Assessment of Wastewater Algae for use in Biofuel Production

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Abstract

In the industrialized world the need for liquid fuel is growing every day. Although new technologies have allowed the attainment of previously untapped fossil fuels these practises are unsustainable and harmful to the environment. Part of the solution to ease the fuel burden is through renewable fuels derived from microalgae as they are a carbon neutral source of fuel. The aim of this research was to assess if algae derived from municipal wastewater sources could be potential biofuel feedstocks by assessing their growth and fatty acid accumulation. When comparing wastewater derived algae to culture collection strain there was no significant difference ($p>0.05$) in terms of growth rates under photoautotrophic and mixotrophic conditions. The strain *Botrydiopsis* B2N under mixotrophic (14mM glucose) possessed the highest growth rate ($2.7x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$) of all the strains tested under the various conditions. It was noted that under mixotrophic growth (14mM glucose) non-axenic algae accumulated significantly higher concentrations of neutral lipids compared to the same algal strains under axenic conditions. The result of which is thought to be caused by bacteria creating a nutrient deprived media causing the algae to become stressed and accumulate fatty acids. Under mixotrophic growth (14mM glucose and 3mM acetate) the organic carbon in the media appeared to shift the composition of fatty acids in most cases increasing the likelihood of an even blend of saturated to unsaturated fatty acids.

Key Words: Biofuel, Algae, Bacteria, Fatty Acids, Feedstock

Dedication

I would like to dedicate this thesis to my uncle Dave Stemmler “aka Great Dave-O” who could not be here to witness the completion of this work. Thank you for always believing in what I did. I will always cherish the time we spent together and I will keep pushing forward in your memory.

Love Kevinski

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List of Abbreviations

CO_2:	Carbon Dioxide
ppm:	Parts per million
GHG:	Green House Gases
DNA:	Deoxyribonucleic Acid
RNA:	Ribosomal Nucleic Acid
ATP:	Adenosine Triphosphate
FAS:	Fatty Acid Synthase
ACP:	Acyl Carrier Protein
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
$NADP^+$:	Nicotinamide Adenine Dinucleotide Phosphate
LCD:	Liquid Crystal Display
CPCC:	Canadian Phycological Culture Collection
FAME:	Fatty Acid Methyl Ester
BAME:	Bacterial Acid Methyl Ester
PCA:	Principle Component Analysis
PAST:	Paleontological Statistics
GC:	Gas Chromatography
GCMS:	Gas Chromatography Mass Spectrometry

Chapter 1. General Introduction

Global energy demands have been on the rise since as early as the industrial revolution. From approximately 1971 to 2010 the energy consumption of the world has been 2.2% per year (Goldemberg and Prado, 2013). Of this energy consumption the world uses 4.4 billion tonnes of petroleum per year to make electricity, power automobiles, and to fuel industries (Roddy, 2013). The result of burning petroleum products some of which are fossil fuels causes the release of carbon dioxide. The excess carbon dioxide as a result of anthropogenic sources in the atmosphere has caused changes globally. For example oceans have begun to acidify and warm which has affected the development of marine life (Padilla-Gamino et al., 2013). Whole ecosystems are also being affected as the change in climates and overall global temperature has caused some insects to switch their feeding onto endangered plant species due to delayed or accelerated emergence (Liu et al., 2011).

1.1 State of Fuels

1.1.1 *Atmospheric Carbon Dioxide Levels*

Atmospheric carbon dioxide (CO_2) has been steadily increasing over the last century, reaching the highest concentration (400 ppm) in human history just recently (NOAA, 2013). The excess carbon dioxide worldwide has led to a 1.31^{0}C increase in temperature globally (Huber & Knutti, 2012). The reason for this is that carbon dioxide absorbs infrared radiation from the earth and directs it back towards the surface (Seinfeld,

2011). Carbon dioxide is a naturally occurring compound that is also a greenhouse gas (GHG), other gases that can absorb infrared light include methane, nitrous oxide, and ozone, however CO_2 is the largest contributor of this warming effect (Huber & Knutti, 2012). Ice core samples have been used to document the rise in temperature as a result of increasing CO_2. Before the industrial revolution CO_2 levels where at 280 parts per million (ppm) whereas levels now have reached 400 ppm (Seinfeld, 2011). The dramatic increase in atmospheric CO_2 levels is largely linked to fossil-fuel burning (Seinfeld, 2011).

1.1.2 *First Generation Biofuels*

In 2008 fossil fuels accounted for 88% of global energy consumption, a figure thought to increase with growing economic progress as well as depleting reserves (Brennan and Owende, 2010). The need for sustainable and carbon neutral fuel like bioethanol from corn or biodiesel from palm plants is important, to reduce the reliance on foreign sources of oil and the GHG levels are not further impacted. This led nations around the world to consider renewable sources of fuel that could offset the dependence on non-renewable sources like fossil fuels. The renewable sources of few were almost always derived from biological sources and were deemed biofuels.

First generation biofuels refers to renewable liquid fuel typically derived from soy, palm, animal fat, and waste cooking oil (Gong and Jiang, 2011). Biofuels are thought to remedy the issues with conventional fossil fuels as they are capable of sequestering atmospheric CO_2 (Singh et al., 2011). This is achieved through the act of photosynthesis where light absorbed by the chlorophyll results in oxidation-reduction reaction in which oxygen and water are produced resulting in the formation of reduced carbon compounds

from the CO_2 first absorbed by the plant (Whittingham, 1952). Additionally since most biofuels are based off of plants like corn and soy they are renewable sources of energy as they can be regrown after harvest. Also the liquid form of the fuel means that the energy is stored chemically giving biofuels the advantage over other renewable sources of energy (solar, tidal, wind) since this fuel type can be easily integrated into current machinery (Amaro et al., 2011; Singh and Gu, 2010).

Global production of biodiesel rose from a level of zero in 1991 to approximately 2 billion gallons in 2006 (Birur et al., 2007). Canada has been slow to adopt biofuel production but there are projects in place that attempt to use biomass from western Canadian forests as a source of solid, liquid, and gaseous fuels. Biomass from forests has been shown to produce charcoal, methanol, and hydrogen. The current practice of biomass mitigation after logging is to collect the residue forest scraps and burn them to prevent forest fires (Sarkar et al., 2011; Kumar et al., 2003). A reason why Canadian efforts at biofuel production are not predominant is due to unfavourable climate and lower solar energy levels available for growth (Park et al., 2012).

Although land based biofuels claim to be carbon neutral (emissions are offset by carbon dioxide sequestered during growth) that statement is often false as there are other obscured effects that would result if the world switched to land based fuels. There are three major concerns that limit the viability of terrestrial biofuels: 1) reduction in biodiversity 2) destruction of arable land, and 3) negative environmental impacts.

If first generation biofuels do not to interfere with current arable land used for crop production, then new areas would have to be converted for agricultural purposes. This proves to be another unsuitable course of action as it will lead to loss of natural

ecosystems as well as the biodiversity associated with the area (Smith et al., 2009). Clearing of rainforests, peat lands, savannas, as well as grassland in Brazil, Southeast Asia, and the United States (the current leaders in biofuel production) would actually result in a carbon debt (Smith et al., 2009). It has been estimated that this conversion of land could potentially release approximately 3.8 billion mega-tons of carbon dioxide into our atmosphere (Koh and Ghazoul, 2008). The release of the carbon dioxide is due to the fact that soil and plant matter actually store carbon within their mass and act as a carbon sink. A popular practise in the tropical areas in the world is through slash and burn in which the natural vegetation is cut down and burnt for monoculture plantations (Ewel et al., 1981). The result of combusting the organic plant matter results in the emission of carbon dioxide. Removal of the native vegetation in the 1930s aided in causing the dust bowl in which the native soil dried out and was aerosolized due to poor land management practices (Cook et al., 2009).

The negative environmental impacts associated with terrestrial biofuels typically deal with the practises implemented to maintain high yields. The most common methods are uses of fertilizers and pesticides which each have negative environmental implications. Typically manure is used as a low cost fertilizer however the environmental impacts that result are acidification, emission of greenhouse gases (from the manure), and eutrophication as excess nitrates and phosphates enter receiving waters (De Vries et al., 2012; Prapaspongsa et al., 2010). The great lakes in Canada and the United states have experienced nutrient enrichment via the initial European settlement from 1850 to 1940, then phosphorus input came from detergent use and intense agricultural development until the 1970s (Han et al., 2012; Richards et al., 2002).

The issue with the increase in nutrient loads into water systems promotes algal blooms, and these blooms have been increasing in Ontario water systems (Winter et al., 2011). Additionally not only are the algal blooms increasing in frequency but the dominating species throughout almost all cases is cyanobacteria. The reason that cyanobacteria are of concern is due to their ability to produce cyanotoxins which can inhibit protein synthesis resulting in genetic damage to cells (Graham et al., 2009). This would pose a threat to the organisms occurring within the affected ecosystem either by reducing their fitness or by killing the species indirectly.

Pesticides like organophosphates and organochlorines are also of environmental concern due to their persistence in the environment and their ability to bioamplify, bioaccumulate, and induce acutely toxic effects (Dau et al., 2001, Karami-Mohajeri and Abdollahi, 2010). Pesticides like organophosphates are harmful to non-target organisms since they lack specificity. During the 1980s there were a large number of bee deaths reported after aerial spraying with organophosphorous compounds (Fletcher and Barnett, 2003). Additionally there appears to be a lack of research into the environmental fate of the pesticides. Approximately over 2000-3000 chemicals on the market have insufficient ecotoxicity and toxicity data, and from what is available only 75% have minimal risk assessment (Ragnarsdottir, 2000).

Lastly biofuels when compared to their fossil fuel counterparts do possess drawbacks (Table 1). Although the environmental impacts of biofuels like ethanol, hydrogen or biodiesel are much lower than their fossil fuel counterparts they are often more expensive, require engine modifications to use and generally do not contain as much energy content thus requiring more fuel to attain the same level of performance.

Table 1: Overview of structure, cost, and challenges in production of fossil fuels like gasoline and diesel compared to their renewable sources (Table adapted from U.S. Department of Energy, 2013)

	Gasoline	Ethanol	Hydrogen	Diesel	Biodiesel
Chemical Structure	C4 to C12	CH3CH2OH	H2	C8 to C25	Methyl esters of C12 to C22
Fuel Material (feedstock)	Non-renewable Crude Oil	Corn, grains, agricultural waste	Electrolysis of water, by product of microbes	Non-renewable Crude Oil	Animal fat, oil from: palm, soy, algae
Gasoline Gallon Equivalent	100%	1 gallon of E85 = 73% to 83%	1 kg of H2 = 100%	1 gallon of diesel = 113%	1 gallon = 103% (only 93% when compared to Diesel)
Energy Content (Lower heating value)	116090 Btu/gal	76330 Btu/gal	51595 Btu/lb	128450 Btu/gal	119550 Btu/gal
Energy Content (Higher heating value)	124340 Btu/gal	84530 Btu/gal	61013 Btu/lb	137380 Btu/gal	127960 Btu/gal
Challenges	Nearly 2/3 is imported	Special hosing and conversions to existing engines may be required	Often require valuable precious metals	Nearly 2/3 is imported	More expensive to convert to a useable fuel
Emissions	Carbon Monoxide, Hydrocarbons, Nitrogen Oxides, Carbon Dioxide, Sulfur Dioxide	Reduction in emissions from burning, but increased Carbon dioxide emissions from processing	Hydrogen, water	Carbon monoxide, Nitrogen oxides, Sulfur Dioxide, Diesel particulate matter	Increased nitrogen oxides, no sulfur dioxide emissions (reduction in all other emissions)
Cost per gallon (US dollars)	1.9[A]	2.5[A]	1.18/kg[B]	0.95[C]	1.8[C]

A: Goldemberg, 2007 B: Sarkar and Kumar, 2009 C: Haas et al., 2006

1.2 Second Generation Biofuels

Despite the advantages of using biofuels, the associated negative implications have greatly diminished their economic viability due to reduction of biodiversity, pesticide use and eutrophication. However, the biofuel sector has moved to other feedstocks and processes. These are known as "second generation' biofuels and algae (micro- and macro-) are being investigated as an important source of these fuels.

1.2.1 Overview of Algae

Algae can be classified as photosynthetic eukaryotes that lack leaves, roots, and organs characteristic of higher plants (Parker et al., 2008). The three main classifications are green algae (*Chlorophyta*), red algae (*Rhodophyta*), and diatoms (*Bacillariophyta*). Additionally algae can be autotrophic in which they require carbon dioxide, salts, and light to perform photosynthesis; heterotrophic algae do not perform photosynthesis so an organic source of nutrients is required (Brennan and Owende, 2010).

Algae are typically aquatic organisms responsible for a majority of the oxygen present on earth today and are responsible for the evolution of eukaryotic organisms (Chapman, 2013). The aquatic algae and cyanobacteria actually account for approximately half of the global carbon fixation (Sasso et al., 2012; Field et al., 1998). Fixing carbon is highly important since algae convert sunlight into organic carbon making them the base of aquatic food webs. A large majority of algae are aquatic based organisms, and carbon dioxide diffuses 10 000 times more slowly into water over air. Algae have adapted by developing carbon concentrating mechanisms which enhances

carboxylation efficiency of ribulose bisphosphate carboxylase oxygenase, this allows inorganic carbon (CO_2) to be fixed to carbon (Graham et al., 2009, Thoms et al., 2001).

Algae as a simple yet ancient group of organisms have allowed scientists to address issues in ecology, evolution, cell biology, biogeochemistry, and as models for higher plants (Sasso et al., 2012). For example the edible seaweed *Eisenia bicyclis* has been shown to protect retinal ganglion cells from oxidative damage. Cells treated with the algal cellular extract showed a reduction in apoptosis and resembled healthy non-treated cells (Kim et al., 2012). This research aims to aid treatment in glaucoma patients as it is a neurodegenerative disease similar to the degenerative effects induced with N-methyl-D-asparate.

Since algae are capable of doubling their biomass several times a day. It has attracted attention to their potential use for biofuel production. Some strains can actually accumulate up to about fifty percent of their mass in lipids or triacyglycerides alone (Singh et al., 2011).

1.2.2 Algal Growth Mechanisms

Photoautotrophic growth is defined as growth utilizing solar energy to convert CO_2, water, and inorganic nutrients into oxygen and complex sugars (Benedict, 1978). Algae which grow strictly in this manner are known as obligate photoautotrophs and have adapted their cellular functions to the fluctuation in the day: night cycle (Benedict, 1978, Price et al., 1998). Algae like plants and animals require nitrogen and phosphorus for production of DNA or to make energy for cellular functions. Certain algae can be found in waters that are low or high in nitrogen and phosphorus, or in conditions that fluctuate

between the extremes (Chu, 1943). When algae become deficient in either nitrogen or phosphorus it decreases cellular division, chlorophyll, and protein contents but increase the levels of carbohydrates and lipids (Amaro et al., 2011, Csavina et al., 2011). Research by Singh and Kumar (1992) demonstrated a 1.6-fold increase in lipid content of *Botryococcus*-spp. under nitrogen limitation versus nitrogen supplementation. Zachleder et al. (1988) discovered that Chlorococcal alga (*Scenedesmus*) when grown in phosphorus deprived media perform no net RNA, DNA, or protein synthesis therefore cellular division halted.

Certain algal strains also possess the ability to grow mixotrophically through the use of an organic carbon source (glycerol, glucose, or acetate) in addition to light exposure (El-Sheekh et al., 2012). Mixotrophic algae are found in nutrient poor environmental conditions and eutrophic areas, additionally the ability to use organic carbon sources provides a competitive advantage over strict photoautotrophs (Subashchandrabose et al., 2013). *Chlorella protothecoides* grown photoautotrophically followed by growth in glucose resulted in a 69% higher lipid yield (Singh et al., 2011). Research by Yan et al. (2012) demonstrated that the cyanobacteria *Synechococcus* sp. PC 7942 displayed energy conversion efficiencies (ATP production) under photoautotrophic, glucose mixotrophic, and acetate mixotrophic cultures were 4.59%, 5.86%, 6.60% respectively.

Heterotrophic growth is defined as the use of dissolved organic compounds for growth in the absence of light (Droop, 1974). Most algae when presented with light exposure usually will preferentially perform photosynthesis over uptake of organic carbon, only when growing under low light conditions for an increased duration will lead

to uptake of organic matter (Markager and Sand-Jensen, 1990). The advantage over photoautotrophic growth is a higher density culture, ease of harvest and lack of photoinhibition (reduction in light to algal cells in the center due to higher cell density surrounding them), but the associated production costs are higher due to the use of organic carbon sources (Kim & Hur, 2013). Azma et al. (2011) found *Tetraselmis suecica* when grown photoautotrophically only reached a concentration of 8.40 $g \cdot L^{-1}$ while under heterotrophic growth was 28.88 $g \cdot L^{-1}$. Research by Liu et al. (2011) demonstrated that *Chlorella zofingiensis* not only experienced an increase in lipid yield under heterotrophic growth versus photoautotrophic growth but also accumulated more fatty acids that can be used for biofuel potential.

1.2.3 Algal Lipid Biosynthesis

Fatty acid synthesis has been characterized in both plants and bacteria allowing the algal synthetic pathway to be theoretically deduced by homologous analysis. In the chloroplast fatty acids are biologically synthesized by a type II fatty acid synthase (FAS) which elongates a growing fatty acid chain by two carbons (Blatti et al., 2013). The first step in the fatty acid biosynthesis pathway utilizes the enzyme acetyl-CoA carboxylase forming malonyl-CoA from acetyl-CoA and CO_2 (Ohlrogge and Browse, 1995). Fatty acid synthesis is achieved through a complex mechanism but has been simplified in Figure 1. The process is broken up into five main reaction points:1) An attachment stage in which acetyl-CoA binds to the acyl carrier protein (ACP) which already has a malonyl-CoA attached, 2) The condensing reaction binds malonyl-CoA to acetyl-CoA causing the CoA group and a CO_2 molecule from the malonyl-CoA to be generated, 3) Next is the

reduction stage where the carbonyl group is reduced by NADPH forming an alcohol group on the molecule (β-hydroxy-acyl-ACP) attached to ACP, 4) The molecule is dehydrated forming a water molecule and a double bond to form on the fatty acid chain, 5) NADPH then converts to $NADP^+$ to remove the double bond thereby allowing for further chain elongation (White et al., 2005).

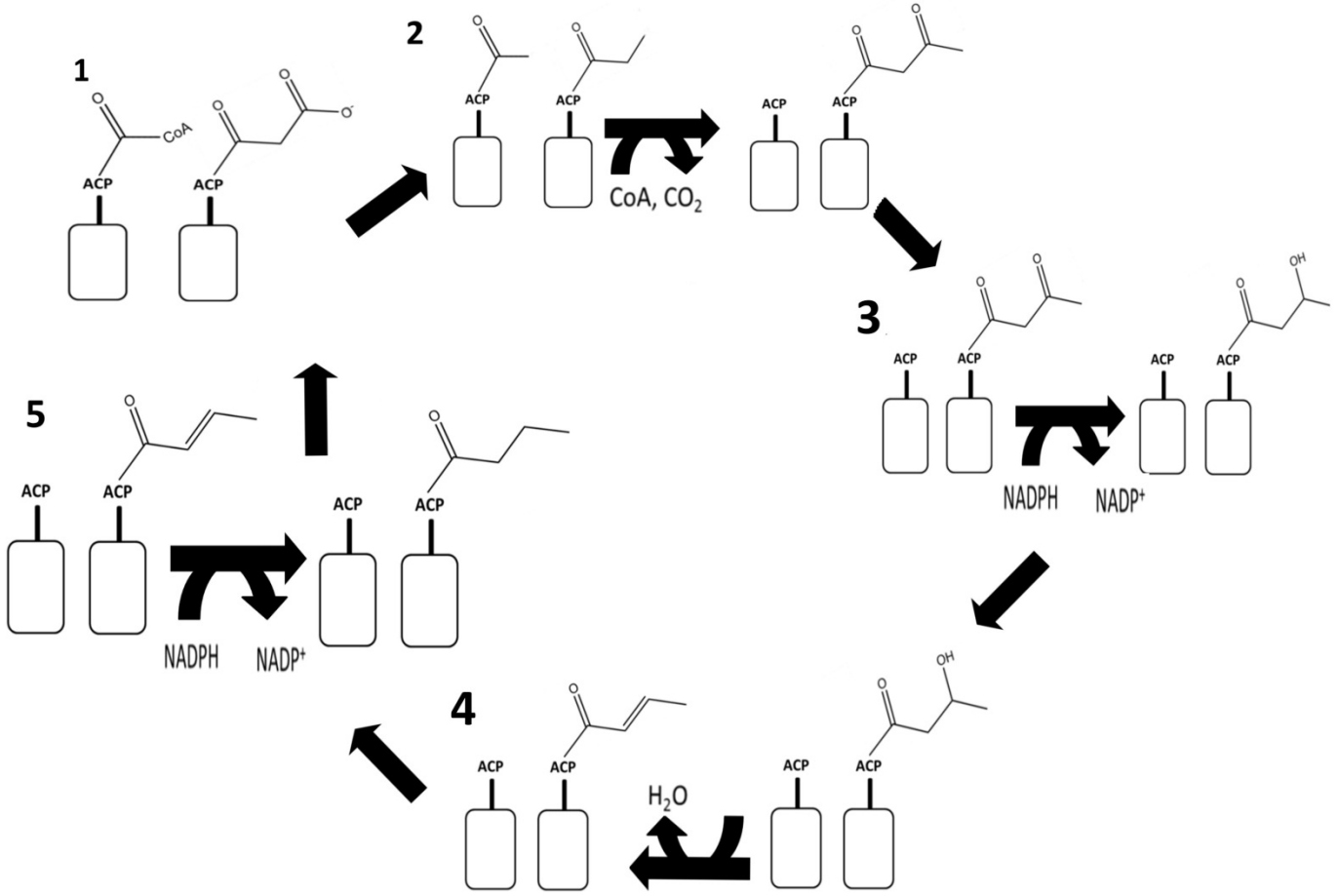
1.3 Algal Biofuels

1.3.1 Algal Biofuels vs. Terrestrial Biofuels (Second Gen vs. First Gen)

Only about one percent (14 million hectares) of the world's arable land is currently used for biofuel production including both first generation and second generation biofuel production (Brennan and Owende, 2010). This means to replace all current fossil fuels utilization of vast amounts of farmland, pesticides, and fertilizers would need to be used. As stated before (First Generation Biofuels) this would have many negative impacts, for terrestrial crops like corn it would take approximately an area of 1000 miles by 1000 miles to displace all the gasoline consumption in the United States (Dismukes et al. 2008). On the contrary algal derived biofuels could displace all the gasoline for the U.S in a fraction of the land requirement. Even at sub-par production the potential area required is roughly equal to that of the corn crop production in the United States during 2006 (Dismukes et al. 2008).

