Adaptation of microalgae bioprospected from stressed environments in Northern Ontario for the production of lipids

by

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Abstract

Photosynthetic green microalgae are a promising bio-feedstock that can be used to generate lipids for transesterification into biodiesel and/or various human health products such as polyunsaturated fatty acids. Unfortunately, due to their cultivation requirements, such as high energy requirements and carbon dioxide (CO_2) as a carbon source, large-scale biomass production generally remains uneconomical. To address this issue, the use of industrial flue gas as a low-cost source of CO_2 and a biorefinery approach to help mend the economic burden of microalgae-based products with an emphasis on creating co-products from lipid-extracted biomass (LEB) are assessed in this thesis.

Microalgae's ability to sequester CO_2 through photosynthesis is also advantageous in mitigating harmful industrial emissions. While these flue gases can have high concentrations of CO_2, they also can contain numerous contaminants (e.g., heavy metals, particulate matter) that discourage microalgae growth, and therefore their ability to fix CO_2. But, the most significant issue can be high nitrous oxide (NO_x) and sulphuric dioxide (SO_2) concentrations within a flue gas that cause acidification when bubbled through liquid media. It is due to this acidification that finding the productive microalgae species to grow in those systems can be problematic.

To address this, bioprospecting acid-tolerant microalgae from low pH environments in active and non-active mining sites was explored and the acidophilic species present identified through DNA sequence analysis. Bioprospected algal species were then grown in acidic conditions similar to those created by bubbling flue gas from a nickel smelter into water (pH 2.5). From this work, it was found that the acid-tolerant green microalgae in the genus *Coccomyxa* acclimated to the acidic conditions with suitable growth rates (0.136 day^{-1}) and biomass production (25.71 mg L^{-1} day^{-1}).

However, anabolic production of target biochemical molecules, such as lipids, is the key step in the bio-product process. It is known that microalgae have the ability to accumulate bioactive compounds when placed in stressed environments, such as high illumination and low nutrient availability, but little is known an about the impact of low pH and in particular the lipid composition of acidophilic microalgae. Research confirmed that the lipid compositions of bioprospected acid-tolerant microalgae was in the target range (13%). However, further work showed that an increased total lipid content (up to 27%), with a desirable rise in the relative level of health beneficial higher polyunsaturated fatty acid, could be achieved by applying dark stress at the end of the exponential growth phase. It is, therefore, proposed that this approach could be an easy, low-cost method to enhance lipid productivity.

Keywords

microalgae, co-products, lipid-extracted biomass, acclimation, acidic environment, *Coccomyxa sp*., polyunsaturated fatty acids, dark stress

Co-Authorship Statement

This thesis was written with myself as the primary author, while various chapters include assistance from numerous co-authors, as outlined below.

Chapter 2 – This chapter is presented as published in *Environmental Reviews*, under the following citation: **Sabrina Marie Desjardins**[a,b], Corey Alfred Laamanen[a,b], Nathan Basiliko[a,b,c] and John Ashley Scott[a,b,c], 2020. Utilization of lipid-extracted biomass (LEB) to improve the economic feasibility of biodiesel production from green microalgae. *Environmental Reviews*, 28(3), pp.325-338.

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SM Desjardins performed the literature investigation and primary manuscript writing; CA Laamanen assisted in manuscript writing and creative inspiration; N Basiliko assisted in manuscript writing; JA Scott assisted in manuscript writing and overall direction.

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Chapter 1

1 Introduction of thesis

1.1 Background

Whilst numerous bio-feedstocks, such as food-crop plants (i.e. corn, sugarcane) and woody waste products (originating from agriculture or forestry industries) have been used in the past to generate various bio-products, microalgae biomass is currently the favoured contender as it can be produced on non-arable lands, and has up to 50 times higher cell growth rate and CO_2 fixation rates (Yen et al. 2013) when compared to terrestrial plants. Microalgae are an exceptionally diverse group of microorganisms, which can be photoautotrophic (Tsuzuki et al. 2019), photoheterotrophic (Khichi et al. 2019), heterotrophic (Pleissner and Rumpold 2018), or mixotrophic (Patel et al. 2019) and either eukaryotic or prokaryotic. Some examples of microalgae include diatoms (Sprynskyy et al. 2017), cyanobacteria (Saka et al. 2020), dinoflagellates (Molina-Miras et al. 2020), green microalgae, and red microalgae.

