	.3568
		100	.46000*	.07903	.001	.1999	.7201
	20	0	-.01567	.07903	1.000	-.2758	.2444
		10	-.07400	.07903	.876	-.3341	.1861
		50	.02267	.07903	.998	-.2374	.2828
		100	.38600*	.07903	.004	.1259	.6461
	50	0	-.03833	.07903	.987	-.2984	.2218
		10	-.09667	.07903	.739	-.3568	.1634
		20	-.02267	.07903	.998	-.2828	.2374
		100	.36333*	.07903	.007	.1032	.6234
	100	0	-.40167*	.07903	.003	-.6618	-.1416
		10	-.46000*	.07903	.001	-.7201	-.1999
		20	-.38600*	.07903	.004	-.6461	-.1259
		50	-.36333*	.07903	.007	-.6234	-.1032
Dunnett t (2-sided)[a]	10	0	.05833	.07903	.870	-.1701	.2868
	20	0	-.01567	.07903	.999	-.2441	.2128
	50	0	-.03833	.07903	.965	-.2668	.1901
	100	0	-.40167*	.07903	.002	-.6301	-.1732

Table 7.4: One-way ANOVA for comparison for growth rates of *Chlamydomonas reinhardtii* cell wall mutant CC-3395 after exposure to a dilution series of 0, 10, 20, 50, and 100 mgL^{-1} naphthenic acid fraction components. n=3.

ANOVA

Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.980	4	.245	223.128	.000
Within Groups	.011	10	.001		
Total	.991	14			

Multiple Comparisons

Dependent Variable:Growth

	(I) NAFC	(J) NAFC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0	10	.38557*	.02705	.000	.2965	.4746
		20	.46936*	.02705	.000	.3803	.5584
		50	.54677*	.02705	.000	.4577	.6358
		100	.78318*	.02705	.000	.6942	.8722
	10	0	-.38557*	.02705	.000	-.4746	-.2965
		20	.08379	.02705	.067	-.0052	.1728
		50	.16121*	.02705	.001	.0722	.2502
		100	.39761*	.02705	.000	.3086	.4866
	20	0	-.46936*	.02705	.000	-.5584	-.3803
		10	-.08379	.02705	.067	-.1728	.0052
		50	.07742	.02705	.097	-.0116	.1664
		100	.31382*	.02705	.000	.2248	.4028
	50	0	-.54677*	.02705	.000	-.6358	-.4577
		10	-.16121*	.02705	.001	-.2502	-.0722
		20	-.07742	.02705	.097	-.1664	.0116
		100	.23640*	.02705	.000	.1474	.3254
	100	0	-.78318*	.02705	.000	-.8722	-.6942
		10	-.39761*	.02705	.000	-.4866	-.3086
		20	-.31382*	.02705	.000	-.4028	-.2248
		50	-.23640*	.02705	.000	-.3254	-.1474
Dunnett t (2-sided)[a]	10	0	-.38557*	.02705	.000	-.4638	-.3074
	20	0	-.46936*	.02705	.000	-.5475	-.3912
	50	0	-.54677*	.02705	.000	-.6250	-.4686
	100	0	-.78318*	.02705	.000	-.8614	-.7050

Table 7.5: *t* values for independent two-tailed t-test (equal variances assumed) performed on absolute and relative phospholipid composition in wild type *Chlamydomonas reinhardtii* and its cell wall mutants CC-400 and CC-3395, as per Tables 4.2-4.5. n=3, df for all tests is (4).

Phospholipid	*t* (Table 4.2) Wild Type		*t* (Table 4.4) CC-400		*t* (Table 4.5) CC-3395	
	Absolute	%	Absolute	%	Absolute	%
14:0	--	--	.529	.111	-19.2	-44.1
16:0	-1.71	-2.30	5.29	2.86	-.078	.533
16:1n-5	7.92	17.2	2.20	-.908	-2.67	-1.40
16:1n-7	9.74	20.9	.561	.159	.265	.507
16:2	13.7	31.2	1.29	-2.54	-.519	-.212
16:3	-1.84	-3.001	.601	-2.51	-.476	-.231
18:0	10.5	36.0	3.77	2.25	-1.92	-.662
18:1n-9	-.013	1.40	6.30	1.72	.181	.433
18:1n-7	.248	1.26	2.26	1.45	-1.50	.055
18:2n-6	-1.54	-3.81	5.51	1.68	-.881	-.401
18:3n-3	-1.64	-2.50	2.07	-3.01	-.874	-.393

	t (Table 4.3)		
	WT	CC-400	CC-3395
mg phosphlipid/g dry sample	-1.18	6.62	-1.25
Peroxidation Index	-2.81	-2.39	-.308
Double bonds/mg saturated phospholipid	.760	-4.88	.134
Double bonds/mg unsaturated phospholipid	.240	-6.22	.513

Table 7.6: *t* values for independent two-tailed t-test (equal variances assumed) performed on lipid composition in wild type *Chlamydomonas reinhardtii* and its cell wall mutants CC-400 and CC-3395, as per Table 4.6. n=3, df for all tests is (4).

t (Table 4.6)

		WT	CC-400	CC-3395
1-2 DAG	Total	-1.43	-1.19	1.85
	16:00	-1.43	.558	.857
	18:00	--	-2.91	2.06
1-3 DAG	Total	-5.67	1.24	.300
	16:00	-4.80	5.18	1.48
	18:00	-3.96	-.663	-.926
	18:1n-9	-5.04	5.20	--
	18:2n-6	-5.02	--	--
	18:3n-3	-4.96	--	--
TAG	Total	-3.84	-.479	-.942
	16:00	-2.35	-.174	.258
	18:00	-5.07	-2.51	-4.72
	18:1n-9	--	5.80	--
	18:2n-6	--	--	-5.89

		WT	CC-400	CC-3395
FFA	Total	-1.50	-3.42	1.32
	16:00	-1.22	-2.10	1.13
	16:1n-5	--	5.05	--
	16:03	--	-5.13	--
	18:00	-2.34	-.471	1.51
	18:1n-7	--	-4.60	--
	18:2n-6	--	-5.84	--
	18:3n-3	--	-6.02	--
CE	Total	-3.00	2.30	.771
	16:00	-2.68	.845	.724
	16:1n-7	--	5.50	.625
	18:00	-3.19	3.27	.651