When compared to terrestrial biofuel crops algae have a 6-12 times greater yearly energy production over corn or switchgrass (Sandefur et al., 2011). Additionally the production of microalgae is non-seasonal, meaning that yields are not limited to one to

Figure 1: Simplified fatty acid synthesis diagram: 1) Attachment phase, 2) Condense phase, 3) Reduction phase, 4) Dehydration phase, 5) Reduction phase. This cycle has the ability to repeat after step five further elongating the carbon chain



two harvests per year (da Silva et al., 2009). This is due to the fact that algae have the inherent ability to double their biomass during their exponential growth phase, sometimes even doubling in as little as three and a half hours (Chisti, 2007; Singh et al., 2011). Additionally algae are not limited to only one fuel derivative. Based on the processing that is undertaken like direct combustion, pyrolysis, or chemical conversion the biomass can be converted to fuels like hydrogen, oil, or even raw electricity (Brennan et al., 2010; Tsukahara and Sawayama, 2005). The advantage of using fuel like biodiesel is the fact that it can be integrated into current diesel engines and can reduce emissions or particulate matter, SO_x, CO, and hydrocarbons (Smith et al., 2009; Sheehan et al., 1998). Bioethanol fuels cannot be easily incorporated into current gasoline engines and actually require modifications to the intake manifold, fuel lines, fuel-filtering system, the compression ratio also needs to be altered along with the catalytic converter (Agarwal, 2006).

1.3.2 Bioreactors vs. Open Pond Systems

Open pond systems are typically referred to as raceway ponds due to their construction resembling a circular racetrack (Chisti, 2007; Brennan and Owende, 2010). The ponds themselves can be built out of concrete or be as low cost as compacted earth lined with a waterproof white plastic (Brennan and Owende, 2010). Although the open pond systems are cheap, easy to operate and can meet large scale demands they do have drawbacks. One issue is that these systems require natural sunlight to function and an issue with that is seasonal variation. Grobbelaar et al. (1996) demonstrated the influence of longer dark cycles and the frequency of light and dark cycles through a pulse

mechanism, which showed that longer dark cycles led to a decrease in photosynthetic rates. Therefore in order for open pond systems to achieve maximum efficiency they would have to be constructed in areas where light fluctuations are minimal year round.

Another issue is that since the system is exposed to the surrounding environment contamination can happen. Often times non-target strains like *Chlorella* or tolerant strains like *Spirulina* will be able to outcompete the alga being used in culture; this requires the strains to be grown in batch or semi-batch modes which require reseeding of the ponds with new growth media (Del Campo et al., 2007; Borowitzka, 1999).

There is another cost effective open pond system which employs the use of a translucent barrier to prevent contamination from the surrounding environment. The semi-closed system typically utilizes plexiglass as it allows the species being grown to remain dominant, extends the growing season (acts like a greenhouse), and lastly if heated can provide a year round growing season (Singh and Sharma, 2012).

The issues inherent to open pond systems can actually be reduced or completely removed when using a closed system. This is due to the fact that since the system is closed foreign contaminants can be managed, cells can be irradiated more effectively through man made lighting, and carbon dioxide as well as inorganic nutrient levels can be adjusted effectively (Sforza et al., 2012). Additionally since the system is closed photobioreactors can actually reduce water loss through evaporation, increase cell concentrations, and since contamination is highly reduced the algae can be used for biopharmaceuticals (Singh and Sharma, 2012).

Many closed system variations exist such as vertical column, flat panel, and tubular photobioreactors (PBRs) (Singh and Sharma, 2012). Although the design and

structuring of a closed system is always being modified and altered to optimize the best mixing and light penetration for optimal growth.

Vertical column PBRs typically implement large cylinders to increase the surface-volume ratio and limit the overall height to four meters to ensure gas transfer is optimal (Wang et al., 2012). The design and construction actually allows the reactor to limit photo inhibition (reduction in light penetration due to high cell density) as the radial mixing of fluid causes cells in the darker core to be continuously moved to the well-lit perimeter of the column (Miron et al. 2002). This is done through the gentle bubbling of carbon dioxide up from the base of the column and allowing gravity to pull the cells within the medium back down. Typically the headspace or 'freeboard' is in place to aid in gaseous transfer of oxygen out of the system.

Flat panel PBRs are of a similar construction to that of a window pane or sheet like structure (Singh and Sharma, 2012). Turbulence within the housing is achieved via the pumping of liquid while airlift flat panel PBRs use compressed air to mix the medium (Wang et al., 2012). The panels are often cost effective as they can be constructed from transparent materials like glass, plexiglass, or even polycarbonate (Singh and Sharma, 2012). When comparing the flat panel PBR to the vertical column PBR research has shown that biomass productivity to be 1.7 times higher in the flat panel model which is attributed to the highly organized mixing of the bioreactor (Degen et al., 2001).

Tubular PBRs are the most common design which uses an array of transparent tubes that can be conformed into straight, bent, or spiral shapes. The tubular design differs only slightly when compared to the previous two PBRs. There is still a transparent tube to allow algal growth, along with a pump to ensure even mixing, but the difference

here is that since the tubes are typically allocated in a horizontal fashion they require a degassing column to provide gas exchange and cooling (Wang et al., 2012). This degassing column is necessary to prevent overheating since the absorption of light is greatly increased.

An issue with closed systems is that they rely heavily on a large capital investment to start up the production process. A Dutch company AlgaeLink N.V. offers algal cultivation systems that are 300 and 1200m^2 which sell for €144,000 and €194,000 (~$200- and $300-thousand CDN) (Holtermann and Madlener, 2011). A life cycle assessment performed by Stephenson et al. (2010) demonstrated the fossil fuel energy input and global warming potential of biodiesel derived from *Chlorella vulgaris* was 85 and 78% lower than that of fossil fuel derived diesel, while tubular bioreactors are 362 and 273% greater. This is extrapolated to be the result of electrical input required for mixing and lighting as well as gaseous fuel burning for heating the solution to optimal temperature.

1.3.3 Culture Conditions and Lipid Optimization

Culture conditions are often dependant on the algal strain being studied as each strain has certain regimes for optimal growth. For example Cho et al. (2007) discovered that *Chlorella ellipsoidea* had the highest growth rate at a temperature of 25°C. Additionally research by Sheehan et al. (1998) found that of 300 strains collected from Utah and Colorado only 15 were able to grow at temperatures ≥30°C. Even considering light exposure regimes will result in different responses based on the algal strains being utilized (Price et al. 1998). The light exposure experiments performed by Price et al.

(1998) revealed that the non-coccolith forming species all performed better when provided with a 14:10 light dark ratio while select strains (*Emiliania huxleyi* 88E, *Cyclococcolithus leptoporus*, and *Cricosphaera carterae*) actually performed better under continuous lighting.

Like terrestrial plants algae also require an input of carbon dioxide, this can either be from the atmosphere or through an external bubbling source. When treated with CO_2 enriched air (750 and 1600ppm CO_2), *Hypnea spinella* experienced enhanced growth by 85.6% and 63.2% (Suarez-Alvarez et al., 2012). External sources such as flue gas from smokestack emissions are being utilized for algal growth production. Chen et al. (2012) discovered that utilizing pressurized carbon dioxide-rich flue gas (500mm Hg) from the Dalin coal-fired power plant in southern Taiwan resulted in a carbon dioxide fixation rate of 2,234 kg per year.

Lipid content (triglyceride concentration) within cells can be altered through changing the available nutrients present in the medium. Research by Mujtaba et al. (2012) found that *Chlorella vulgaris* increased lipid content from 14.5% in fresh media to 24.6% in nitrate depleted media, corollary to this when nitrate levels were kept at a steady state the lipid content remained at 14-16%. This starvation of the cell however needs additional time being required to attain maximum lipid concentration due to slower growth rates and will additionally lead to lower biomass production since cellular division slows or halts upon starvation (Das et al. 2011). The starvation can be offset by adding in a sugar substrate to change the photoautotrophic culture to a mixotrophic culture. Das et al. (2011) demonstrated that with addition of glycerol fatty acid methyl

ester (FAME) content increased by 30%, allowing for increased biomass and lipid content of the culture.

Changes to algal lipid composition (types of fatty acids: triglycerides vs. glycolipids) can also be induced by temperature stress. It is believed that changes in Temperature results in changes in the lipid profile of the cellular membrane to protect cellular functions (Somerville, 1995). There is a wide variation as some strains exhibit changes in lipid content with decreasing temperature while others increase lipid content with increasing temperature. Another proposed method is to use pH-based stress to induce lipid accumulation. Stress via pH has not been significantly researched but it is thought to be easier to implement and scale up over nutrient or temperature stress (Skrupski et al., 2012). Gardner et al. (2011) demonstrated that pH change induced lipid accumulation in both *Scenedesmus* and *Coelastrella* strains. Differences of incremental pH change vs. static pH change have also been investigated. Three strains FGP5, OS1-3, and OS4-2 isolated from Alberta and Saskatchewan Canada accumulated oil under these varying conditions. The strain FGP5 accumulated oil in quantities of 18% of its dry weight (double that over normal conditions) in both incremental and constant pH stress, however OS1-3 and OS4-2 accumulated 47 and 45% oil per dry weight when pH was incrementally changed over control (constant pH stress resulted in slightly higher yields) (Skrupski et al., 2012).

Although lipid alteration can be achieved by changing the growth conditions the algae are being exposed to, genetic manipulation can be used to increase yields. By combining UV mutagenesis with flow cytometry sorting, researchers isolated cells which displayed enhanced lipid content in *Isochrysis* affinis *galbana*, in which the fatty acid

content ranged from 262 mg total fatty acids $(gC)^{-1}$ to 409 mg total fatty acids $(gC)^{-1}$ without affecting maximum growth rate (Bougaran et al., 2012). Increasing the gene expression of Acetyl CoA Carboxylase led to no increased lipid production indicating that direct manipulation of the fatty acid synthesis pathway may not be possible (Gong and Jiang, 2011).

1.4 Water Requirements

1.4.1 Fresh Water Issues

By the year 2025 it is predicted that two out of every three persons in the world could be residing in regions without access to drinking water (Mehanna et al., 2010). The seriousness of this issue is not only due to human consumption of surface and groundwater sources but also due to the increase in global temperatures. Sandstrom (1995) showed that a 15% reduction in precipitation due to climate change resulted in a 40-50% reduction in aquifer water recharge. The need for water for human consumption is already dire in parts of the world and this issue is further strained as farmers will require more water for the production of crops. Currently the consumption for crops is roughly 2700 km^3 of drinking water, this number is projected to increase to 4000 km^3 of water required by 2050 (Chinnasamy et al., 2010). This is disturbing for the future of algal biofuels as large scale cultivation of algae would require approximately 1.5 million liters of water per hectare, if grown in open ponds the evaporation loss would be 7-11 million liters of water per hectare per year (Chinnasamy et al. 2010). A viable alternative is to utilize water that cannot be put forth for drinking or farming purposes.

1.4.2 Wastewater

After industry or farming operations consume fresh water, the discharged wastewater enters surface waters, contributing to eutrophication or contamination of local ecosystems (Foley et al., 2012). Eutrophication is often the result of excess nitrogen and phosphorus concentrations. Nitrogen and phosphorous concentrations can be 10-100 mg/L in municipal wastewater and >1000 mg/L in agricultural wastewater (de la Noue et al., 1992). This will result in algal blooms that will alter dissolved oxygen levels or release toxins which can decrease species richness. Li-Na et al. (2011) found a reduction in molluscs from 83 species and 7 subspecies in the 1940s to only 16 species and one sub species in the early 1990s as a result of phosphorus accumulation in Lake Dianchi in China. This is achieved due to hypoxia developing in which the rate of oxygen use exceeds the rate of replenishment. A large hypoxic dead zone that is well documented is formed around the Louisiana shelf in the Gulf of Mexico and is the result of increased transport of nutrients from anthropogenic sources such as fertilizers, sewage, and livestock-derived runoff (Osterman et al., 2009). In 1995 two scientists reported 44 dead zones globally but in 2004 the United Nations Environment Programme reported there are 150 recurring and permanent dead zones as the result of hypoxia (WWF, 2005).

The concept of recycling wastewater for other uses is not a new idea as the ancient Greeks (300-500 BCE) actually had public toilets which drained into piping that carried the waste away from the city to agricultural fields where it was used as a fertilizer for crops (Lofrano & Brown, 2010). Wastewater poses an interesting avenue for algal biofuels as the high levels of nitrogen and phosphorus provide ideal nutrient levels for algal growth.

Sandefur et al. (2011) has actually shown that algal strains utilizing wastewater discharge were able to alter phosphorus concentrations from 0.165 mg/L to 0.095 mg/L and nitrogen from 9.20 mg/L to 7.56 mg/L. Additionally when algae are grown in farm effluent like piggery wastewater An et al. (2003) showed that the wastewater containing 788 mg/L NO_3 had a removal of 80% of the NO_3 content using *Botryococcus braunii*. A paper by Amaro et al. (2011) outlined the fact that marine and fresh water species of microalgae can possess oil levels between 20-50%. This makes the option for using naturally occurring algal strains derived from wastewater treatment plants more alluring as they do not have to be cultivated over multiple generations to select the highest productivity phenotype as their environmental stressors have been doing the isolation.

1.5 Knowledge Gaps

Although the use of wastewater is not a novel idea, since research has shown that algae are effective at removing nitrogen and phosphorus (Sandefur et al., 2011) from waste effluent much has yet to be considered. Few researchers isolate naturally occurring algal species from the effluent being studied. Typically the algae isolated are from lakes, rivers, or naturally occurring water bodies (Zhou et al. 2011, Park et al. 2012, Liu et al. 2012). Abou-Shanab et al. (2011) showed microalgae derived from naturally occurring sources ranged anywhere from 21 to 58% lipid content. Although this supports the validity of using environmentally isolated strains it does not verify if those strains are more effective lipid producers over laboratory strains. Lastly Zhou et al. (2011) has stated that only a few strains in the *Chlorella* genus and *Scenedesmus* genus have actually been

analyzed for their ability to grow in wastewater. This indicates there is a lack of testing of various algal strains.

Most research on the topic of using algae grown in wastewater typically sterilizes the algae to remove bacteria from the solution through either filter sterilization, application of antibiotics, or through chlorination (Zhou et al., 2011, Chinnasamy et al. 2010, Liu et al. 2012). In doing this the natural algal bacterial interaction is no longer present which may impact not only growth but lipid accumulation.

1.6 Purpose

The purpose of this research was to determine if there are microalgae strains from municipal wastewater systems that can serve as candidates for feedstock in biofuel production. The rationale behind this is that microalgae from wastewater effluent would be tolerant of wastewater conditions and also effective at removing nitrogen and phosphorus from wastewater. This was accomplished by the following objectives:

1. Isolate algal strains from municipal wastewater
2. Grow algal isolates under photoautotrophic, mixotrophic, and heterotrophic conditions to assess metabolic capacity
3. Analyze the lipid content and composition of each strain under varying conditions
4. Assess if bacterial presence or absence changes the lipid content/composition, or growth
5. Verify whether the culture collection strains or algal isolates from wastewater treatment plants are more effective lipid producers

I hypothesized that the environmentally isolated strains would outperform the culture collection strains under the various growth conditions. I theorize heterotrophic growth would yield the best lipid content and biomass; in addition the presence of bacteria would also greatly improve the growth and lipid accumulation of all the strains isolated.

Chapter 2. Isolation and Growth Characteristics of Wastewater Algae

2.1 Introduction

Currently the largest collection of freshwater algae resides at the University of Coimbra, Portugal maintaining over 4000 strains with only a few hundred strains studied for their chemical contents (Duong et al., 2012). This means that environmentally isolated algae may have untapped potential for uses in the biofuel sector. There is need for high lipid yield and high biomass production of algae which if perfected would ultimately lead to an increase in production. As stated previously lipid production can be induced by various means but lipid production under normal growth needs to be determined before considering growth under adverse conditions (Lim et al., 2012). For biofuel production to be successful, strains need to be productive, tolerant of the local climate/growth medium and possess the desired end products (Mutanda et al., 2011). Yang et al. (2000) stated that to improve the overall efficiency of an algal culture the growth media and light intensity have to be optimized first.

A multitude of methods exist for isolating microalgae: single cell isolation, agar plate isolations, atomized cell spray technique, dilution techniques, gravimetric isolation, and more advanced techniques such as flow cytometry (Andersen, 2005). Single cell isolation involves the transfer of a singular cell using a Pasteur pipette. Agar plate isolations utilizes spread plate methods or pour plates methods to grow single colonies directly on or inside the agar. Atomized cell spraying forces algal cells through a small opening scattering cells onto agar plates. Dilution involves reducing the initial inoculum to a small enough amount to ensure a single cell is all that remains at the end of the

process. Gravimetric isolation involves the use of gravity to settle algal cells based on size (larger heavier cells sink to the bottom while smaller lighter cells stay towards the top). Flow cytometry enables the sorting of cells based on intracellular components or based on outer cellular characteristics through the use of a laser.

2.2 Materials and Methods

2.2.1 Strain Collection and Isolation

Wastewater samples were collected from three municipal wastewater treatment plants: Nonquon River Lagoons Wastewater Pollution Control Plant (Port Perry, ON), Corbett Creek Water Pollution Control Plant (Whitby, ON), and Woodward Avenue Wastewater Treatment Plant (Hamilton, ON). Six one-liter Nalgene bottles were used to collect samples of raw effluent after secondary treatment (after biological treatment) and before final treatment (before chlorination). The samples were then placed into a cooler and stored at 4°C until enrichment cultures were prepared. Once the samples were ready to be processed 100mL of raw effluent was placed into sterile Erlenmeyer flasks for both stages of the wastewater treatment. Triplicate flasks were then placed on a light table (12:12 light cycle) to encourage algal growth in effluent.

Once growth was established and confirmed via either naked eye or microscopic examination, 5 mL of the sample was transferred to a 250 mL Erlenmeyer flask containing either autoclaved BG 11 (Rippka et al., 1979) or CHU 10 (Stein, 1973) medium as a first step. After growth was established in media the sample was subcultured by transferring 5 mL of the old culture to the new medium and allowed to grow for two

weeks. This process was repeated two successive times to ensure the strength of the algal culture in lab conditions (Andersen, 2005). Isolations of the consortium was undertaken by serial dilutions and transferring 100 µL of diluted culture to 1.5% agar for spread plating. Once single colonies were formed they were transferred with the use of a flame sterilized loop to a well in a 24 microwell plate containing 1 mL of either medium. When growth was present and unialgal cultures were confirmed microscopically via the Evos XL core inverted LCD microscope, the cultures that were unialgal were then transferred to fresh media. Algal isolates were identified through the use of dichotomous keys and various online algal databases. Before any experimentation each culture was re-assessed for unialgal status to ensure only the presence of single strain. All chemicals were purchased from Sigma Aldrich (Canada).

Reference strains were obtained from the Canadian Phycological Culture Collection (CPCC). *Scenedesmus acutus* – CPCC strain 10, isolated by P.M. Stokes from Boucher Lake, Falconbridge, ON, Canada, June 1970. The strain is copper and nickel tolerant. *Chlorella kesslerii* – CPCC strain 266, isolated by R. Pratt pre-1946, location unknown other than freshwater habitat in USA. Originally deposited as *C. vulgaris* but later identified as *C. kesslerii* by Kessler and Huss, 1992. Relatives: UTEX 263, CCAP 211/11h, SAG 211-11h, ATCC 11468.

2.2.2 Growth Conditions

Algae were grown in an environmental growth chamber (Algaetron Photon System Instruments, Czech Republic) with a built in shaker table. Growth conditions in

the literature varied greatly: Park et al. (2012) used 175 rotations per minute (rpm) at 22°C and an illumination of 75µmol photons m^{-2} s^{-1}. Kirkwood et al. (2003) used no shaking, 26°C, 46.3µmol photons m^{-2} s^{-1} as well as 8µmol photons m^{-2} s^{-1}. Da Silva et al. (2009) stirred the solution with a bubbler at 25°C and 150µmol photons m^{-2} s^{-1}. Csavina et al. (2011) used 300 rpm, 25°C, and 10 µmol photons m^{-2} s^{-1}. Based on the wide range of literature values, I set my growth experiments at 200 rpm and 150 µmol photons m^{-2} s^{-1}. A temperature of 22°C was used to match the ambient temperature in the lab to minimize any variations encountered when sampling.

To assess which condition would be optimal for growth and fatty acid accumulation all three growth conditions were tested (photoautotrophic, mixotrophic, and heterotrophic). For the photoautotrophic conditions, all organics were removed from the BG 11 media (citric acid, ferric ammonium citrate, and sodium EDTA) and the iron was replaced with ferric chloride similar to the method described by Kirkwood et al. (2003). The mixotrophic conditions utilized the organic reduced medium from the photoautotrophic trials with the addition of either glucose (14mM) or acetate (3mM). Additionally the heterotrophic trials utilized the same medium as the mixotrophic trials with the exception of being placed in a box to eliminate light from illuminating the culture.

Cultures for experimentation were grown in 50 mL Erlenmeyer flasks in triplicate. To standardize the growth, implementation of an exponential phase algal inoculum (~day 5 of growth) was used over a specific cellular density. The reasoning for this was we wanted to ensure the algal strains were in early exponential growth similar to the method described by Kirkwood et al. (2003). During the growth cycle for each strain

under each growth regime cell counts were performed via a Bright-Line Haemocytometer slide (Hausser Scientific) and the absorbance was taken via the Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific) every day of the experiment to establish growth rates. Standard curves for each strain was developed based on cellular counts and optical density readings. This was done to ease in future experiments for determining cellular density. The experiments lasted a total of seven days.

2.2.3 Statistical Analysis

Growth rates for each strain under each of the three conditions were calculated based on the exponential growth slope determined by linear regression analysis using MS Excel 2010. An Analysis of Variance (ANOVA) was performed on the growth rate data using Sigma Plot 12 to determine significant differences between treatments. A Shaprio-Wilk test was performed to determine if the data were normally distributed and all pairwise multiple comparisons were performed with the Holm-Sidak method. An alpha of 0.05 was used to determine the overall statistical significance of each of the treatments. A Two way ANOVA was also performed to assess the differences between each strain in comparison to the culture collection strains (the data was Ln transformed).

Growth rates for each strain were input into the Paleontological Statistics (PAST) programme for Cluster analysis using the Bray-Curtis similarity measure.

2.3 Results

2.3.1 Strain Collection and Isolation

In order to study the fatty acid profiles and growth characteristics of the algae isolated from municipal wastewater first the proper medium needed to be chosen. A comparison between CHU10 and BG11 media showed that after 5-10 days of growth that algae grown in CHU10 started to exhibit a yellow colour while BG11 resembled healthy algae green in colour (Figure 2).