In particular, photosynthetic green microalgae have consistently shown high biomass and bio-active compound yields. While the accumulation of biomass and the resulting bio-active compounds are highly dependent on species and culture conditions (Arumugam et al. 2020), these high yields give green microalgae immense potential as a bio-feedstock for numerous beneficial bio-products such as biofuel, bio-plastics, and health products. Unfortunately, due to their high production costs, green microalgae are currently less economical than other feedstocks. This thesis aims to bridge a gap between the production of microalgae and economic feasibility by focusing on a biorefinery approach to a well-established biodiesel industry.

Biodiesel is generated from lipids removed from biomass. Typically, food-crop plants are used as feedstock to produce biodiesel, as 376 million L yr^{-1} of biodiesel (mostly from canola sources) is consumed in Canada, resulting in 5.5 $MtCO_2$ eq. yr^{-1} of avoided greenhouse gas (GHG) emissions (Wolinetz et al. 2019). However, green microalgae can accumulate up to 80% lipid content per dry weight of biomass (Chungjatupornchai et al. 2019), increasing its potential as a bio-feedstock for biodiesel. The production of microalgae-sourced biodiesel is reviewed in Chapter 2, along with an analysis of the manufacturing of co-products from the lipid-extracted biomass – typically seen as a waste stream.

Manufacturing additional end-products is a suitable use of the waste stream created from the production of biodiesel, however waste streams from other industries can also be used throughout the cultivation of microalgae. For example, wastewaters from numerous sources, including municipal, agricultural, and industrial (Mat Aron et al. 2020) have been used as low-cost sources of nitrogen and phosphorus, which are essential for microalgae growth. Additionally, the use of industrial flue gasses has been used as a low-cost source of energy (heat and circulation that facilitates even access to light) and CO_2, which are essential for operational cultivation of phototrophic microalgae. Unfortunately, while industrial flue gasses have the necessary CO_2, pressure and heat (in cold climates at least) needed for supporting rapid photosynthesis in batch reactors, they also have increased concentrations of SO_2 and NO_x, which causes acidification of the liquid media. Acidic conditions are normally not explored, as these environments are hazardous towards neutrophilic microalgae, which are currently used as the bio-feedstocks at large-scale. Hence, research on acid-tolerant or acidophilic microalgae is lacking. Chapter 3 explores the effects of acidification on the growth of acid-tolerant green microalgae and its effect on community structure of a wild sample.

Another area of biodiesel research that can benefit from low-cost alternatives is at the end of cultivation when the system has reached the maximum biomass density and a stressor is applied to induce lipid accumulation. Lipid accumulation is typically induced through environmental stress, and involves either an increase in energy, an added stressor that will need to be addressed in the downstream process, or both. Chapter 4 explores the use of dark stress, which reduces the need for extra energy and does not affect any downstream processes. The use of dark stress could potentially be used to induce lipid accumulation in biomass intended for food supplements as an end-product.

1.2 Research objectives and thesis structure

The primary objective of this thesis is to document and explore various areas of the cultivation of microalgae biomass in order to improve its economic feasibility at large-scale. Numerous research questions will be addressed herein, including a) where are the existing limitations of the development of bio-products, b) what areas of cultivation can be improved, c) how does acidification of growing media influence microalgae growth, and d) can we improve the accumulation of bioactive molecules without disrupting the downstream process.

This thesis is organized into five distinct chapters, with chapters' 2-4 each having their own hypotheses and results, and therefore some repetition in respect of contextual information may occur. Chapter 2 provides information on the co-production of numerous commodities from lipid-extracted biomass, and is a published review article in *Environmental Reviews* (DOI: 10.1139/er-2020-0004). Chapter 3 explores the effects of acidified growing media on microalgae growth, and how acidification can be used to select for acid-tolerant microalgae species from bioprospected samples. This work has been submitted and is currently in review at *Extremophiles*. Chapter 4 explores dark stress as a technique to induce lipid accumulation in acid-tolerant microalgae biomass in the stationary phase of growth. This work is intended for publication in *Applied Environmental Microbiology*.

Chapter 2

2 Utilization of lipid-extracted biomass (LEB) to improve the economic feasibility of biodiesel production from green microalgae

2.1 Abstract

Photosynthetic green microalgae are eukaryotic microorganisms, which can mitigate anthropogenic carbon dioxide and generate lipids as a feedstock for production of biodiesel. Biodiesel production may not, however, compete economically with fossil fuel sourced diesel, but obtaining additional value from the biomass left after lipid-extraction has the potential to help make the overall process more cost-effective. This review focuses on these additional value-added options that obtain and utilize either whole lipid-extracted biomass (LEB), which typically constitutes 60-70% of total cell mass, or specific non-biodiesel lipid components such as polyunsaturated fatty acids, carbohydrates, and proteins.