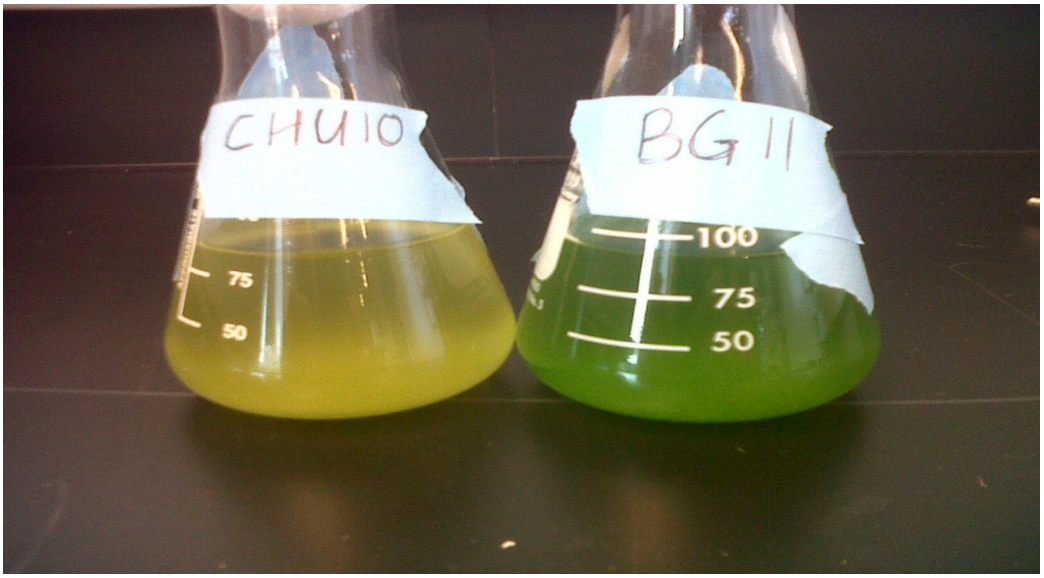


Figure 2: Algae grown in CHU10 and BG11 media over the course of 10 days

After comparing both CHU10 and BG11 media to the concentration of phosphorus and nitrogen derived from the wastewater treatment plants it was determined that the concentrations for CHU10 were much too low (see Table 2). The concentration for BG11 in terms of phosphorus was acceptable however the total nitrogen was not. The nitrogen concentration needed to be reduced by a factor of ten to bring the concentration

down to a level that more closely resembled wastewater conditions. This nitrogen overloading was also noted by Kirkwood et al. (2003), where the nitrogen concentration was also reduced by a factor of 10.

Table 2: Comparison of total nitrogen and phosphorus concentrations derived from the wastewater treatment plants to the concentrations found in the growth media

Average Effluent Concentrations per year				Media Type			
Plant 1		Plant 2		CHU 10		BG11	
Total P (mg/L)	Total N (mg/L)	Total P (mg/L)	Total N (mg/L)	Total P (mg/L)	Total N (mg/L)	Total P (mg/L)	Total N (mg/L)
3.66	31.74	3.69	27.60	1.80	6.80	5.00	250.00

*The data derived from the wastewater treatment plants is kept confidential; therefore the location just appears as plant 1 and plant 2.

From the consortium of algae grown in wastewater strains were isolated from each site via spread plate methods. Table 3 provides a list of all the isolates, the media used to isolate each strain, and the sample site. There was a much higher presence of both *Chlorella* and *Scenedesmus* strains across all wastewater treatment plants.

2.3.2 Growth Conditions

Each strain was tested under photoautotrophic, mixotrophic, and heterotrophic growth. Utilizing the growth data over the seven day experiment growth rates were calculated and plotted to compare the treatments to one another see Figures 2-23. The major finding was only a few select strains (*Chlorella kessleri*, *Chlorella* 19CC, *Scenedesmus* 39CC, and *Dictyochloris* F2.2N) could tolerate the heterotrophic conditions, all of the remaining strains had a low or negative growth rate. The *Scenedesmus* strain which possessed the highest growth rate was under mixotrophic

growth conditions. The growth rate for S3N under 3mM acetate mixotrophic conditions reached a rate of $4.12\pm0.57x10^3$ cells$\cdot L^{-1}\cdot day^{-1}$. While the lowest growth rate was found in the heterotrophic growth. S1B, S4N, and S6H all had negative growth rates under heterotrophic growth. The *Chlorella* strains overall possessed the second highest growth rate of any strains tested with the maximum growth rate of $1.72\pm0.29x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$ for C4C under 14mM glucose mixotrophic conditions. The tolerance under heterotrophic growth was much higher as only two strains (C2H and C3N) could not grow sufficiently under heterotrophic conditions. The highest growth rate of any of the strains was B2H possessing a growth rate of $2.7\pm0.14x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$ under 14mM glucose mixotrophic conditions. However the *Botrydiopsis* strains possessed low growth rates or led to cell death under heterotrophic conditions. *Ellipsoidon* displayed the highest growth under 14mM glucose mixotrophic conditions with a growth rate of $4.87\pm0.59x10^3$ cells$\cdot L^{-1}\cdot day^{-1}$ for strain E2C. Again under heterotrophic conditions the growth was significantly ($p<0.05$) lower than that of photoautotrophic growth or mixotrophic growth. *Dictyochloris* showed to have the highest growth rate under 14mM glucose heterotrophic conditions with a rate of $1.65\pm0.22x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly ($p<0.05$) greater than growth under photoautotrophic or mixotrophic growth. The *Microcystis* strain actually had the highest growth rate under photoautotrophic conditions with a rate of $9.53\pm2.27x10^2$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different than the negative/low growth rate under heterotrophic conditions.

To assess the performance of each strain in relation to the culture collection strains the growth rates were Ln transformed and each wastewater strain was compared to

both *Scenedesmus acutus* (S1B) and *Chlorella kesslirii* (C1U) (Table 3). What can be noted was that under both photoautotrophic growth and mixotrophic growth (3mM Acetate) there were no significant differences ($p>0.05$) except for strain C2H. Under mixotrophic growth (14mM Glucose) again C2H is significantly ($p\leq0.05$) lower in growth when compared to both C1U and S1B, but in addition M1H, S6H, and S4N are also significantly lower. All remaining strains display no significant differences. Under heterotrophic growth (14mM Glucose) C1U displayed one of the highest growth rates resulting in significant differences among S2N, S3N, M1H, S6H, S4N, S5N, B1N, C3N, E1N, S7H, S8C, S9C, C2H, E3N, and even S1B. The growth under heterotrophic conditions (3mM Acetate) also yielded similar results as only D1N, S3N, C5C, E2C, and S9C were the only strains which were not significantly different ($p>0.05$) when compared to C1U and S1B.

Under each of the conditions the strains with the highest growth rates were selected as potential strains for biofuel feedstocks and analyzed for significance in relation to the other wastewater strains.

1) Photoautotrophic: C6C with a growth rate of $6.19\pm0.21\text{x}10^{3}$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different ($p\leq0.05$) in growth only to C3N and C2H
2) Mixotrophic (14mM glucose): B2H with a growth rate of $2.7\pm0.14\text{x}10^{4}$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different ($p\leq0.05$) in growth to S4N, C2H, S6H, M1H, S5N, C3N, S7H, S8C, and S3N.
3) Mixotrophic (3mM acetate): D1N with a growth rate of $8.59\pm1.07\text{x}10^{3}$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different ($p\leq0.05$) in growth to S6H, S4N, and C2H.

4) Heterotrophic (14mM glucose): D1N with a growth rate of $1.65\pm0.22x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different ($p\leq0.05$) in growth to S4N, S6H, M1H, E3N, C2H, B1N, C3N, S3N, S2N, S5N, E1H, S7H, S9C, S8C, B2H, E2C.
5) Heterotrophic (3mM acetate): C6C with a growth rate of $3.8\pm1.79x10^3$ cells$\cdot L^{-1}\cdot day^{-1}$ which was significantly different ($p\leq0.05$) in growth to S4N, B2H, C2H, E3N, S2N, C3N, C4C, S7H, S6H, B1N, M1H, S5N, S8C, E1H, S3N, E2C, S1B.

Based on the growth rate data a cluster analysis was performed to look at the similarities in growth patterns among strains (Figure 25). A tentative pattern of percent similarities does form typically among the *Scenedesmus* and *Chlorella* strains. Interestingly enough the *Microcystis* M1H and *Ellipsoidon* E3N two taxonomically and morphologically dissimilar algal strains possessed very similar growth patterns under photoautotrophic, mixotrophic, and heterotrophic growth. Also C2H a *Chlorella* strain possessed no similarity to any of the other *Chlorella* strains. Additionally E1H was very similar to S2N as was C3N to S7H.

Table 3: The algal strains isolated from each sample site along with the initial media used to isolate, the attributed strain ID and if the algal strain could be classified as axenic. N/A= Not Applicable

Algal strain	**Isolating Medium**	**Treatment stage**	**Sample site**	**Strain ID**	**Axenic**
Scenedesmus acutus	Unknown	N/A	Boucher Lake, ON	S1B	Y
Scenedesmus F3.6N	CHU 10	Final Treatment	Nonquon	S2N	Y
Scenedesmus F4.3N	CHU 10	Final Treatment	Nonquon	S3N	Y
Scenedesmus S1.2N	CHU 10	Secondary Treatment	Nonquon	S4N	N
Scenedesmus F4.3*N	CHU 10	Final Treatment	Nonquon	S5N	Y
Scenedesmus M2.2H	BG 11	Secondary Treatment	Hamilton	S6H	Y
Scenedesmus M3.3H	BG 11	Secondary Treatment	Hamilton	S7H	Y
Scenedesmus 18CC	CHU 10	Secondary Treatment	Corbett Creek	S8C	N
Scenedesmus 39CC	CHU 10	Secondary Treatment	Corbett Creek	S9C	N
Chlorella kesslirii	Unknown	N/A	Unknown (USA)	C1U	Y
Chlorella F2.4H	BG 11	Final Treatment	Hamilton	C2H	N
Chlorella S4.3N	CHU 10	Secondary Treatment	Nonquon	C3N	Y

Chlorella 03CC	CHU 10	Secondary Treatment	Corbett Creek	C4C	N
Chlorella 04CC	CHU 10	Secondary Treatment	Corbett Creek	C5C	N
Chlorella 19CC	CHU 10	Secondary Treatment	Corbett Creek	C6C	N
Botrydiopsis S4.2N	CHU 10	Secondary Treatment	Nonquon	B1N	Y
Botrydiopsis F1.3H	BG 11	Final Treatment	Hamilton	B2H	N
Ellipsoidon F2.3H	BG 11	Final Treatment	Hamilton	E1H	Y
Ellipsoidon 23CC	CHU 10	Secondary Treatment	Corbett Creek	E2C	N
Ellipsoidon F3.3N	CHU 10	Final Treatment	Nonquon	E3N	N
Dictyochloris F2.2N	CHU 10	Final Treatment	Nonquon	D1N	Y
Microcystis M2.3H	BG 11	Secondary Treatment	Hamilton	M1H	N

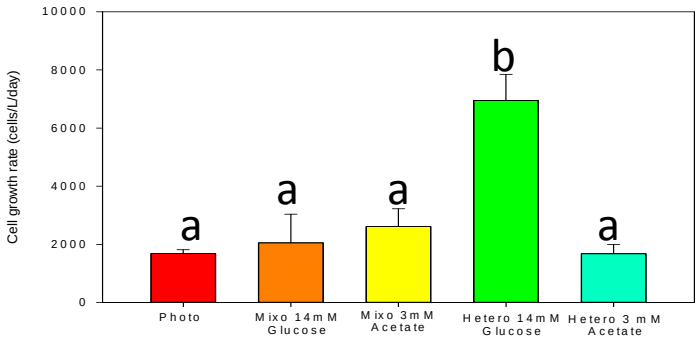


Figure 3: *Chlorella kessleri* tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

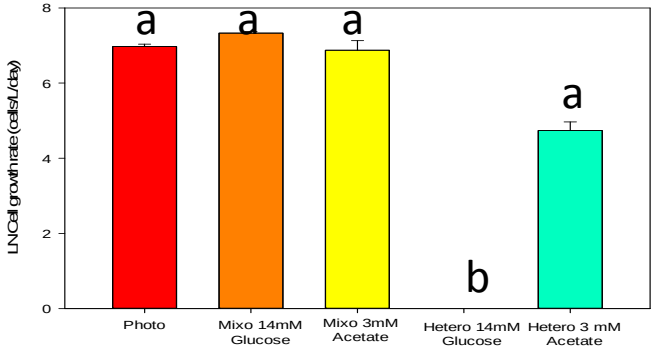


Figure 4: *Scenedesmus actus* tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was ln transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

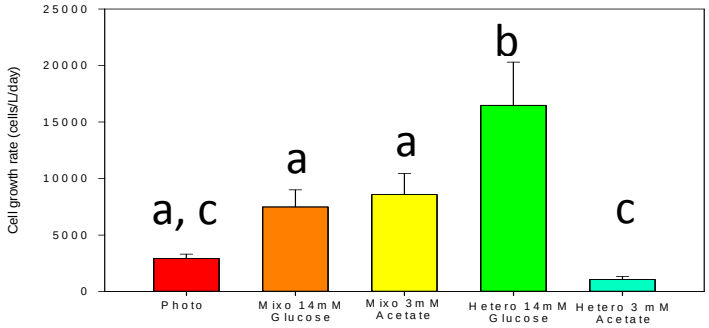


Figure 5: *Dictyochloris* F2.2 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

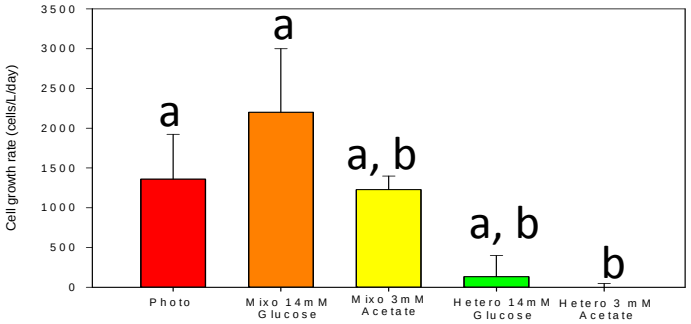


Figure 6: *Scenedesmus* F3.6 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

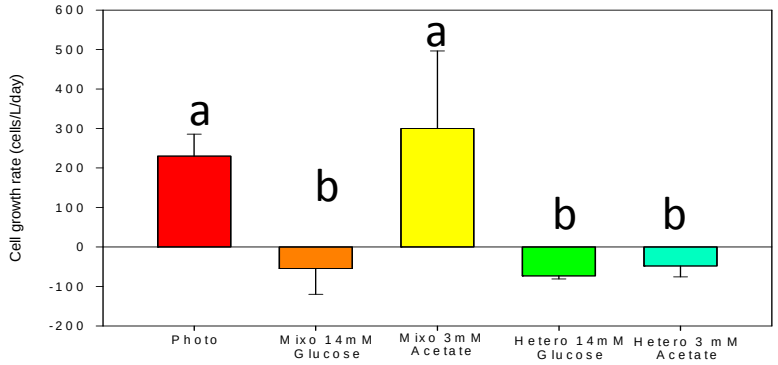


Figure 7: *Scenedesmus* FT4.3* Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was ln transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

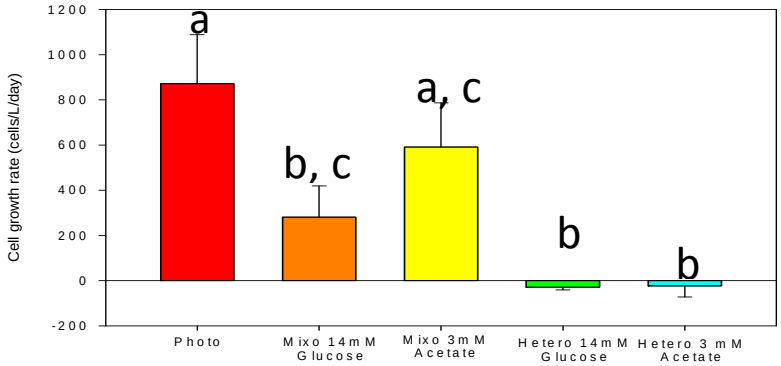


Figure 8: *Scenedesmus* FT4.3 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

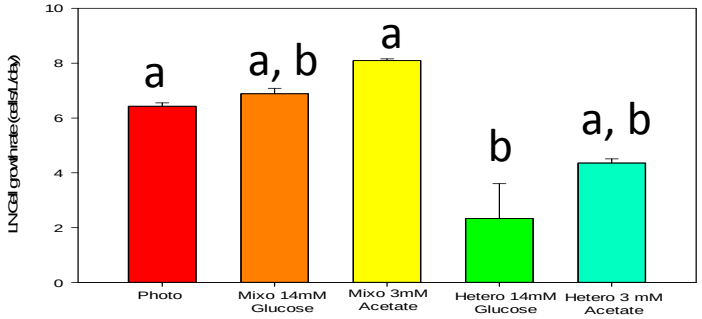


Figure 9: *Scenedesmus* S1.2 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

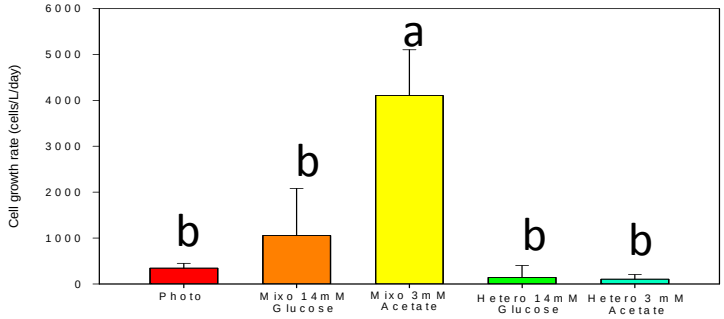


Figure 10: *Ellipsoidon* S3.3 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

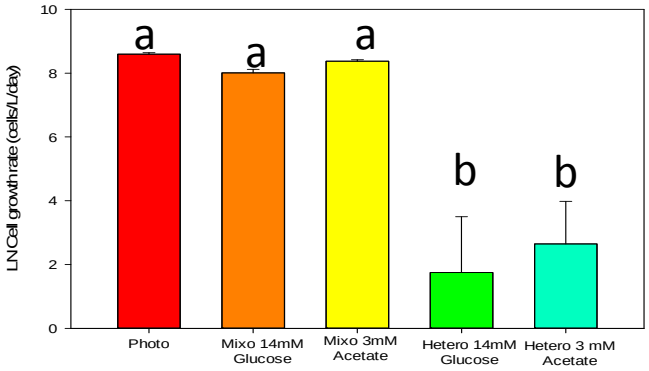


Figure 11: *Botrydiopsis* S4.2 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was ln transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

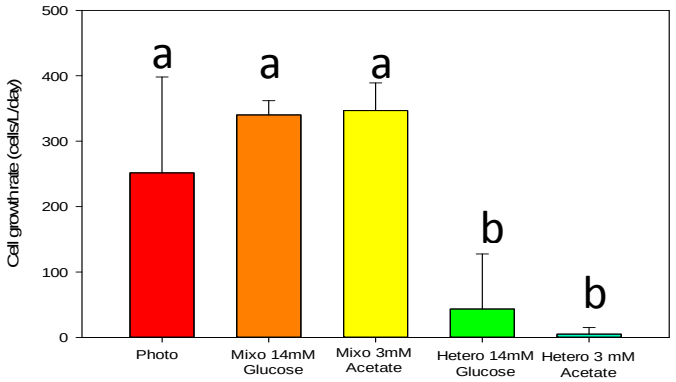


Figure 12: *Chlorella* S4.3 Nonquon tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was log transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

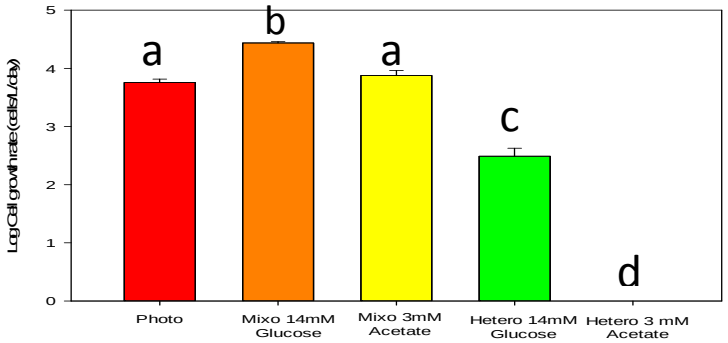


Figure 13: *Botrydiopsis* F1.3 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was log transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

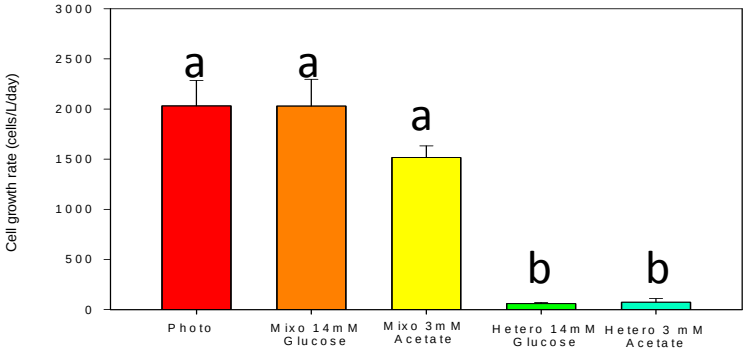


Figure 14: *Ellipsoidon* F2.3 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

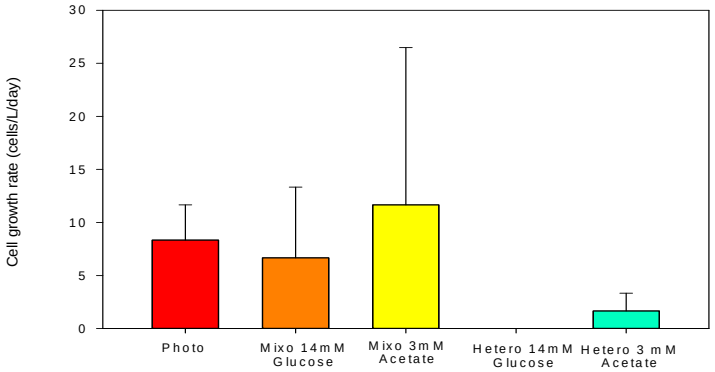


Figure 15: *Chlorella* F2.4 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. There was no significant difference found between any of the treatments. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

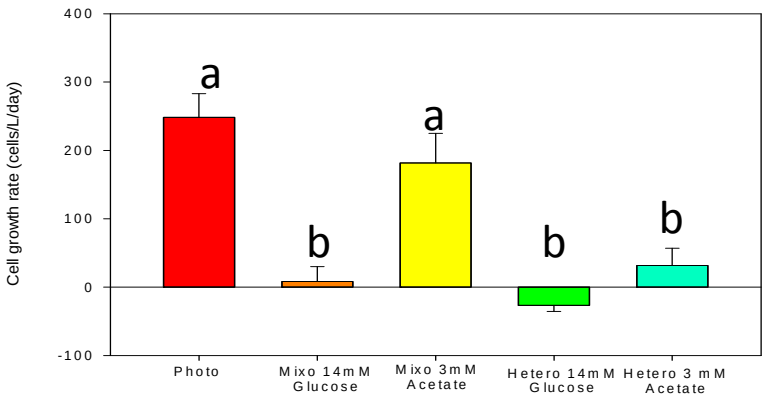


Figure 16: *Scenedesmus* M2.2 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

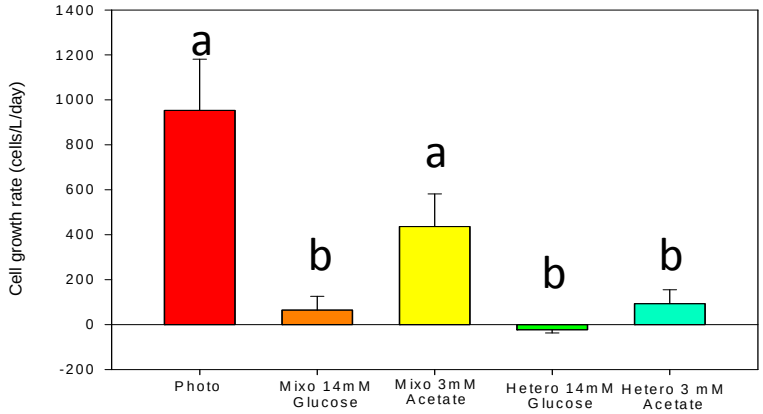


Figure 17: *Microcystis* M2.3 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

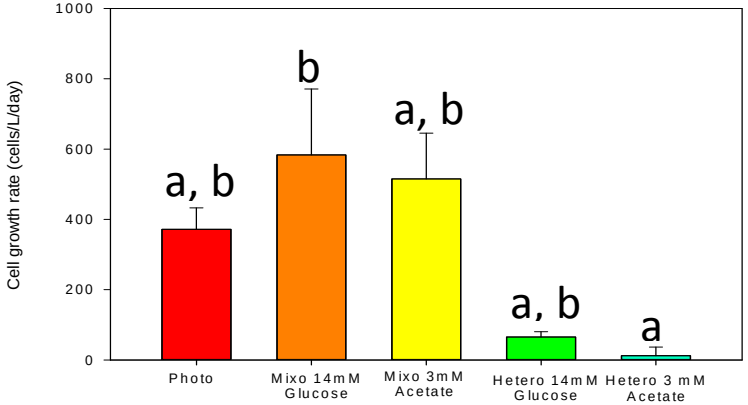


Figure 18: *Scenedesmus* M3.3 Hamilton tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

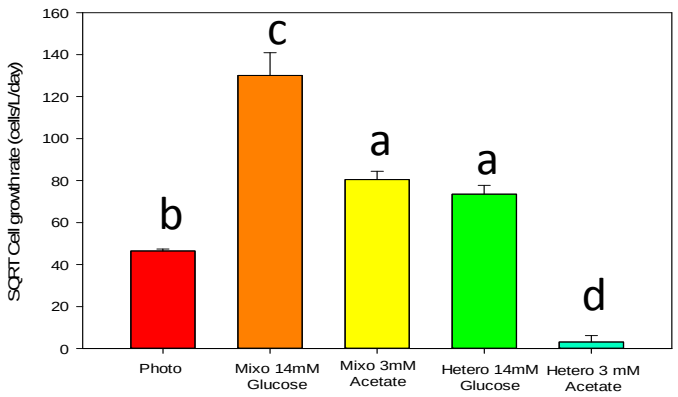


Figure 19: *Chlorella* 03 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was transformed by taking the square root of the raw data to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

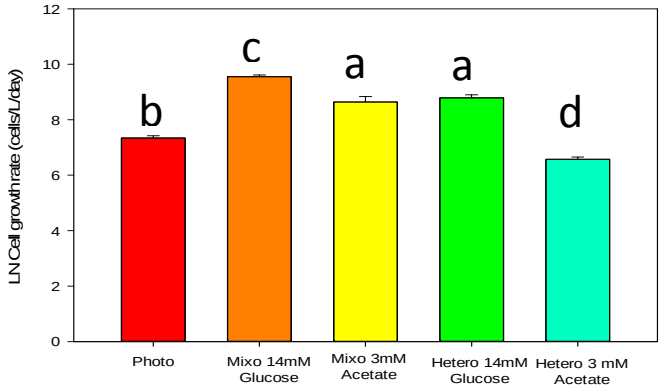


Figure 20: *Chlorella* 04 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Data was ln transformed to ensure normal distribution. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

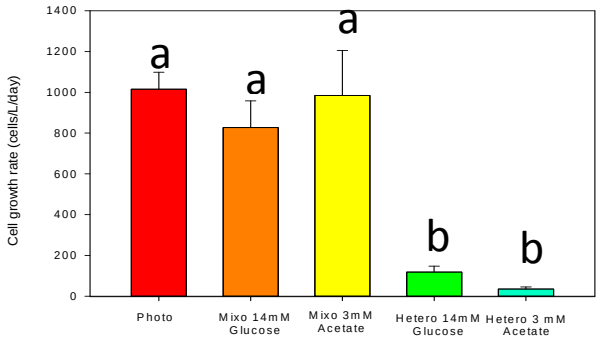


Figure 21: *Scenedesmus* 18 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

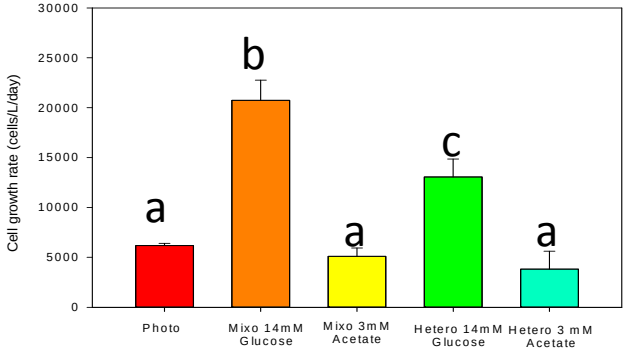


Figure 22: *Chlorella* 19 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

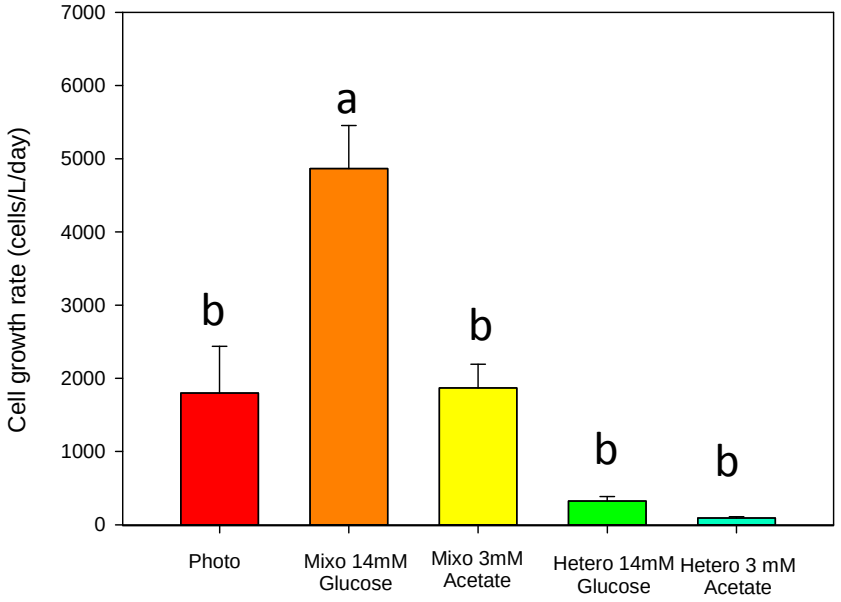


Figure 23: *Ellipsoidon* 23 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

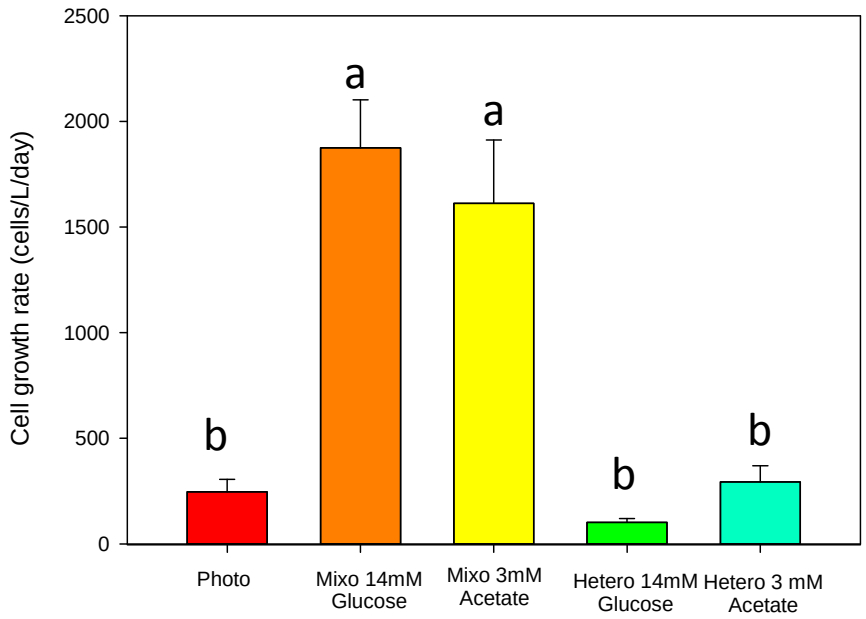


Figure 24: *Scenedesmus* 39 Corbett Creek tested under the three conditions photoautotrophic, mixotrophic, and heterotrophic growth. Significant differences are denoted by different letters. Samples were run in triplicate under each condition simultaneously in the algaetron growth chamber.

Table 4: The Ln growth rate (cells·L^{-1}·day^{-1}) of algal strains under the various conditions: P= Photoautotrophic, M=Mixotrophic, and H= Heterotrophic where G= glucose and A=Acetate. Each strain had each condition run at the same time with an n=3.

Strain ID	Growth Rates				
	P	M 14mMG	M 3mMA	H 14mMG	H 3mMA
C1U	**7.43±0.05^{ab}**	**7.55±0.26^{ab}**	**7.85±0.15^{ab}**	**8.84±0.08^{a}**	**7.41±0.11^{a}**
S1B	**6.97±0.06^{ab}**	**7.33±0.04^{ab}**	**6.88±0.25^{ab}**	**0.00±0.00^{b}**	**4.74±0.23^{b}**
D1N	7.98±0.07^{ab}	8.91±0.11^{ab}	9.04±0.13^{ab}	9.69±0.13^{a}	6.95±0.14^{ab}
S2N	7.16±0.24^{ab}	7.64±0.24^{ab}	7.11±0.08^{ab}	3.02±1.75^{b}	1.34±1.34^{c}
S3N	6.43±0.12^{ab}	6.88±0.20^{ab}	8.09±0.06^{ab}	2.34±1.26^{b}	4.36±0.15^{ab}
M1H	6.79±0.29^{ab}	2.74±1.51^{c}	5.93±0.42^{ab}	0.00±0.00^{b}	3.28±1.66^{b}
S6H	5.49±0.15^{ab}	2.04±1.11^{c}	5.13±0.29^{ab}	0.00±0.00^{b}	2.46±1.29^{b}
S4N	5.42±0.14^{ab}	0.00±0.00^{c}	5.55±0.40^{ab}	0.00±0.00^{b}	0.00±0.00^{c}
S5N	5.82±0.18^{ab}	4.93±2.47^{ab}	8.30±0.14^{ab}	3.10±1.76^{b}	3.39±1.70^{b}
B1N	8.60±0.04^{ab}	8.01±0.11^{ab}	8.37±0.05^{ab}	1.75±1.75^{b}	2.65±1.33^{b}
C3N	5.36±0.46^{ab}	5.83±0.04^{ab}	5.84±0.07^{ab}	2.18±1.46^{b}	1.44±0.79^{c}
E1H	7.60±0.13^{ab}	7.60±0.14^{ab}	7.32±0.08^{ab}	4.07±0.17^{c}	4.04±0.51^{b}
S7H	5.89±0.18^{ab}	6.24±0.37^{ab}	6.18±0.25^{ab}	4.11±0.28^{c}	2.30±1.18^{b}
C4C	7.68±0.04^{ab}	9.72±0.16^{ab}	8.77±0.10^{ab}	8.59±0.11^{ab}	1.48±1.48^{b}
C5C	7.34±0.08^{ab}	9.55±0.06^{ab}	8.64±0.20^{ab}	8.79±0.11^{a}	6.57±0.08^{ab}
S8C	6.92±0.08^{ab}	6.69±0.18^{ab}	6.83±0.25^{ab}	4.72±0.23^{c}	3.47±0.29^{b}
C6C	8.73±0.04^{ab}	9.93±0.09^{ab}	8.51±0.16^{ab}	9.46±0.14^{a}	8.04±0.44^{a}
E2C	7.28±0.51^{ab}	8.47±0.12^{ab}	7.50±0.17^{ab}	5.74±0.18^{a}	4.48±0.20^{ab}
S9C	5.45±0.23^{ab}	7.52±0.13^{ab}	7.34±0.21^{ab}	4.59±0.17^{c}	5.60±0.28^{ab}
B2H	8.65±0.13^{ab}	10.22±0.05^{ab}	8.93±0.20^{ab}	5.73±0.32^{a}	0.00±0.00^{c}
C2H	1.98±0.37^{c}	1.00±1.00^{c}	1.77±1.07^{c}	0.00±0.00^{b}	0.54±0.54^{c}
E3N	6.75±0.14^{ab}	5.55±0.30^{ab}	6.35±0.18^{ab}	0.00±0.00^{b}	1.13±1.13^{c}

Significant differences are denoted by superscript letters, all wastewater derived strains are compared to culture collection strains found in bold.

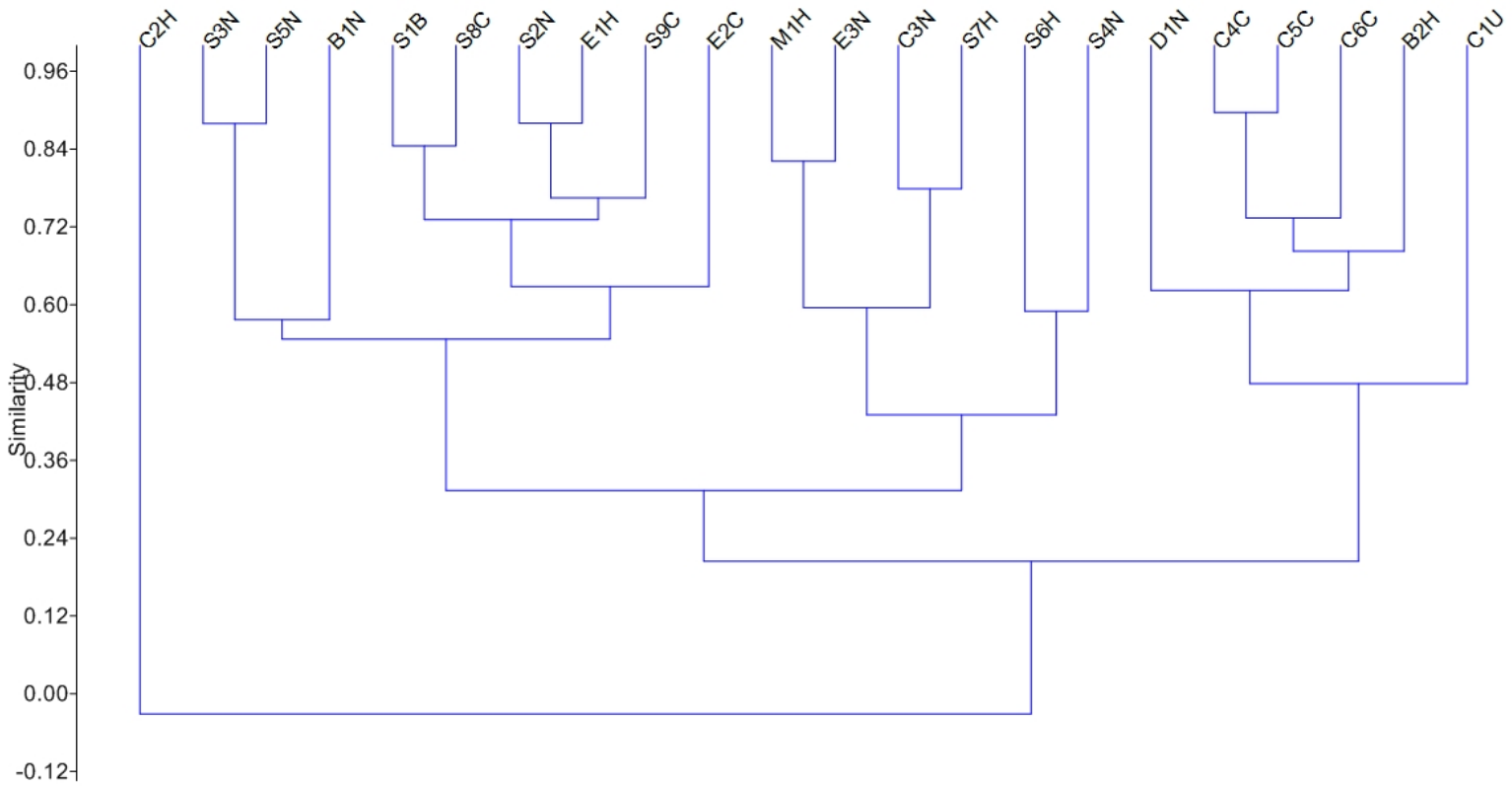


Figure 25: Cluster analysis of strains using the Bray-Curtis similarity measure to provide percent similarity to visually depict how similar strains are based on their growth patterns under photoautotrophic, mixotrophic, and heterotrophic conditions.

2.4 Discussion

2.4.1 Strain Collection and Isolation

The strains that were isolated from this study were *Dictyochloris, Scenedesmus, Microcystis, Botrydiopsis, Ellipsoidon,* and *Chlorella*. The reason why more strains were not found was due to two reasons: The first is the fact that laboratory conditions are not always representative of the natural habitat the algae are derived from so the algal population of certain strains may die off and be populated by a strain that can tolerate varying conditions, secondly these algae are the strains that have been selected for by the abiotic and biotic conditions of the wastewater environment (Wilkie et al., 2011).

2.4.2 Growth Conditions

The *Chlorella* strains were found in high amounts across the three sample sites. This may be due to their natural tolerance to low oxygen conditions (Shanthala et al., 2009). Park et al. (2012) commented that both *Scenedesmus* and *Chlorella* are among the most commonly isolated species from wastewater treatment waters (Table 3), which explains their high prevalence in all the sample sites. The composition of the algal community in the treatment plant is subject to changes depending on the time samples are taken however. It has been shown in the Hamilton harbour that the composition of algae can change from chlorophytes (*Scenedesmus*), to diatoms (*Fragilaria*), to cyanobacteria (*Microcystis*) just based on seasonal changes alone (Gudimov et al., 2010). Since most of the industrial scale operations are open pond systems this poses a problem (Borowitzka,

1999). This also shows the importance of sampling period as different algal strains can be found during different seasons.

The growth data provided a picture of how the media itself can influence changes in growth (Figure 3-24). As was seen across almost all strains the inclusion of either glucose or acetate improved the growth rate. This is not uncommon as mixotrophic medium for *Nannochloropsis* sp. increase the growth rate from 27 mg/L/day to 87 mg/L/day with the application of glucose (Das et al., 2011). Yan et al. (2012) actually demonstrated that the addition of either glucose or acetate actually increased the energy conversion efficiencies over the photoautrophic growth. The idea is that media supplement with organic carbon sources experiences less biomass loss during the dark phase (Brennan and Owende, 2010).

Heterotrophic growth was also assessed to see if it would be a viable method for future biofuel production. Miao and Wu (2006) demonstrated that *C. protothecoides* grown heterotrophically possessed lipid content four times higher than photoautotrophic cells. It is also stated that select algae can achieve higher biomass production under heterotrophic conditions reaching upwards of 4-20 g/L/day (Shen et al., 2010). This effect was not seen in the strains that were isolated from the wastewater systems. Only *Chlorella kessleri* C1U, *Chlorella* C6C, *Scenedesmus* S9C, and *Dictyochloris* D1N were able to tolerate the heterotrophic growth conditions. It is not surprising that the Chlorella strains performed so well during heterotrophic growth as *Chlorella protothecoides*, *Chlorella vulgaris*, and *Chlorella regularis* all demonstrate the ability to grow heterotrophically (Xu et al., 2006; Griffiths, 1970; Endo et al., 1977). *Dictyochloris* was discovered to possess the ability to grow heterotrophically by Parker et al. in 1961. Also

Scenedesmus obliquus has been found to be able to survive when supplied external carbon sources under heterotrophic growth (El-Sheekh et al., 2013). In most cases under heterotrophic growth the initial algal inoculation either 'crashed' resulting in cell death or showed little to no growth. This is due to the fact that algae derived from naturally occurring water bodies have adapted their cellular processes to daily light fluctuations and are therefore unable to grow strictly heterotrophically (Price et al., 1998). Since the cellular densities were so low by day seven it was decided that heterotrophic trials not be pursued as the cultivation of cells for fatty acid analysis would require consuming the whole sample preventing any additional sampling.

The highest growth rate achieved was from *Botrydiopsis* with a rate of $2.74\pm0.14x10^{4}$ cells$\cdot L^{-1}\cdot day^{-1}$ which proves difficult to compare this strain to literature as research into this strain is highly limited. Most research into *Botrydiopsis* covers the zoosporogenesis or the carbon dioxide concentrating mechanism found within this strain (Lokhorst and Segarr, 1989; Beardall and Entwisle, 1984). Comparisons were made to the culture collection strains (Table 4). The most interesting fact was during growth under photoautotrophic and mixotrophic (3mM acetate and 14mM glucose) the least amount of statistically significant differences ($p>0.05$) was denoted. What this translates to is that the strains isolated from wastewater treatment plants under these conditions are comparable to culture collection strains further increasing the validity of utilizing naturally occurring strains. The strains with the highest growth rates were also analyzed and it was found that the *Dictyochloris* D1N and *Chlorella* C6C strains possessed the highest rates under not one but two different growth conditions. This fact increases the

versatility and usefulness of these strains as they are not strictly limited to one growth condition like B1N.

The cluster analysis (Figure 25) revealed very similar metabolic profiles based on photoautrophic, mixotrophic, and heterotrophic growth for *Microcystis* M1H and *Ellipsoidon* E3N. The *Microcystis* strain had a high growth rate and distinct growth characteristics under the varying growth conditions. The similar growth pattern of the *Ellipsoidon* cannot be explained as there is very little research that has been performed with this particular strain. The research that has been performed has typically been centered on the usefulness of the bioactive compounds for treating the replication of haemorrhagic septicaemia virus and African swine fever virus (Fabregas et al., 1999). Ultimately the subsequent information derived about this strain will provide the basis for not only growth characteristics in various media but will also provide lipid fingerprint through the fatty acid analysis. The strain C2H which was a *Chlorella* strain appeared to be distinct from all other algae even the others of the *Chlorella* identified. However Post et al. (2004) showed that two different ecotypes of *Chlorella vulgaris* were isolated from wastewater oxidation ponds one with a rapid growth rate and the other a slower growth rate due to the use of different metabolic pathways. A similar outcome may be occurring here as C2H was the strain with the lowest growth overall of the algal isolates (Table 4).

Chapter 3. Fatty Acid Content and Profiles of Wastewater Algae

3.1 Introduction

3.1.1 Algal Harvesting Techniques

A large obstacle facing algal biofuel production is the biomass collection of the algae for processing. This step is complicated by the fact that algal cultures are typically grown in an aqueous environment. Since microalgae have a small diameter and a surface charge, harvesting requires a substantial proportion of energy which can range as high as 2500 kWh·m^3 (Brentner et al., 2011). This actually accounts for a large proportion of the total algal production cost which can be as high as 30% (Singh and Dhar, 2011). The most common methods for algal recovery are centrifugation, filtration, and flocculation.

Centrifugation uses gravitational force to spin the solid matter contained in the aqueous culture to the bottom. The overall size of the cells will affect how much force is required to push the cells out of the water, larger colonial cells will settle more readily than their smaller single celled counterparts (Singh and Dhar, 2011). Utilizing a centrifuge method for recovery is rapid but energy intensive as well since power estimates range from 0.3 to 8 kWh per m^3 (Grima et al., 2003).

Filtration involves the use of very small pores that prohibit the algae from passing through. Membrane filtration is typically cheaper and less energy intensive than centrifuges (Rickman et al., 2012). The drawback is that filtration is often prone to fouling which is essentially the pores becoming blocked. This is typically caused by shearing of the microalgal cells due to the high pressure or vacuum conditions used to

collect the algae, this releases smaller particles and dissolved organic matter which clogs the pores (Bilad et al., 2012).

Flocculation is typically used in conjunction with centrifugation to aid in reducing the overall energy burden of the process. Microalgae carry negative cell surface charge and addition of chemicals (typically metal salts) eliminates this charge (Singh and Dhar, 2011). This causes the algae to clump and settle out of water more easily when centrifuged. Research performed by Beach et al. (2012) showed that addition of chitosan (material derived from crustacean exoskeletons) resulted in 95% recovery of algae versus only 25% recovery when using traditional ferric sulfate.

3.1.2 Identification of Lipid Bodies Within Algal Cells

Traditional methods for identifying or even quantifying neutral lipids typically involve time consuming methods of extraction, purification, and concentration (Sitepu et al. 2012). The term neutral lipids simply refers to the polarity of the fatty acid within the cell, unlike hydrophobic or hydrophilic fatty acids neutral lipids carry no charge. Neutral lipids are primarily composed of triacylglycerols, steryl esters, and wax esters (Athenstaedt and Daum, 2006). Overall the lipid content of the algal cells is the key factor in determining the value a strain possess for biofuel production. Methods like gravimetric determination and chromatography exist for determining lipid contents the methods as stated before are time consuming and sometimes require use of organic solvents like chloroform (Doan and Obbard, 2011). An alternative method is using *in vivo* fluorometric techniques to identify the lipids in the algal cells either microscopically or spectrofluorometrically. The most common dye use is a lipid-selective fluorescent dye

known as Nile Red which only produces yellow-gold fluorescence when the dye interacts with neutral lipid globules (Feng et al., 2013; Doan and Obbard, 2011). The reason for this is Nile Red is a lipid soluble dye that penetrates into intracellular neutral lipid globules (Doan and Obbard, 2011).

3.1.3 Lipid Extraction Methods and Transesterification

Typically after harvesting of the algal cells occurs the process of gaining access to fatty acids needs to be undertaken thereby separating the algae from the valuable lipids. There exist three well known methods that are currently implemented to extract oil from algal cells: pressing, solvent extraction, and supercritical fluid extraction (Demirbas, 2011). Other methods have been attempted such as sonication, microwaving, and autoclaving but are less common than the first three (Prabakaran and Ravindran, 2011).

Pressing as the name suggests utilizes mechanical cell disruption through rupturing the cell wall or forcing the algae through a small hole which results in shearing ultimately releasing the oil (Mercer and Armenta, 2011). Bead beating also falls into this category as it induces direct disruption of cells through fine beads that rub against the cells shearing or tearing them open (Lee et al., 2010). These methods are often used as it reduces chemical contamination of cells and actually improves lipid extraction (Viswanathan et al., 2012).

Solvent extraction typically uses organic solvents such as a combination of chloroform and methanol to completely extract the oils present within the algae, this is problematic since chloroform is toxic and the technology is only used at the laboratory scale (Yao et al., 2012). The downside to using solvent extraction often requires the algal

biomass to be completely dry. If the cells are still wet limited extraction of lipids can occur since the algal cells are surrounded by water preventing solvents from penetrating the cell wall (Samori et al., 2013).

New research utilizing dimethyl ether as a solvent has proven to have advantages such as the high affinity it has for oily chemicals, it can mix partially with water, and is a safe extraction solvent for use in the food industry (Kanda et al., 2013). The ability to mix partially with water is especially attractive as this ability would allow for lipid extraction with wet biomass reducing the cost of drying the algae. Alcohols have also been considered as another solvent for extraction as the oil solubility can be changed by varying the temperature and water content in the alcohol, this allows for ease of oil recovery as when the mixture is cooled the oil will separate into a distinct phase (Yao et al., 2012).

Supercritical fluids are typically gases that a put under enough pressure that they are at an interface between liquid and gas causing them to act much like solvents (Hardardottir and Kinsella, 1988). This is an alternative to traditional solvent extractions as the process avoids contaminating the final product, mass transfer is enhanced due to the liquid/gas like properties, and the degree in which single chemicals are solvated can be accomplished through adjusting the pressure and temperature (Couto et al., 2010).

The sample in which fluids are to be extracted from (in the case of algae fatty acids) is placed into the extraction column. Typically CO_2 is used as the supercritical fluid which travels to the feed valve and preheats in the mixture vessel. This then flows through the bottom inlet to the extraction column where the desired product is solvated out of the original mixture. The mixture then exits from the top outlet to the extract

collector where the CO_2 can be exhausted off or condensed and recollected for further use.

Research by Halim et al. (2011) shows that in eighty minutes of supercritical fluid extraction with CO_2 resulted in more lipid yield over traditional hexane (solvent) extraction over five and a half hours. However research performed by Santos et al. (2012) indicates that using a supercritical fluid reactor resulted in considerably lower yields over solvent extraction but the extraction time was much lower only being ten minutes.

Once the fatty acids are extracted from the cells the process of transforming them into biodiesel can occur. The transformation process is referred to as transesterification by which the fatty acids (also known as triglycerides) are converted into methyl esters or more commonly referred to as biodiesel (Demirbas, 2011). Essentially triglycerides are combined with methanol to produce glycerine and methyl esters. The reaction typically sits in equilibrium and as a result methanol is used in excess to drive the reaction to produce the biodiesel. This excess methanol can be recovered later and reused for subsequent process of future triglycerides (Demirbas, 2011).

3.2 Materials and Methods

3.2.1 Antibiotic Treatment of Algal Isolates

In order to test for fatty acids the algal strains needed to be purified to axenic strains to limit background fatty acids derived from bacteria. This was accomplished through the application of streptomycin and penicillin following the methods outlined by

Droop (1967). The procedure utilizes six test tubes run in parallel in which the antibiotic concentration greatest at tube one and decreasing by half in each subsequent tube. The pure antibiotic was filter sterilized before being added to tube one. A 2 mg sample of streptomycin was used as streptomycin can inhibit algae at concentrations ranging from 0.5 to 150 mg/L (Kviderova and Henley, 2005). Additionally to the streptomycin 75 mg of penicillin was added as penicillin and streptomycin both possess different modes of action providing us with a broader spectrum of bacterial treatment. Penicillin is a beta-lactam which interferes with cell wall synthesis while streptomycin is a protein synthesis inhibitor. To verify axenic status after 24 hours of incubation in the antibiotic media a 1 mL aliquot of each dilution was transferred to 1.5% agar containing peptone via pour plate method. The plates were then placed on the light table and monitored over the course of 48 hours for bacterial growth. If no growth was visible on the plates microscopic examination was performed to verify axenic status. If a sampled failed to achieve axenic status the procedure was repeated.

To ensure standardization among all the algal strains the absorbance of each culture was taken to obtain a cellular density, which was then input into the following formula to standardize the cellular density to $x10^5 cells \cdot L^{-1}$:

$$Cell\ inocula = \frac{(Volume\ of\ media\ in\ 1st\ tube\ *\ Dilution\ factor) * Volume\ total}{Cellular\ density}$$

*Volume total is how much media you want to reside in each test tube along with the addition of algal transfer.

Antibiotics were purchased from Bioshop (Canada) and peptone was purchased from Sigma Aldrich (Canada).

3.2.2 Quantification of Neutral Lipids

A calibration curve utilizing triolein was calculated relating the fluorescence response to the concentration of triolein in solution. This standard curve (Appendix A Figure 1) was used to quantify the lipids of each strain. A small subsample (~1.5mL) of algae from the growth samples were stored in -20°C for examination under the staining of Nile Red dye as it was a high throughput method. Research by Stigum (2012) showed that Nile Red fluorescence emissions do not change with freezing algal samples. Methods from Chen et al., (2009), Feng et al. (2013), and Sitepu et al., (2012) were attempted for determination of neutral lipids but fluorescence under the defined protocols was not observed. Due to the constrains of the filter set available in the lab I decided to troubleshoot the issue and discovered an excitation of 590 and emission of 640 nanometers worked best due to the strong fluorescent response of the Nile Red at these wavelengths. The Nile Red was dissolved in HPLC grade acetone at a concentration of 500 µg/mL, and was stored in complete darkness in a vial covered in tinfoil to prevent photodegradation. In a black opaque 96 microwell plate 150 µL of algal sample was aliquoted into the wells in triplicate along with triplicate blanks. Following the addition of the algae to the wells 9 0µL of Nile Red was added to the treatment wells to dye the algal cells. The microwell plate was covered and incubated in darkness at room temperature for 10 minutes to ensure penetration of the dye into the cells. After incubation, the plate was immediately analyzed with the Synergy HT microwell plate spectrophotometer under the fluorescence function. All chemicals and standards were purchased from Sigma Aldrich (Canada).

3.2.3 Lipid Extraction for Analysis of Fatty Acid Methyl Esters

The growth experiments were re-run in the absence of bacteria in the Algaetron growth chamber under the conditions listed in the previous section titled Growth Conditions. The lipid extraction procedure followed was a direct FAME synthesis in O'Fallon et al. (2007). In short, 6-7 mL of algae with a cellular density typically above $x10^5$ cells/mL was collected at day seven of the experiment in Kimax screw-cap tubes. These samples were centrifuged using a the Sorval ST 16 centrifuge (Thermo Scientific) at 1690 g force for 5 minutes. The supernatant was discarded and the samples were stored in -20°C until freeze dried.

Once freeze dried (typically 24-48 hours) samples could be processed. The first addition was 0.7 mL of 10 N KOH in water along with 6.3 mL of MeOH. The tubes were incubated in 55°C water bath for 1.5 hours followed by hand shaking every 20 minutes. Once this step was complete samples were cooled using a cold water bath allowing for the addition of 0.58 mL of 24 N H_2SO_4 in water. A precipitate formed that needed to be mixed via shaking. Again the sample was incubated in 55°C water bath for 1.5 hours with hand shaking every 20 min.

The tubes were cooled a final time in the cold water bath and 3 mL of hexane was added followed by vortexing the sample. Once all samples were mixed they were placed in the centrifuge and spun at 1690 g force for 5 min to ensure proper phase separation. A 900 µL sample of the hexane layer (top layer) was extracted and placed into GC vials followed by addition of 100 µL of the 1000 ppm nanodecanoic acid methyl ester in hexanes (C 19 standard). Each vial was capped and placed in -20°C until analyzed via gas chromatography mass spectrometry.

3.2.4 Gas Chromatography Mass Spectrometry Analysis

The fatty acids derived via the lipid extraction method was determined by capillary Agilent J&W GC column (30 m·0.25 mm·0.25 µm) installed in a Varian 450 gas chromatograph. The initial oven temperature was 135°C held for 4 min followed by an increase to 250°C at a rate of 4°C $\cdot min^{-1}$, followed by a hold at this temperature for 10 min. The front injector was 250°C and held for 20 min, the middle was 110°C for 1 min, and the rear injector was held at 180°C for 20 min. A mixture of Helium and Nitrogen was used as the carrier gas with a split ratio of 10:1 at a flow rate of 1mL$\cdot min^{-1}$ for 3.5 min then shut off. The retention times of each sample were analyzed via the Varian 240-MS IT Mass-Spectrometer via an electron impact ion trap mass spectrometer. The standards used for analysis were carbon chain 8 to carbon chain 24 (C8-C24) along with a Bacterial Acid Methyl Ester (BAME) fatty acid mix. Fatty acids were identified through the use of MS workstation through comparing their retention times to known fatty acid methyl standards. Each chromatograph was then analyzed manually and any peaks that were above the background noise threshold were library searched to determine if the peak was an associated fatty acid. All standards were purchased from Sigma Aldrich (Canada).

3.2.5 Statistics

Fatty acid concentration for each strain under each of the three conditions was calculated based on the calibration curve for triolein determined by linear regression analysis using MS Excel 2010. This data was coupled with the cellular density data known for each sample and input into Sigma Plot 12. For strains that had an axenic and

non-axenic result the conditions were compared to each other through Sigma Plot 12 to assess if the differences were statistically significant.

For each strain the peak areas of fatty acids identified in the FAME analysis were center-standardized prior to statistical analyses using the Paleontological Statistics (PAST) programme. Principal Component Analysis (PCA), were run to visualize similarity in FAME profiles of each strain. A PCA is computed by determining the covariance of vectors to their individual values, this allows the measurement of how much the dimensions (x, y, z, etc.) in an ordination plot differ from each other (Jeong et al., 2008) PCA axis scores were regressed against fatty acid concentrations to determine the most important fatty acids contributing to the variation in each axis. Peak areas were also exported to MS Excel 2010 and the percent relative fatty acid composition was calculated by dividing each fatty acid by total fatty acid measured.

3.3 Results

3.3.1 Antibiotic Treatment of Algal Isolates

Application of antibiotic treatment resulted in, nine of the 20 strains being classified as axenic, (Table 3) Culture collection strains *Chlorella kessleri* and *Scenedesmus actus* were axenic prior to and throughout experimentation. A second culture of all axenic strains was kept prior to application of antibiotics to enable comparison of axenic and non-axenic conditions. The strains classified as 'non-axenic strains' had the application of antibiotics but the result was one of two scenarios: either the antibiotics proved ineffective at killing the bacteria or the antibiotics prevented algal

growth after application (Table 3). The antibiotic concentration was increased for both penicillin and streptomycin individually and in combination resulting in no change. Additionally the incubation time was increased from 24 hours to 48 hours resulting in again no net change.

3.3.2 Quantification of Neutral Lipids

Under photoautrophic conditions the algal strains treated with antibiotics showed that eight out of the nine strains had a greater neutral lipid concentration over the reference strains from the culture collection (Figure 26). Additionally axenic strains C3N and S7H exhibited very high neutral lipids in relation to their overall cellular density. The axenic strain E1H although appears to have a very high neutral lipid concentration in relation to cellular density actually had a cellular density too low to be detected spectrophotometrically so a zero value was assigned. This was due to the absorbance value falling outside of the range of detection on the growth curve for that particular strain. Algal strain B1N with bacteria had a very high cell density over the antibiotic treatment but a lower neutral lipid concentration with the opposite occurring for axenic S7H. Interestingly the strain E1H increased in cellular density in the presence of bacteria however the two strains S4N and C2H had cellular densities too low to be detected spectrophotometrically. As explained earlier this was not surprising as C2H possessed the lowest growth rate of any algal strains studied meaning the cellular density would not be large enough to be detected spectrophotometrically.

When growing the algal strains under mixotrophic conditions with 14mM glucose, most strains exhibited an increase in neutral lipid concentration over culture

collection strains, with the exception of S8C, E2C, S9C, C2H, and E3N (Figure 27). Several strains had neutral lipid concentrations above 200 $\mu g \cdot mL^{-1}$ including: axenic S6H, E1H, S7H, and non-axenic S2N, and C5C. All other strains had a greater neutral lipid concentration when treated with antibiotics. The most interesting ratio was found in strain M1H which had a cellular density of ~580 $cells \cdot L^{-1}$ and a neutral lipid concentration of ~165 $\mu g \cdot mL^{-1}$ which is a high lipid concentration for such a low cellular density.

Growth in 3mM acetate under mixotrophic conditions revealed that only the following strains had a lower neutral lipid concentration over the culture collection strains: C5C, S8C, E2C, S9C, and E3N (Figure 28). Although strains E2C, S9C, and E3N appear to have neutral lipid concentrations of zero this is only because their fluorescence values fell outside of the limits of detection meaning that their concentrations cannot be accurately measured but are less than 3.5 $\mu g \cdot mL^{-1}$.

To assess the influence of bacteria the nine strains that resulted in axenic strains were tested also under non-axenic conditions. It can be seen (Appendix A Table 1) that S2N under photoautotrophic conditions is the only strain which displays no statistically significant difference ($p>0.05$) between axenic and non-axenic trials. Under photoautotrophic conditions axenic algae which possessed higher neutral lipid content which was significantly different ($p\leq0.05$) compared to their non-axenic counterpart were: S6H, S5N, B1N, and C3N. The opposite results were seen in the following strains D1N, S3N, E1H, and S7H. Mixotrophic growth (14mM glucose) in which non-axenic algae had higher neutral lipid content found to be significantly different ($p\leq0.05$) compared to their axenic counterpart were: D1N, S2N, S3N, and B1N. The opposite

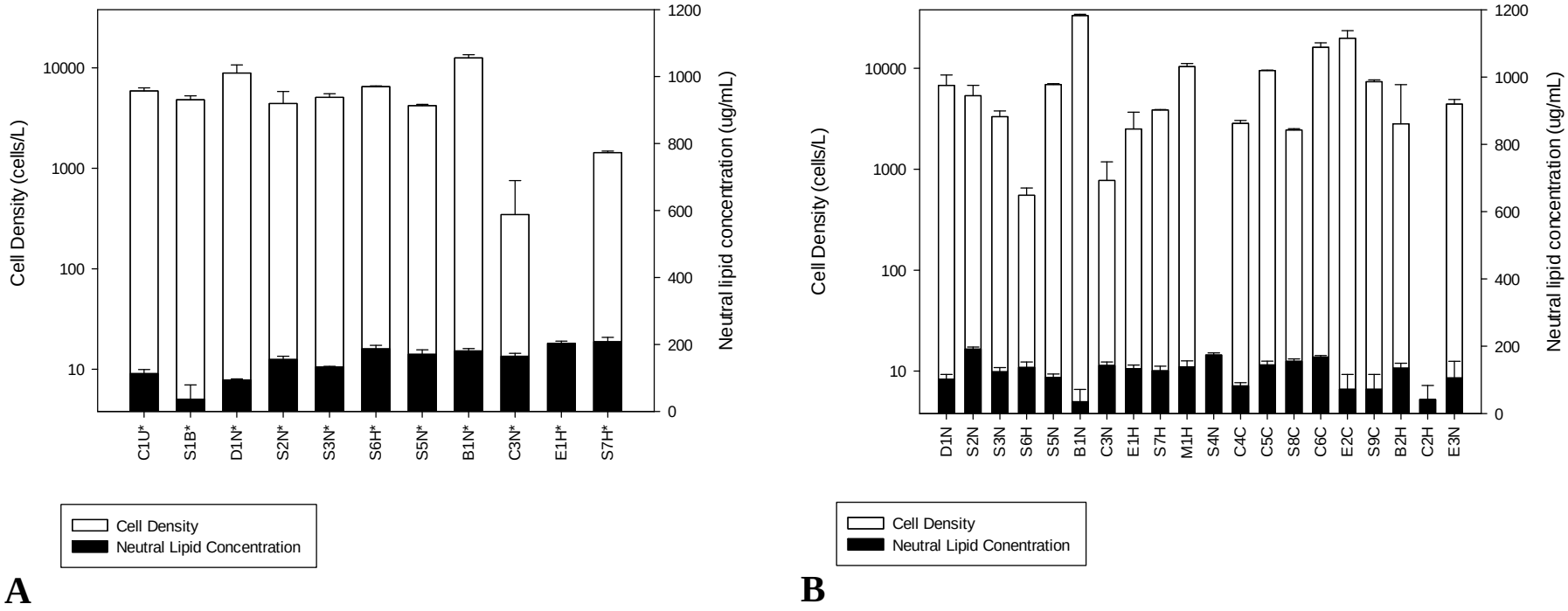
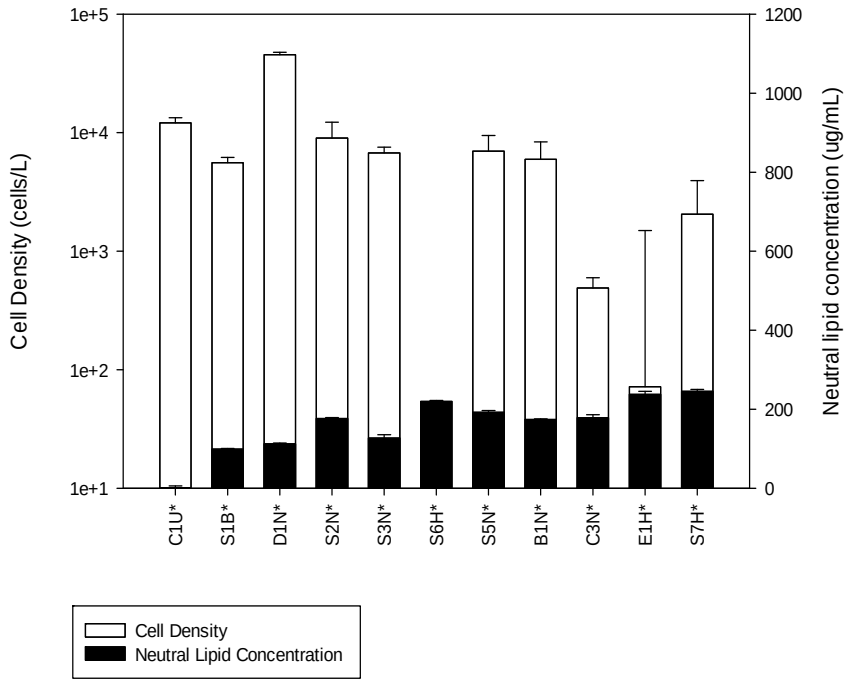
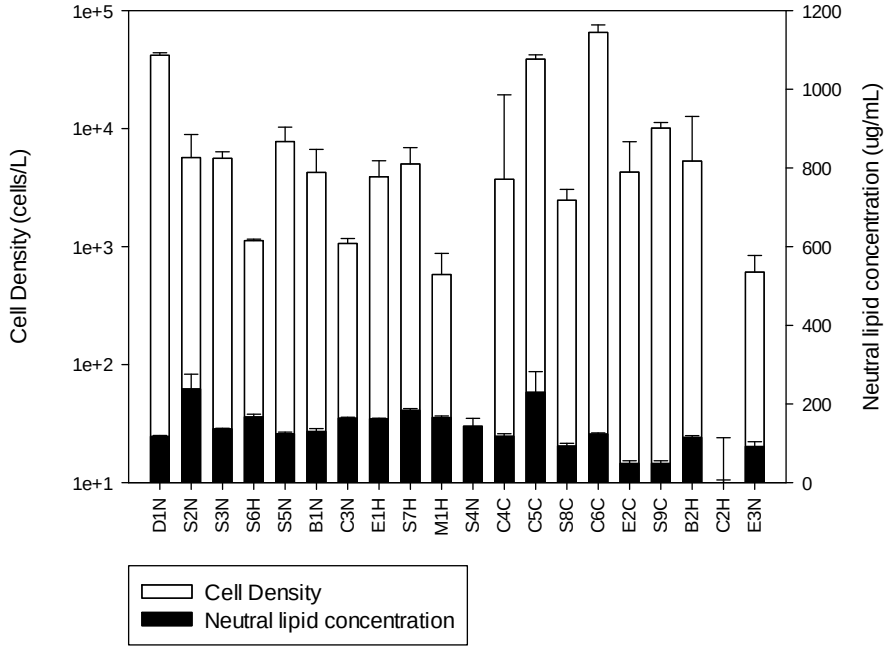


Figure 26: Cellular density of each strain under photoautotrophic conditions in comparison to the neutral lipid concentration measured via Nile Red dye. A axenic algal cells, B is non-axenic. Strains have been abbreviated by a letter designation C=*Chlorella,* S=*Scenedesmus,* D=*Dictyochloris,* B= *Botrydiopsis,* E= *Ellipsoidon, and* M=*Microcystis.* Measurements were taken in triplicate for both the cellular density and Nile Red measurements.



A



B

Figure 27: Cellular density of each strain under mixotrophic conditions (14mM glucose) in comparison to the neutral lipid concentration measured via Nile Red dye. A axenic algal cells, B is non-axenic. Strains have been abbreviated by a letter designation C=*Chlorella,* S=*Scenedesmus,* D=*Dictyochloris,* B= *Botrydiopsis,* E= *Ellipsoidon, and* M=*Microcystis.* Measurements were taken in triplicate for both the cellular density and Nile Red measurements.

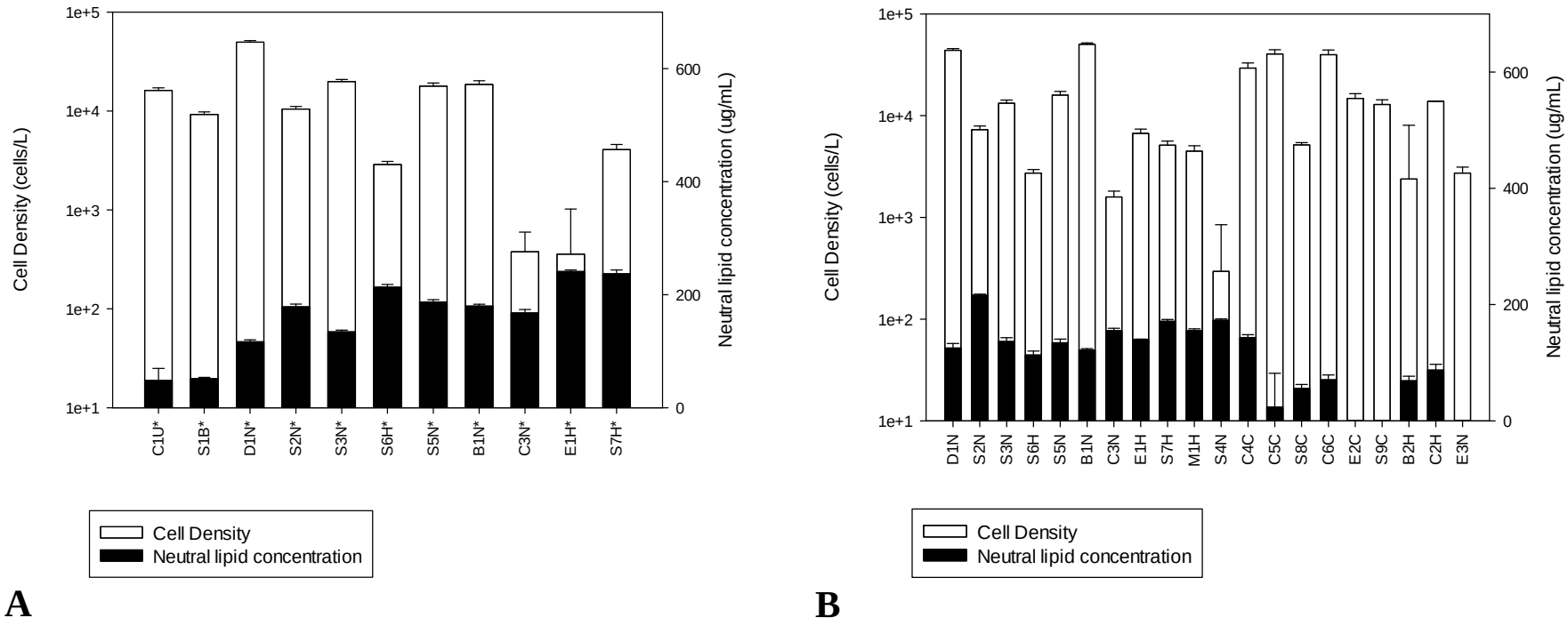


Figure 28: Cellular density of each strain under mixotrophic conditions (3mM acetate) in comparison to the neutral lipid concentration measured via Nile Red dye. A axenic algal cells, B is non-axenic. Strains have been abbreviated by a letter designation C=*Chlorella,* S=*Scenedesmus,* D=*Dictyochloris,* B= *Botrydiopsis,* E= *Ellipsoidon, and* M=*Microcystis.* Measurements were taken in triplicate for both the cellular density and Nile Red measurements.

results were found for: S6H, S5N, E1H, S7H and C3N. Under mixotrophic growth (3mM acetate) axenic algae which possessed higher neutral lipid content which was significantly different ($p \leq 0.05$) compared to their non-axenic counterpart were: S3N, S6H, B1N, E1H, and S7H. The opposite results were seen for the following non-axenic algal strains: D1N, S2N, S5N, and C3N.

3.3.3 Gas Chromatography Mass Spectrometry Analysis

Gas chromatography (GC) revealed diverse fatty acid profiles for each strain under photoautotrophic, mixotrophic 14mM glucose, and mixotrophic 3mM acetate conditions. A principle component analysis was performed (see Figure 29-31) to assess the variation in lipid composition among strains. This is achieved by the fact that PCA analysis takes the smallest number of components that account for the most variation in the data and display it in terms of a biplot. Under photoautrophic treatment (Figure 29) axenic strains (those with asterisks) clustered together suggesting that they possessed very little variation in fatty acid profiles.

Additionally B2H, B1N, S7H, S6H, and E1H all possessed dissimilar FAME profiles based on their position relative to other strains on the biplot. Ultimately 29.51% of the variation in the data set can be explained by component 1 while 20.93% of the variation can be explained by component 2. This in turn means that 50.44% of the variation in the data can be described by this plot. In order to assess the separation of data points along the principle component axis for each different fatty acid they were compared to the principle component axis scores. Any of the fatty acids with an R^2 value

above 0.5 was considered to be an important fatty acid for determining differences between the algal strains (Table 5). Based on the data the fatty acids that determine variation among the samples is linoleic acid methyl ester, oleic acid methyl ester (cis), palmitic acid methyl ester, 12-methyltetradecanoic acid methyl ester, erucic acid methyl ester, and behenic acid methyl ester.

When the strains were tested under 14mM glucose mixotrophic conditions a more abundant fatty acid profile was detected overall (Figure 30). Additionally like the photoautotrophic results a majority of the strains clustered together notably the axenic strains. There were two strains that were very different than the rest S9C and S6H based on their overall location on the biplot. A few additional strains that deviated from the centralized cluster were B1N, C4C, D1N* and S6H*. Based on the PCA 58.86% of the variation in the data set can be explained by this plot. To assess the separation of data points along the principle component axis for each different fatty acid the R^2 values were assessed as they were under photoautotrophic conditions. Table 6 displays the major fatty acids that determined the variation among the samples.

When the media conditions were 3mM acetate mixotrophic growth the fatty acid profile changed comparatively to that of the glucose, resembling the photoautotrophic results in terms of the fatty acids detected (Figure 31). Although the number of fatty acids detected appears to be the same as those from the photoautotrophic results the actual fatty acids detected are very different. Axenic algal strains under acetate conditions displayed a more diverse set of fatty acids as S3N*, S2N* and D1N* are different than other axenic algae in terms of fatty acid composition.

However M1H, C4C, and E3N all appear to be very different in terms of fatty

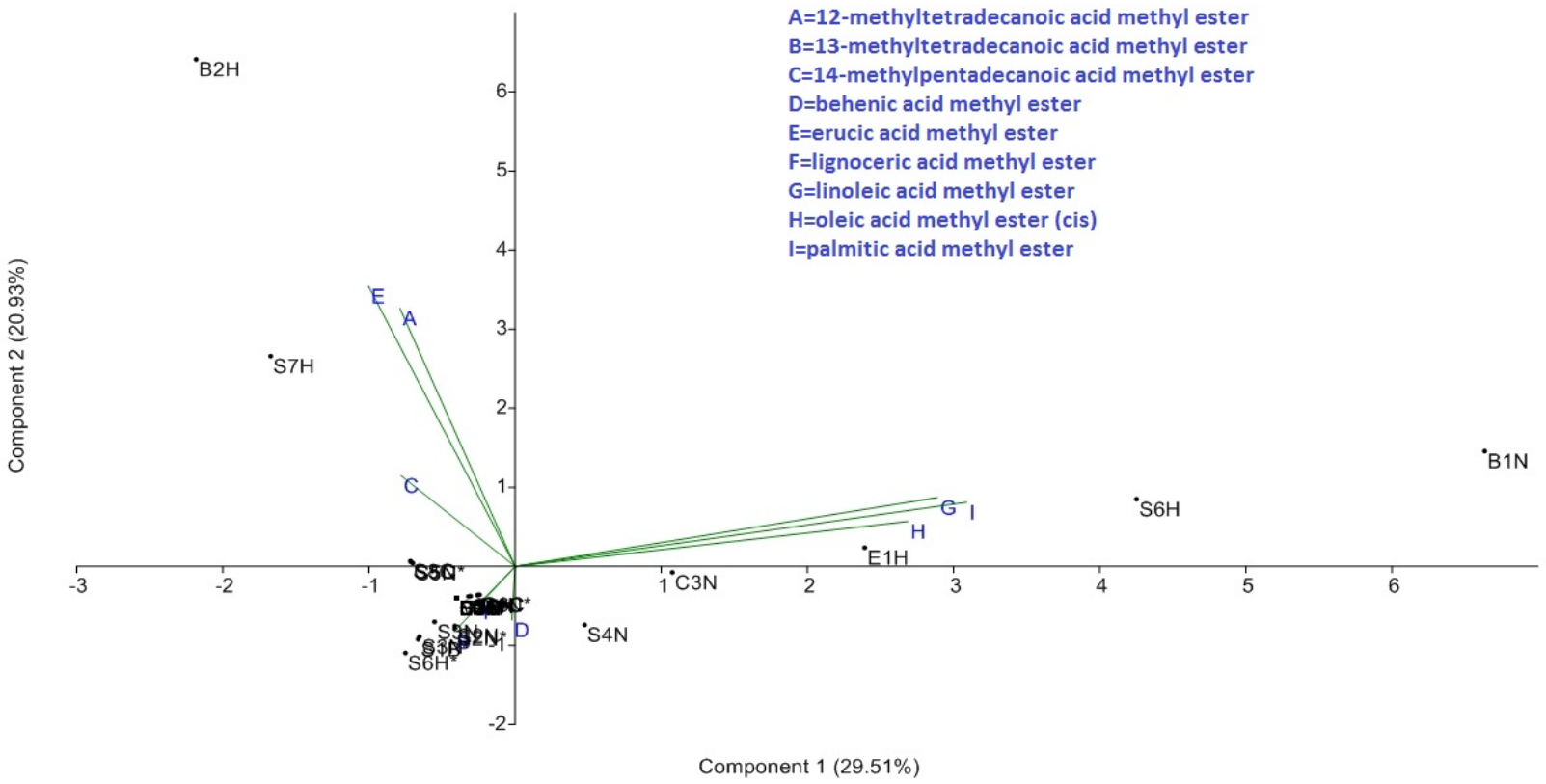


Figure 29: A principle component analysis of fatty acid methyl esters in algal strains grown under photoautotrophic conditions. Fatty acids are denoted by the blue lettering and algal strains are indicated by their alpha numeric coding. The asterisk that follows the strain lettering denotes axenic algal strains.

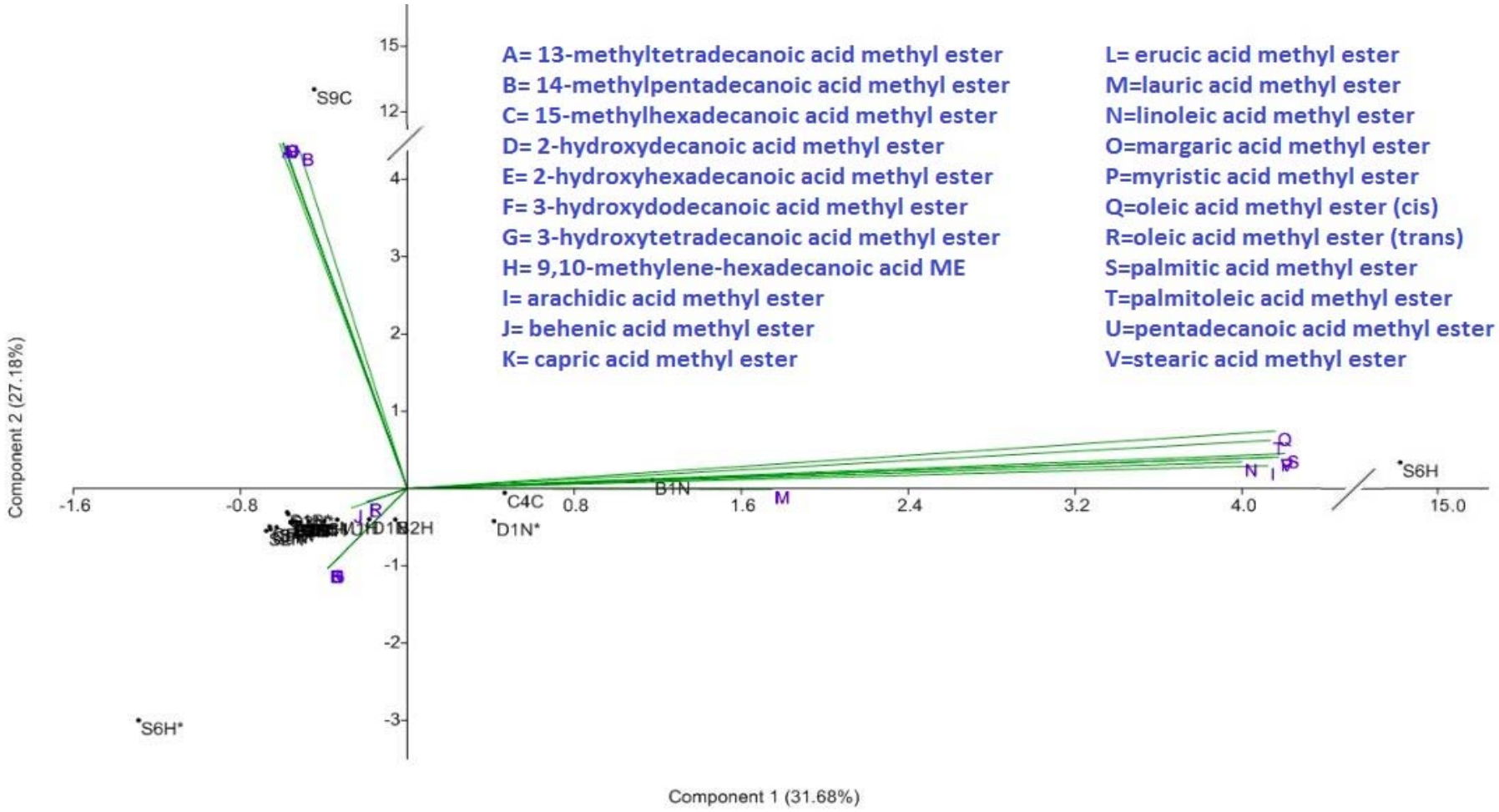


Figure 30: A principle component analysis of fatty acid methyl esters in algal strains grown under 14mM glucose mixotrophic conditions. Fatty acids are denoted by the blue lettering and algal strains are indicated by their alpha numeric coding. The asterisk that follows the strain lettering denotes algal strain treated with antibiotics.

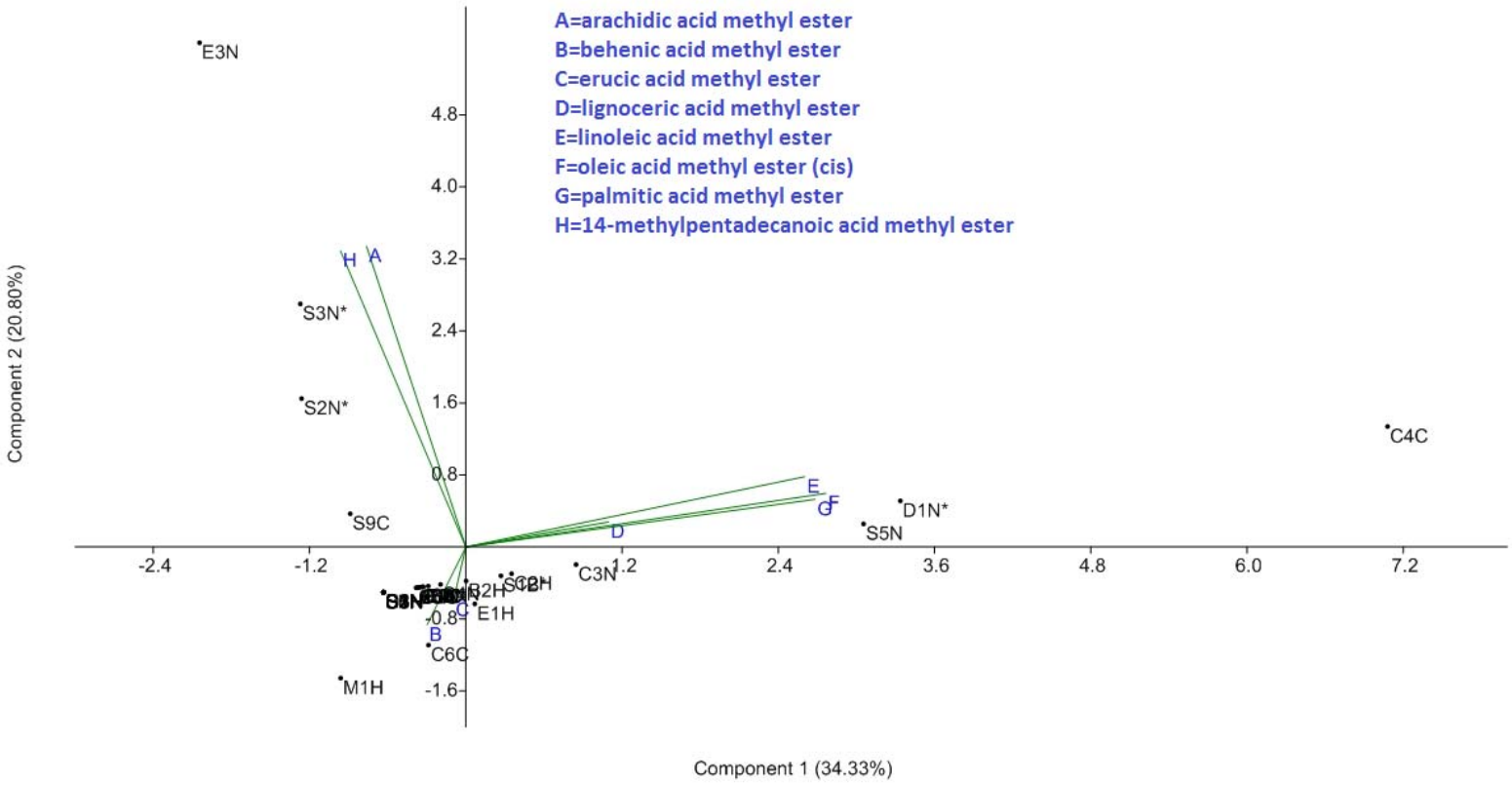


Figure 31: A principle component analysis of fatty acid methyl esters in algal strains grown under 3mM acetate mixotrophic conditions. Fatty acids are denoted by the blue lettering and algal strains are indicated by their alpha numeric coding. The asterisk that follows the strain lettering denotes algal strain treated with antibiotics.

acid profiles when compared to the majority of strains. From the PCA results 55.13% of the variation in the data set can be explained in this plot. To assess the separation of data points along the principle component axis for each different fatty acid the R^2 values were assessed. The results from Table 7 do in fact support the fatty acids with the longest vectors found in Figure 31 are the most significant fatty acids.

Table 5: Regression coefficients from linear regression analysis of principle component axis scores and fatty acid concentrations under photoautotrophic conditions. Only statistically significant coefficients are reported ($p \leq 0.05$).

PC 1		
Fatty acid	**Letter Designation**	**R^2**
linoleic acid methyl ester	G	0.8
oleic acid methyl ester (cis)	H	0.7
palmitic acid methyl ester	I	0.92
PC 2		
Fatty acid	**Letter Designation**	**R^2**
12-methyltetradecanoic acid methyl ester	A	0.72
erucic acid methyl ester	E	0.86
PC 3		
Fatty acid	**Letter Designation**	**R^2**
behenic acid methyl ester	D	0.62

R^2= Regression Coefficient

Table 6: Regression coefficients from linear regression analysis of principle component axis scores and fatty acid concentrations under mixotrophic (14mM glucose) conditions. Only statistically significant coefficients are reported ($p \leq 0.05$).

PC 1		
Fatty acid	**Letter Designation**	**R^2**
arachidic acid methyl ester	I	0.94
linoleic acid methyl ester	N	0.88
myristic acid methyl ester	P	0.96
oleic acid methyl ester (cis)	Q	0.95
palmitic acid methyl ester	S	0.98
palmitoleic acid methyl ester	T	0.94
stearic acid methyl ester	V	0.96
PC 2		
Fatty acid	**Letter Designation**	**R^2**
13-methyltetradecanoic acid methyl ester	A	0.94
14-methylpentadecanoic acid methyl ester	B	0.93
15-methylhexadecanoic acid methyl ester	C	0.94
9,10-methylene-hexadecanoic acid ME	H	0.94
margaric acid methyl ester	O	0.94
pentadecanoic acid methyl ester	U	0.94
PC 3		
Fatty acid	**Letter Designation**	**R^2**
3-hydroxydodecanoic acid methyl ester	F	0.94
3-hydroxytetradecanoic acid methyl ester	G	0.94
capric acid methyl ester	K	0.94

R^2= Regression Coefficient

Table 7: Regression coefficients from linear regression of principal components axis scores and fatty acid concentrations under 3mM acetate mixotrophic conditions. Only statistically significant coefficients are reported ($p \leq 0.05$).

PC 1		
Fatty acid	**Letter Designation**	**R^2**
linoleic acid methyl ester	E	0.76
oleic acid methyl ester (cis)	F	0.87
palmitic acid methyl ester	G	0.81
PC 2		
Fatty acid	**Letter Designation**	**R^2**
arachidic acid methyl ester	A	0.76
14-methylpentadecanoic acid methyl ester	H	0.74

R^2= Regression Coefficient

The diverse fatty acid composition for each strain is presented in Appendix A Tables 2-7. The results demonstrate that although lipids were detected and could be quantified by means of Nile Red fluorescence. Some strains displayed zero values for the fatty acids being screened. This was a common occurrence not only in the axenic strains but in the non-axenic strains as well. Additionally this commonality was seen across all the various growth conditions. Since Nile Red looks a neutral lipids and screening through mass spectrometry is selecting specific fatty acids this result was not unexpected.

The main differences in fatty acid profiles can be seen not only in comparing axenic to non-axenic strains but also by viewing the ratio of saturated fatty acids to unsaturated fatty acids. Table 8-13 display the various growth conditions as well as the grouping of strains based on their axenic status. The results from the photoautotrophic trials for axenic/non-axenic strains (Table 8) shows the fatty acid content for approximately half of the strains is 100% saturated fatty acids. When the non-axenic (Table 8-9) strains were tested about seven out of twenty strains had 100% saturated fatty acids. An additional seven out of the twenty strains had a mixture of saturated to unsaturated fatty acids. The remaining strains also had no detectable fatty acids. The only strains that can be compared between axenic and non-axenic status (Table 8) are S2N, S6H, and S5N. Only S6H exhibited a change in fatty acid saturation when tested with the presence of bacteria.

Under the 14 mM glucose mixotrophic conditions (Table 10) S2N*, S6H*, and C3N* all had purely saturated fatty acids while S5N* had 100% unsaturated fatty acids. Additionally C1U* and D1N* possessed an almost even split of saturated to unsaturated fatty acids. The remaining strains displayed no fatty acid profiles. However under non-

axenic conditions the level of unsaturated fatty acids in comparison to saturated fatty acids increases with strains D1N, B1N, E1H, (Table 10) and C4C (Table 11) reaching an almost 50/50 ratio. The strain S5N (Table 10) which possessed 100% unsaturated fatty acids under axenic conditions displayed 86% saturated fatty acids and 14% unsaturated fatty acids. While S6H possessed (Table 10) 100% saturated fatty acids under axenic conditions the ratio split to 63% saturated 37% unsaturated under non-axenic conditions.

When testing the algal isolates under 3 mM acetate mixotrophic conditions (Table 12) over half of the strains fatty acids could not be detected while S1B* and D1N* were the only two strains which displayed a mixture of saturated and unsaturated fatty acids. While C1U*, S2N*, and S3N* all possessed 100% saturated fatty acids. For the strains that could be classified as both non-axenic and axenic there was no change in fatty acid saturation (Table 12). For the purely non-axenic strains (Table 13) 10/20 strains possessed 100% saturated fatty acids while 6 strains displayed mixtures between unsaturated and saturated fatty acids. The remaining four strains possessed no fatty acid profile.

Table 8: Percent saturation of fatty acids for axenic/non-axenic algae under photoautotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
C1U*	-	-
S1B*	100	-
D1N*	-	-
D1N	-	-
S2N*	100	-
S2N	100	-
S3N*	100	-
S3N	-	-
S6H*	100	-
S6H	86.42	13.57
S5N*	100	-
S5N	100	-
B1N*	-	-
B1N	78.91	21.09
C3N*	-	-
C3N	45.73	54.27
E1H*	-	-
E1H	66.34	33.66
S7H*	-	-
S7H	87.81	12.19

Table 9: Percent saturation of fatty acids for non-axenic algae under photoautotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
M1H	-	-
S4N	71.25	28.75
C4C	100	-
C5C	100	-
S8C	100	-
C6C	100	-
E2C	-	-
S9C	-	-
B2H	62.88	37.12
C2H	100	-
E3N	-	-

Table 10: Percent saturation of fatty acids for axenic/non-axenic algae under 14mM glucose mixotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
C1U*	45.86	54.14
S1B*	-	-
D1N*	56.18	43.82
D1N	51.31	49.69
S2N*	100	-
S2N	-	-
S3N*	-	-
S3N	93.24	6.76
S6H*	100	-
S6H	63.12	36.88
S5N*	-	100
S5N	85.61	14.39
B1N*	-	-
B1N	59.26	40.74
C3N*	100	-
C3N	100	-
E1H*	-	-
E1H	47.91	52.09
S7H*	-	-
S7H	-	-

Table 11: Percent saturation of fatty acids for non-axenic algae under 14mM glucose mixotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
M1H	100	-
S4N	100	-
C4C	45.29	54.71
C5C	9.59	90.41
S8C	100	-
C6C	79.7	20.3
E2C	100	-
S9C	89.98	10.02
B2H	89.19	10.81
C2H	-	-
E3N	100	-

Table 12: Percent saturation of fatty acids for axenic/non-axenic algae under 3mM acetate mixotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
C1U*	100	-
S1B*	88.89	11.11
D1N*	59.93	40.07
D1N	-	-
S2N*	100	-
S2N	-	-
S3N*	100	-
S3N	100	-
S6H*	-	-
S6H	100	-
S5N*	-	-
S5N	76.94	23.06
B1N*	-	-
B1N	100	-
C3N*	-	-
C3N	69.03	30.97
E1H*	-	-
E1H	66.66	33.34
S7H*	-	-
S7H	-	-

Table 13: Percent saturation of fatty acids for non-axenic algae under 3mM acetate mixotrophic growth conditions

Strain	Total saturated %	Total unsaturated %
M1H	100	-
S4N	100	-
C4C	67.15	32.85
C5C	100	-
S8C	100	-
C6C	94.5	5.41
E2C	100	-
S9C	-	-
B2H	100	-
C2H	64.11	35.89
E3N	100	-

3.4 Discussion

3.4.1 Antibiotic Treatment of Algal Isolates

Penicillin is a beta-lactam antibiotic that inhibits peptidoglycan synthesis of prokaryotic cell walls. The antibiotic streptomycin is an aminoglycoside antibiotic that blocks protein synthesis (Kviderova and Henley, 2005). Application of a combination of these antibiotics was an attempt at broad-spectrum treatment of algal strains. However, the application of antibiotics only caused half of the algal strains to be purified to axenic status. The presence of bacterial contamination in the remaining samples (Table 3) can be explained through the prevalence of antibiotic resistance. Research by Akter et al. (2012) revealed that 24 *E.coli* isolates derived from hospital wastewater were resistant to β-lactams (ampicillin, cefalexin, ceftazidine, cloxacillin,), fluoroquinolone (ciprofloxacin),

and tetracycline (doxcyclin). This also means that the antibiotic resistance could be found in the municipal wastewater tested in this study as antibiotics including amoxicillin, ampicillin, penicillin G, ceftazidime, ceftriaxone (β-lactams) tetracycline, and doxycycline (tetracyclines) have been extracted from wastewater treatment plants in Romania (Opris et al., 2013). Not only are the antibiotics detected in the wastewater but ampicillin resistance genes in bacteria have been found to increase in urban wastewater as well as wastewaters receiving hospital discharges in Germany (Volkmann et al., 2004; Rizzo et al., 2013). Samples from Hebei Province in China discovered that bacteria isolated from a wastewater treatment plant and downstream of the plant possessed high resistance to beta-lactam antibiotics such as penicillin (Li et al., 2009). The bacteria also may avoid exposure to the antibiotics completely as homogenous mixtures of bacteria can attach to the surface of algal cell or reside in their mucilage thereby preventing exposure to the bacteria completely (Fisher et al., 1998). Bacteria can also evade the effects antibiotics by entering the stationary phase or by slowing growth therefore preventing the effects of both penicillin and streptomycin (Tuomanen et al., 1986).

The unialgal culture may in fact represent a natural algal-bacterial consortium formed between microalgae and their bacterial counterparts (Subashchandrabose et al., 2011). Disruption of this natural symbiotic relationship could actually have side effects as the mutualistic benefits have been cut off for the algae. For example when bacteria are grown with *Thalassiosira rotula* (a diatom) bacterial isolates promoted growth under different media conditions (Grossart and Simon, 2007). Additionally Abed (2010) discovered co-culturing the bacteria *Pseudomonas* with the cyanobacterium *Synechocystis* led to an eight fold increase in cyanobacterial biomass. Ideally the algal

bacterial symbiotic relationship is beneficial as the molecular oxygen produced by algae through photosynthesis is used as an electron acceptor by bacteria which then degrade organic matter in wastewater, ultimately releasing carbon dioxide as a waste product but further fueling algal photosynthesis (Subashchandrabose et al., 2011). It also may be in fact impossible to eliminate bacteria completely from some strains as it has been demonstrated in *Volvox carteri* contained endosymbiotic bacteria which occur within the cytoplasm as well bacteria can escape antibiotic exposure through refuge within the mucilage (Cole, 1982).

3.4.2 Quantification of Neutral Lipids

The strains S4N, C2H, and E1H* all exhibited zero values for their cellular densities which can be explained based on their calibration graphs, the spectrophotometric values taken for these strains lie outside the limits of detection. Since the spectrophotometric value fell outside of this limit an accurate cell density cannot be attributed so a value of zero was attributed to eliminate bias. Under photoautotrophic growth there was an even split between which condition produced a greater concentration of neutral lipids per cell per liter. This made determining the effect of bacteria on algae difficult to determine under photoautotrophic conditions.

Under mixotrophic growth (14mM glucose) axenic algae possessed a higher neutral lipid concentration which relates back to the fact that there is a lack of stress in one system over the other. The algal systems containing bacteria possess a natural system in which the bacteria are utilizing the organic content in the media (Subashchandrabose et al., 2011). The presence of bacteria can actually cause a deficiency in available nutrients

for the algae. Liu et al. (2011) discovered that when glucose is added to the growth media it stimulates bacterial growth reducing availability of nutrients to the algae. A similar result was also discovered with *Scenedesmus obliquus* when grown with a natural bacterial community resulted in limitation of phosphorus and nitrogen at the plateau of the growth cycle (Daufresne et al., 2008). It is well known that when algae are stressed by their system it triggers the accumulation of neutral lipids (Saha et al., 2013). Much of the research to make algal biofuels feasible involves growing algae under conditions with abundant nutrients increasing the overall biomass followed by a shift to a nutrient poor medium (nitrogen or phosphorus depleted) to trigger the accumulation of fatty acids (Ratha et al., 2013).

When analyzing the mixotrophic (3mM acetate) growth conditions only four out of the nine strains displayed non-axenic algae possessing higher neutral lipid concentrations over the axenic algae. The higher level of neutral lipids found in the axenic algae does not make sense as the bacteria would consume the organic carbon before the algae could uptake it. Wright and Hobbie (1966) who demonstrated that radio labelled glucose and acetate is less than 10% of the total uptake even if the algal biomass is orders of magnitude greater than the bacteria. As previously stated the competitive inhibition induced through resource consumption by the bacteria produces nutrient deprived conditions for the algae. Unfortunately the axenic algae of five out of the nine strains displays higher neutral lipid content which cannot be explained based on current results. This can be explained through the photoassimilation of acetate by algae mainly is allocated to the lipids. This was proven by Ihlenfeldt and Gibson (1977) who demonstrated that cyanobacteria *Synechococcus* and *Aphanocapsa* distributed acetate

from the growth media to lipid portions of their cells. *Chlorella vulgaris* has been shown to incorporate acetate into the glycerol fractions of its cell (glycerol is the alcohol compound that links fatty acids together in algae) (Neilson and Lewin, 1974). Due to the lack of the bacteria in these situations it allowed for untapped organic carbon that could be utilized by the algae to increase their neutral lipid concentrations.

It should be noted that there are limitations and benefits associated with utilizing Nile Red for the determination of neutral lipids. The dye only produces a yellow-gold fluorescence when bound to neutral lipids and not glycol- or phospholipids increasing the selectivity of the dye (Doan and Obbard, 2011). The fluorescence intensity can be affected by factors like emission wavelength, dye concentration, cell density and staining time; all conditions which must be optimized for each individual algal strain (Feng et al., 2013). Additionally it should be noted that Nile Red typically exhibits a stronger fluorescence intensity when bound to unsaturated fatty acids than saturated fatty acids (Fowler et al., 1987). Chen et al. (2009) has even stated that Nile Red is inefficient for some green algal species due to the impermeability of the cell wall. Although the Nile Red protocol is not ideal for all the strains it has provided information when comparing not only the differences found within the different growth conditions but the differences found between the algal strains that were both axenic and non-axenic.

3.4.3 FAME Profiles of Wastewater Algae

Typically when algae are assessed for potential as a biofuel feedstock it involves a labour intensive process to identify the significant fatty acids or individual strains that differ from the larger collection of strains. This can make screening larger quantities of

algal strains difficult as trends such as which fatty acid is most significant may be overlooked or missed when displaying the breakdown of the fatty acid composition as percent relative fatty acid. Utilizing a Principle Component Analysis based on the fatty acids measured by gas chromatography mass spectrometry allowed for a visual examination of the important fatty acids driving the variation in FAME profiles among strains. For example, under photoautotrophic conditions there is tight clustering of axenic algae indicating the fatty acid profiles they possesses are highly similar. Under the photoautotrophic conditions B2H, S7H, S6H, and B1N were all distributed further away in the biplot from the remaining strains suggesting a unique fatty acid profile. Also when the vectors are displayed this would allow researches a rapid visual examination of potential strains for future experimentation as strains high in specific fatty acids can quickly be located. The PCA can also provide valuable insight into which fatty acids are significant in determining the differences between strains.

This rapid analysis of the fatty acid data also displays trends for both the acetate and glucose mixotrophic conditions. Under the 14 mM glucose media two strains (S6H* and S9C) displayed distinct fatty acid compositions that were largely separated from other algal strains on the biplot. With the correlation of fatty acid variables to principle components axis scores the difference that exist from growth condition to growth condition can be elucidated. For example there are a greater number of significant fatty acids found in 14 mM glucose mixotrophic growth over that of photoautotrophic and 3mM acetate mixotrophic growth. This result was also seen by Wang et al. (2012) in which total lipid content and lipid productivity was found to be 2.8-4.6 times greater with glucose than that of control while starch and sodium acetate had no significant difference

compared to the control. Although Laliberte and Noue (1993) reported that utilizing acetate under mixotrophic growth actually caused lower lipid percentages but an increase in protein in *Chlamydomonas humicola*. This matches the results we found as photoautotrophic growth resulted in a more diverse set of fatty acids over acetate and glucose containing the most diversity in overall fatty acids. Another advantage this method of observation offers is that the PCA data can identify which fatty acids are responsible for the variation among strains.

Analyzing the strains under the three different conditions showed that under antibiotic treatments there was a slight shift in fatty acid composition. For example under photoautotrophic conditions S6H when axenic possessed 100% saturated fatty acids but when non-axenic the saturated fatty acids shifted down to 86%. A similar result was noted with *C. vulgaris* and *C. sorokiniana* when grown with the bacterium *A. brasilene* which caused the variation in fatty acids to change from five to eight different fatty acids with increasing amounts of unsaturation (de-Bashan et al., 2002). However S3N and S5N experienced no change in saturation under axenic or non-axenic trials. This cannot be explained presently other than the fact that saturation and unsaturated fatty acids can vary from species to species even when grown under the same growth conditions. Gatenby et al. (2003) demonstrated that *N. oleoabundans, P. tricornutum, and B. grandis* all grown under the same conditions and in the same media had varying profiles, as *N. oleoabundans* had higher unsaturated fatty acids while *P. tricornutum* had C 16 saturated and mono-unsaturated fatty acids and *B. grandis* contained high percentages of C 18 polyunsaturated fatty acids.

Findings in the literature show that adding glucose to solution changes the lipid composition, significantly increasing oleic acid concentration over photoautotrophic grown algae (Sunja et al., 2011). This was as also found in my experiments, where the number of strains with C 19:2 changed from five under photoautotrophic conditions to nine under 14 mM glucose mixotrophic conditions. Additionally Cherisilp and Torpee (2012) have confirmed that algae grown under mixotrophic conditions using glucose showed an overall increase in lipid content over photoautotrophic growth and heterotrophic growth. Under 14 mM glucose mixotrophic conditions S6H and S5N experienced changes in saturation, as S6H under axenic conditions possessed 100% saturated fatty acids while S5N possessed 100% unsaturated fatty acids. S6N experienced a shift to unsaturated fatty acids under non-axenic conditions while S5N shifted more towards saturated fatty acids under non-axenic conditions. The finding for S5N follows what was reported by Wang et al. (2012) as they demonstrated that under glucose growth, fatty acid unsaturation decreased for *C. kessleri*. Metabolically this makes sense that the degree of unsaturated fatty acids is lower in the presence of bacteria, as the bacteria may be utilizing the glucose before the algae can. Research performed by Wright and Hobbie (1966) have demonstrated that when bacteria are present with algae their uptake of glucose was higher than that of algae even when the algal biomass is orders of magnitude greater than the bacterial biomass. Similar results have also been found showing that bacteria preferentially uptake dissolved glucose over carbon sources that are released from algae (Kisand and Tammert, 2000).

Two strains (D1N and C3N) did not experience changes in their fatty acid profiles under 14 mM glucose mixotrophic growth under axenic or non-axenic conditions. This

can be explained by the fact that some strains of algae lack the capabilities to uptake organic carbon sources. *Dunaliella tertiolecta* actually could not utilize glucose even when incubating whole cells in the media and the lack of the ability to growth mixotrophically is attributed to membrane impermeability (Kwon and Grant, 1971). Additionally this lack of change can be attributed to the fact that these strains utilize organic glucose differently. For example Wan et al. (2011) demonstrated that *N.oculata* CCMP 525 increased in protein content with little effect to lipid productivity while the strain *C.sorokiniana* significantly increased in both protein and lipid content. This further illustrates the importance of phycoprospecting as individual strains of algae do not grow or accumulate fatty acids the same ways as other strains.

Under 3 mM acetate mixotrophic growth there was no difference in fatty acid saturation from axenic to non-axenic cultures. The only strain in which fatty acids were detected in both axenic and non-axenic cultures was S3N and like under photoautotrophic growth the saturation did not change. As stated previously depending on the growth media certain strains can utilize the organic carbon and some strains cannot. The results were very similar to the photoautotrophic results in the amount of strains which possessed 100% saturated fatty acids versus those with unsaturated fatty acids.

Although not entirely conclusive by our results bacteria are of importance as they release transparent exopolymer particles that cause the algal cells to aggregate together and sink out of solution (Grossart et al., 2006). This will aid in scaling the algae for biofuel production up to the industrial scale as collecting algae out of water requires large amounts of energy and can cost up to 30% of total cost for processing (Brentner et al., 2011; Singh and Dhar, 2011). Additionally the lack of fatty acids being registered by the

mass spectrometer El-Sheekh et al. (2012) actually showed that the production of lipid by algae depends on the specific algal species, culture conditions, and concentration of the reducing sugar. Additionally saturation of fatty acids can be altered by what season algae are cultivated during, further demonstrating the importance of not only temperature but light exposure to the algal cells (Olofsson et al. 2012). The reason why some strains have their fatty acids detected by Nile red dye but not mass spectrometry can be explained by the specific lipids being screened for. For example, one study that looked at *Anabaena doliolum, Anacystics nidulans,* and *Chlorella vulgaris* showed that the two blue green algae *A.doliolum* and *A.nidulans* possessed only glycolipids while *C.vulgaris* had glycolipids and significant amounts of neutral and phospholipids (Sakthivel et al. 2011).

The most predominant fatty acid across all three growth conditions was C17 which is ideal for a biofuel feedstock. Hu et al. (2008) actually states that fatty acids possessing carbon chain lengths from 16 to 18 units are ideal precursors for biodiesel production. Not only does carbon chain length provide a basis for deriving biodiesel but specifically the saturation versus unsaturation provides characteristics that make the fuel more versatile. Biodiesel derived from microalgae with more saturation provide a higher cetane number (CN), would have lower NO_x emissions, and have shorter ignition delay time (Cherisilp and Torpee, 2012). This comes at a cost since when temperatures are lowered; saturated fatty acids tend to form a solid due to their high melting point (Dogan and Temur, 2013). Unsaturated fatty acids require less heating and are often liquids at room temperature, however the higher the presence of double bonds the more prone the fuel is to producing NO_x emissions (Gopinath et al., 2010). Gopinath et al. (2010)

actually states that having a 50/50 blend of saturated and unsaturated fatty acids produces better thermal efficiency and reduces NO_x emissions.

Based on the parameters of saturated and unsaturated fatty acids it makes sense to select algal strains that have a relatively equal mixture of both. Therefore under photoautotrophic conditions the top three candidates are C3N, E1H, and B2H. Under mixotrophic conditions utilizing 14mM glucose, D1N, B1N, E1H, and C4C have promise as biofuel feedstock. Under mixotrophic growth utilizing acetate, D1N*, C3N, E1H, and C2H should be selected. It should be noted that of all the strains selected the best ratios were found in those that possessed the resident bacterial community indicating to researchers the added benefit of having non-axenic cultures. To maintain axenic cultures of algae requires the input of large amounts of time for screening cultures and re-screening to ensure cultures do not become contaminated. Additionally large amounts of money need to be spent on antibiotics to keep the algae axenic. This provides industrial start-up companies an added cost savings as non-axenic algae will provide a better fatty acid profile over axenic algae.

Chapter 4: General Conclusion

The objective of this research was ultimately to assess the potential of naturally occurring algae derived from municipal wastewater treatment plants for their potential as a biofuel feedstock. This was accomplished by first isolating and purifying algae from the raw effluent obtain from the three treatment plants: Nonquon River Lagoons Wastewater Pollution Control Plant (Port Perry, ON), Corbett Creek Water Pollution Control Plant (Whitby, ON), and Woodward Avenue Wastewater Treatment Plant (Hamilton, ON). This was followed by analysing the growth patterns under different media conditions along with assessing the fatty acid composition and amount under these varying growth parameters. As a consequence of not attaining axenic status for all of my strains, I was able to investigate the effect of naturally occurring bacteria on the lipid production in algal culture.

Through the course of the experiment 20 algal isolates were discovered falling into one of six genera: *Chlorella, Scenedesmus, Dictyochloris, Botrydiopsis, Ellipsoidon, and Microcystis*. Additionally a proper rationale for the choice of media was made by analyzing the composition of the naturally occurring levels of nitrogen and phosphorus found in wastewater a step often unreported or overlooked by much of the published research. It was discovered that growth of most of these strains under heterotrophic conditions could not result in a great enough biomass for future analysis of fatty acid content as the cellular density was much too low. When comparing the culture collection strains *Scenedesmus acutus* and *Chlorella kesslirii* to the wastewater derived strains it was discovered there was very little statistically significant differences found in the growth rates under photoautotrophic and mixotrophic (14mM glucose and 3mM acetate)

conditions. The most profound change was noted under heterotrophic conditions but this was explained as the culture collection *Chlorella kesslirii* is a strain that has been proven to grow under heterotrophic conditions. Additionally two distinct strains *Dictyochloris* D1N and *Chlorella* C6C were discovered to be strains with the highest growth rates under two different conditions. This further increases their potential as biofuel feedstock as they are not limited to one growth condition further promoting their versatility. The highest growth rate of all the strains was found to be from *Botrydiopsis* with a rate of $2.7\pm0.14x10^4$ cells$\cdot L^{-1}\cdot day^{-1}$. The lack of research into this strain provides future avenues of research.

The difficulty of utilizing wastewater algae was the fact that half the strains being worked with could not be purified of bacterial contamination. This was due to the fact that antibiotic resistant bacteria are found throughout municipal and medical wastewater sources indicating that the consortium associated with the algal strains had innate resistances to both penicillin and streptomycin. Additionally bacteria can harbor on algae or within their mucilage escaping the effects of the antibiotics all together. Under photoautotrophic conditions it was difficult to determine the effect of bacteria on the algae but the effect became evident under mixotrophic growth (14mM glucose). As the bacteria are present in the system they consume the freely available organic and inorganic compounds creating a nutrient deprived condition. The lack of nutrients induces stress on the algae causing the strains to accumulate neutral lipids. The opposite effect was seen with acetate but the higher neutral lipid content can be explained through how algae utilize photoassimilation of acetate directly into their fatty acid stores. Since bacteria outcompete algae for free organic carbon the axenic algae have access to acetate in the

media and assimilate it into their cells. This process results in a higher neutral lipid content under axenic conditions.

It should be noted that the use of principle component analysis should be implemented when screening large numbers of algal strains. Not only can the PCA depict which fatty acids are generally more abundant in a certain strain but it can also display similarity among strains. This data however should always be coupled with a breakdown of percent relative fatty acids in order to display the levels of saturated, monounsaturated, or polyunsaturated fatty acids. Both saturated and unsaturated fatty acids have disadvantages and advantages but it has been stated that a 50/50 blend would allow for the benefits of both to be present while reducing the drawbacks.

When selecting the best biofuel candidate it is difficult to decide as once again there are trade-offs between fatty acid content and composition. For the highest yields the following should be pursued:

1) photoautotrophic conditions
    - axenic *Chlorella* *S4.3N (C3N)
    - axenic *Scenedesmus** M3.3H (S7H)
    - *Scenedesmus* M2.2H (S6H)
    - *Chlorella* S 4.3N (C3N)
2) 14 mM glucose mixotrophic conditions
    - *Microcystis* M2.3H (M1H)
    - *Scenedesmus* F3.6N (S2N)
3) 3 mM acetate mixotrophic conditions
    - *Dictyochloris* F2.2N (D1N)

- *Scenedesmus* F3.6N (S2N)

For an even blend of saturated to unsaturated fatty acids should be considered:

1) photoautotrophic conditions
    - *Chlorella* S4.3N (C3N)
    - *Ellipsoidon* F2.3H (E1H)
    - *Botrydiopsis* F1.3H (B2H)
2) 14 mM glucose mixotrophic conditions
    - *Dictyochloris* F2.2N (D1N)
    - *Botrydiopsis* S4.2N (B1N)
    - *Ellipsoidon* F2.3H (E1H)
    - *Chlorella* 03CC (C4C)
3) 3 mM acetate mixotrophic conditions
    - axenic *Dictyochloris* F2.2N (D1N*)
    - *Chlorella* S4.3N (C3N)
    - *Ellipsoidon* F2.3H (E1H)

Chapter 5: Future Directions

The future directions for this work are very diverse as this project has merely established a foundation for further work. As the most novel findings of this research was first isolating naturally occurring algae from wastewater treatment plants and second the way in which naturally occurring bacterial communities influence fatty acid composition this in particular needs to be confirmed and pursued by other researchers. To date a vast majority of research into algal biofuels treats the algae with antibiotics and it has been demonstrated in this study that the fatty acid composition can change with bacterial presence.

A new method of neutral lipid quantification should also be pursued as there have been reported issues with the specific calibration of not only the wavelengths used to take readings but the specificity of concentration of dye, incubation time, and permeability. Other options do exist such as gravimetric determination and a new dye known as BODIPY.

Growth did not appear to be an issue in this study however few strains could tolerate heterotrophic growth. Perhaps the strains that did not experience detrimental growth rates under dark conditions could be analyzed for their fatty acid content and composition. Trials were discontinued as not enough strains exhibited enough growth under these conditions. Perhaps by comparing these strains to strains that are known to be effective at heterotrophic growth new results can be attained.

This experiment was only conducted under a strict set of growth conditions. The variability of increasing or decreasing temperature as well as light intensity may alter the lipid profile and may enhance growth of the feedstock further. Additionally to this the

experiment was conducted at a lab scale with small volumes meaning that further work needs to be done at the lab scale with larger volumes and eventually at pilot scale bioreactors.

Lastly since these strains are derived from wastewater treatment plants it would be highly beneficial to test their growth and lipid characteristics when grown in effluent. Not only is this research a future avenue but it is imperative that these naturally occurring algal communities be considered for their potential as biofuel feedstocks.

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Appendix A

Figure A1: Calibration curve of triolein in relation to the absorption units detected spectrofluorometrically

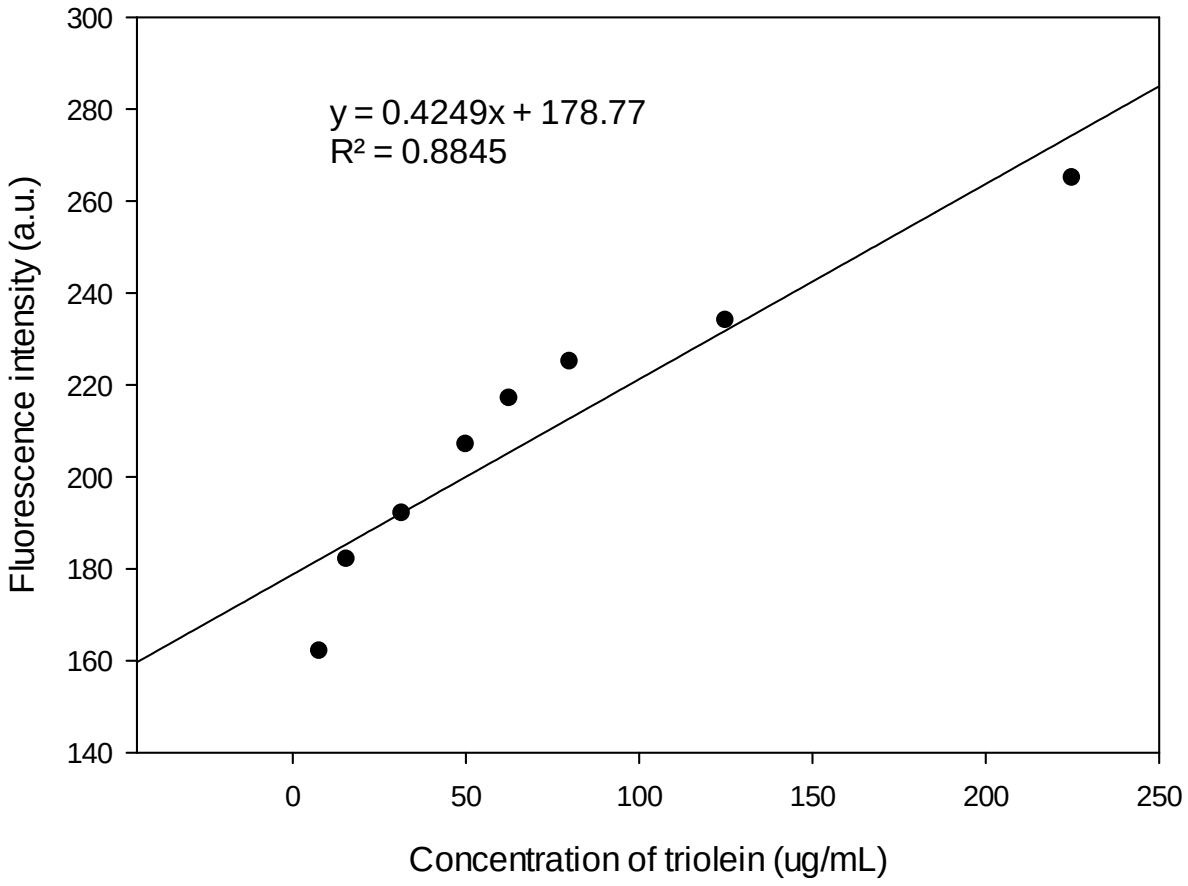

Table A1: Assessment of significant differences between axenic and non-axenic algal strains in terms of neutral lipid content. Significant differences ($p \leq 0.05$) are denoted by superscript lettering (same letters = no significant difference, different letters= significant differences).

Strain ID	Neutral Lipid content per cell ($\mu g \cdot cell^{-1} \cdot L^{-1}$)		
	Photo	**14mM G**	**3mM A**
D1N*	$106.0 \pm 0.2 \times 10^{2a}$	$24.7 \pm 0 \times 10^{2a}$	$23.5 \pm 0.0 \times 10^{2a}$
D1N	$151.0 \pm 1.2 \times 10^{2b}$	$28.1 \pm 0 \times 10^{2b}$	$28.6 \pm 0.1 \times 10^{2b}$
S2N*	$352.0 \pm 2.3 \times 10^{2a}$	$195.0 \pm 0.2 \times 10^{2a}$	$170.0 \pm 1.3 \times 10^{2a}$
S2N	$356.0 \pm 1.7 \times 10^{2a}$	$420.0 \pm 4.9 \times 10^{2b}$	$296.0 \pm 1.0 \times 10^{2b}$
S3N*	$261.0 \pm 1.2 \times 10^{2a}$	$188.0 \pm 2.1 \times 10^{2a}$	$67.6 \pm 0.2 \times 10^{2a}$
S3N	$373.0 \pm 10.2 \times 10^{2b}$	$245.0 \pm 0.5 \times 10^{2b}$	$103.0 \pm 0.7 \times 10^{2b}$
S6H*	$288.0 \pm 30.0 \times 10^{2a}$	$232000.0 \pm 17200.0 \times 10^{2a}$	$743.0 \pm 16.1 \times 10^{2a}$
S6H	$2480.0 \pm 390.0 \times 10^{2b}$	$1490.0 \pm 292.0 \times 10^{2b}$	$415.0 \pm 12.7 \times 10^{2b}$
S5N*	$405.0 \pm 45.0 \times 10^{2a}$	$276.0 \pm 0.5 \times 10^{2a}$	$105.0 \pm 0.3 \times 10^{2a}$
S5N	$155.0 \pm 13.4 \times 10^{2b}$	$160.0 \pm 0.3 \times 10^{2b}$	$84.2 \pm 0.4 \times 10^{2b}$
B1N*	$144.0 \pm 1.0 \times 10^{2a}$	$292.0 \pm 0.2 \times 10^{2a}$	$96.7 \pm 0.2 \times 10^{2a}$
B1N	$10.5 \pm 0.4 \times 10^{2b}$	$305.0 \pm 1.0 \times 10^{2b}$	$24.3 \pm 0.0 \times 10^{2b}$
C3N*	$4770.0 \pm 98.9 \times 10^{2a}$	$3650.0 \pm 255.0 \times 10^{2a}$	$4440.0 \pm 122.0 \times 10^{2a}$
C3N	$1840.0 \pm 46.6 \times 10^{2b}$	$1540.0 \pm 34.8 \times 10^{2b}$	$977.0 \pm 17.8 \times 10^{2b}$
E1H*	$0.0 \pm 0.0 \times 10^{2a}$	$33000.0 \pm 182.0 \times 10^{2a}$	$6740.0 \pm 26.4 \times 10^{2a}$
E1H	$533.0 \pm 5.0 \times 10^{2b}$	$414.0 \pm 0.4 \times 10^{2b}$	$209.0 \pm 0.2 \times 10^{2b}$
S7H*	$1450.0 \pm 388.0 \times 10^{2a}$	$1200.0 \pm 2.8 \times 10^{2a}$	$582.0 \pm 7.5 \times 10^{2a}$
S7H	$329.0 \pm 91.3 \times 10^{2b}$	$366.0 \pm 0.9 \times 10^{2b}$	$335.0 \pm 2.2 \times 10^{2b}$

Table A2: Photoautotrophic trial of axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**										
	C1U*	S1B*	D1N*	S2N*	S3N*	S6H*	S5N*	B1N*	C3N*	E1H*	S7H*
C 15	0	70	0	0	0	100	0	0	0	0	0
C 17	0	0	0	0	0	0	100	0	0	0	0
C 19:1 (cis)	0	0	0	0	0	0	0	0	0	0	0
C 19:2	0	0	0	0	0	0	0	0	0	0	0
C23	0	30	0	100	0	0	0	0	0	0	0
C 23:1	0	0	0	0	0	0	0	0	0	0	0
C 25	0	0	0	0	100	0	0	0	0	0	0
TS%	-	100	-	100	100	100	100	-	-	-	-
TM%	-	-	-	-	-	-	-	-	-	-	-
TP%	-	-	-	-	-	-	-	-	-	-	-
TN	113.3±11.8	36.6±42.9	94.0±3.5	155.3±9.3	132.6±2.0	187.1±10.4	170.6±13.3	180.8±6.9	164.9±8.5	203.5±6.1	208.1±13.3

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$)

Table A3: Photoautotrophic trial of non-axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**																			
	D1N	S2N	S3N	S6H	S5N	B1N	C3N	E1H	S7H	M1H	S4N	C4C	C5C	S8C	C6C	E2C	S9C	B2H	C2H	E3N
C 15	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	62.88	0.00	0.00
C 17	0.00	0.00	0.00	86.42	100.00	78.9	45.73	66.34	87.81	0.00	57.44	100	100.00	100.00	100.00	0.00	0.00	0.00	100	0.00
C 19:1 (cis)	0.00	0.00	0.00	7.35	0.00	13.7	54.27	33.66	0.00	0.00	28.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 19:2	0.00	0.00	0.00	6.23	0.00	7.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C23	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	13.81	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 23:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	37.12	0.00	0.00
C 25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TS%	-	100.00	100.00	86.42	100.00	78.9	45.73	66.34	-	-	71.25	100	100.00	100.00	100.00	-	-	62.88	100	-
TM%	-	-	-	7.35	-	13.7	54.27	33.66	12.19	-	28.75	-	-	-	-	-	-	37.12	-	-
TP%	-	-	-	6.23	-	7.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TN	101.9 ±13.9	191.1 ±6.5	124.1 ±12.4	137.1 ±15.7	107.0 ±10.4	34.9 ±36	142.8 ±10.3	133.2 ±11.0	126.9 ±13.9	138.8 ±17.5	174.0 ±5.9	82.1 ±9.7	144.5 ±11.1	155.3 ±6.7	167.8 ±4.3	71.8 ±44	71.8 ±44	134.9 ±14.5	41.8 ±41	105.3 ±49.8

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$)

Table A4: 14mM Glucose mixotrophic trial of axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**										
	C1U*	S1B*	D1N*	S2N*	S3N*	S6H*	S5N*	B1N*	C3N*	E1H*	S7H*
C11	0.0	0.0	0.0	0.0	0.0	50.7	0.0	0.0	0.0	0.0	0.0
C13	0.0	0.0	9.7	0.0	0.0	3.2	0.0	0.0	0.0	0.0	0.0
C 14	0.0	0.0	0.0	0.0	0.0	26.2	0.0	0.0	0.0	0.0	0.0
C15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	37.5	0.0	0.0
C16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 17	45.9	0.0	46.5	71.4	0.0	19.5	0.0	0.0	62.5	0.0	0.0
C18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19:1 (cis)	42.8	0.0	28.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C19:1 (trans)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19:2	11.4	0.0	15.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C21	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0
C23	0.0	0.0	0.0	28.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 23:1	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
TS%	45.9	-	56.2	100.0	-	100.0	-	-	100.0	-	-
TM%	42.8	-	28.6	-	-	-	100.0	-	-	-	-
TP%	11.4	-	15.2	-	-	-	-	-	-	-	-
TN	0.0±6.0	99.1±1.5	112.7±2.0	176.3±2.6	126.9±8.7	220.0±2.5	192.2±4.4	174.0±1.5	178.6±7.6	237.6±7.9	245.5±4.4

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$)

Table A5: 14mM Glucose mixotrophic trial of non-axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**																			
	D1N	S2N	S3N	S6H	S5N	B1N	C3N	E1H	S7H	M1H	S4N	C4C	C5C	S8C	C6C	E2C	S9C	B2H	C2H	E3N
C11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C13	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.2	0.0	0.0
C 14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C15	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0
C16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0
C 17	51.3	0.0	93.2	58.8	85.6	59.3	100.0	47.9	0.0	100.0	0.0	45.3	9.6	100.0	79.7	100.0	44.4	80.0	0.0	100.0
C18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.6	0.0	0.0	0.0
C 19	0.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19:1 (cis)	33.2	0.0	0.0	34.0	14.4	35.9	0.0	47.5	0.0	0.0	0.0	43.0	0.0	0.0	20.3	0.0	10.0	10.8	0.0	0.0
C19:1 (trans)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19:2	15.5	0.0	6.8	2.9	0.0	4.8	0.0	4.6	0.0	0.0	0.0	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C21	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 23:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TS%	51.3	-	93.2	63.1	85.6	59.3	100.0	47.9	-	100.0	100.0	45.3	9.6	100.0	79.7	100.0	90.0	89.2	-	100.0
TM%	33.2	-	-	34.0	14.4	35.9	-	47.5	-	-	-	43.0	90.4	-	20.3	-	10.0	10.8	-	-
TP%	15.5	-	6.8	2.9	-	4.8	-	4.6	-	-	-	11.7	-	-	-	-	-	-	-	-
TN	117.3±2.5	238.2±37.5	136.6±1.5	167.2±6.5	124.6±4.0	129.7±7.5	163.8±2.5	162.1±1.5	183.7±4.4	165.5±4.0	143.4±20.2	117.8±6.5	229.7±52.2	92.8±6.9	123.5±2.6	48.6±7.1	48.6±7.1	115.0±4.5	0.0±6.7	91.7±12.4

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$)

Table A6: 3mM mixotrophic trial of axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**										
	C1U*	S1B*	D1N*	S2N*	S3N*	S6H*	S5N*	B1N*	C3N*	E1H*	S7H*
C 17	100.0	88.9	56.3	100.0	94.6	0.0	0.0	0.0	0.0	0.0	0.0
C 19:1 (cis)	0.0	11.1	22.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 19:2	0.0	0.0	17.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C21	0.0	0.0	0.0	0.0	5.4	0.0	0.0	0.0	0.0	0.0	0.0
C23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 23:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 25	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TS%	100.0	88.9	59.9	100.0	100.0	-	-	-	-	-	-
TM%	-	11.1	22.2	-	-	-	-	-	-	-	-
TP%	-	-	17.8	-	-	-	-	-	-	-	-
TN	48.6±20.8	51.4±2.3	116.7±3.4	178.6±4.6	134.3±3.0	213.2±4.9	187.1±4.1	179.7±3.4	167.8±6.0	241.0±2.6	237.0±6.7

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$)

Table A7: 3mM mixotrophic trial of non-axenic algal strains displaying percent relative fatty acids in terms of their carbon chain short form. Algal strains appear as their short form designation. Lipids are further broken down into saturated, monounsaturated and polyunsaturated percentages.

Fatty Acid	**Algal Strain**																		
	D1N	S2N	S3N	S6H	S5N	B1N	C3N	E1H	S7H	M1H	S4N	C4C	C5C	S8C	C6C	E2C	S9C	B2H	C2H	E3N
C 17	0.0	0.0	100.0	100.0	76.9	100.0	69.0	61.0	0.0	0.0	100.0	67.2	100.0	100.0	94.6	100.0	0.0	100.0	64.1	79.1
C 19:1 (cis)	0.0	0.0	0.0	0.0	23.1	0.0	31.0	33.3	0.0	0.0	0.0	23.6	0.0	0.0	0.0	0.0	0.0	0.0	35.9	0.0
C 19:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.9
C23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.7	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C 23:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.4	0.0	0.0	0.0	0.0	0.0
C 25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TS%	-	-	100.0	100.0	76.9	100.0	69.0	66.7	-	100.0	100.0	67.2	100.0	100.0	94.6	100.0	-	100.0	64.1	100.0
TM%	-	-	-	-	23.1	-	31.0	33.3	-	-	-	23.6	-	-	5.4	-	-	-	35.9	-
TP%	-	-	-	-	-	-	-	-	-	-	-	9.2	-	-	-	-	-	-	-	-
TN	125.2±7.8	216.0±2.0	136.6±6.5	113.3±6.9	134.3±6.4	121.8±2.6	155.3±4.0	140.0±0.6	171.2±3.4	155.3±3.2	172.9±2.6	143.4±4.9	23.6±58	55.9±6.5	70.7±8.4	0.0±18.2	0.0±18.2	69.0±7.7	87.2±10	0.0±5.0

TS%= Total Saturated%, TM%= Total Monounsaturated%, TP%=Total Polyunsaturated%, TN=Total Neutral lipids (measured in $\mu g \cdot mL^{-1}$