2.2 Introduction

Photosynthetic microalgae-sourced lipids for conversion into biodiesel, are an attractive biofuel option. As carbon in the biodiesel is assimilated through algal photosynthesis, from either atmospheric carbon dioxide (CO_2) or from anthropogenic CO_2 found in industrial off-gases (Hosseini et al. 2018a), the net global warming potential of biodiesel is lower than fossil fuels (Maity et al. 2014). Depending on the species, microalgae have been shown to produce 20-80%

of lipid content per dry weight of biomass (Chisti 2007; Bandopadhyay et al. 2013) and can be grown year-round in suitable environmental conditions. Microalgae also have considerably greater (>85%) biodiesel production rates per unit area (L/ha) compared to terrestrial plant bio-feedstocks such as palm and jatropha (Mata et al. 2010; Maity et al. 2014).

In comparison to fossil fuel sourced diesel, biodiesel exhibits no sulphur emissions (Gouveia and Oliveira 2009; Mutanda et al. 2011) and a sizeable reduction in other harmful emissions, such as carbon monoxide and particulate matter (Islam et al. 2017). However, both increased (Islam et al. 2015; Hossain et al. 2017) and decreased (Al-lwayzy and Yusaf 2017) nitrogen oxide emissions from biodiesel, when compared to petrochemical diesel, have been reported. These contradictory reports could be attributed to different combustion conditions related to the additives (Khalife et al. 2017) or oxygen content (Singh et al. 2017) of the biodiesel. Unfortunately, vehicles that are not optimized for biofuels have shown a 9% decrease in energy per liter when utilizing biodiesel compared to petrochemical diesel, resulting in an increase amount of fuel used to travel the same distance (Wolinetz et al. 2019).

Many reviews have examined microalgae growth and lipid extraction methods for biodiesel production (Halim et al. 2012; Vanthoor-Koopmans et al. 2013; Mubarak et al. 2015; Enamala et al. 2018). However, various economic life cycle analyses have shown that this fuel may at present not be economically competitive with fossil fuel sources (Chauton et al. 2015; Carneiro et al. 2017; Chia et al. 2018). The U.S. Department of Energy (2018) reported an average biodiesel price in July 2018 of $3.55 USD/gallon, whereas the average price of petrochemical diesel was $3.24 USD/gallon. Unfortunately, depending on the process used, the biomass concentration produced, the lipid content of the microalgae, and the scale of production, microalgae biodiesel production can cost upwards of $13.31USD/gallon (Chen et al. 2018).

The high cost of biodiesel production is due primarily to purchasing and supplying additional CO_2 to boost biomass levels, and harvesting methods (Chia et al. 2018). However, other factors such as lipid-extraction processes (Bennion et al. 2015; Gerber et al. 2016), cultivation systems (Richardson et al. 2012), and spatiotemporal effects (Davis et al. 2014), have an impact on production economics. For example, the cost of producing saline microalgae in coastal areas is more than double the cost of freshwater microalgae, but saline operations can double the biomass productivity compared to freshwater (Davis et al. 2018b). Additionally, the high capital and operating costs of microalgae fuel technologies, and its consequential high energy demand, make competition with petrochemical and other bio-feedstock-sourced fuels challenging (Christiansen et al. 2018). Furthermore, the production of algal biodiesel has been reported to use more energy than the biofuel can deliver (Carneiro et al. 2017). While comparing different microalgae production routes for biodiesel production is complicated by variability in the estimation techniques used for generating capital costs (Gebremariam and Marchetti 2018).

However, irrespective of the production route, an opportunity to increase economic competitiveness may be realized in co-recovering with lipids for biofuels, other value-added algal products as well as co-treatment of waste streams. That is, cell disruption techniques employed for lipid removal could act as a pre-treatment step for recovery of lipid-extracted biomass (LEB) and other useful co-products, thereby potentially improving the overall process economics (Sari et al. 2013; Chia et al. 2018). Recent works support the viability of using biochemical fractionation, which can be used to isolate and convert multiple constituents into co-products (Laurens et al. 2017; Leow et al. 2018). Further economic benefit may be also gained by coupling microalgae biomass production to treatment of waste streams, such as using it to mitigate carbon dioxide (CO_2) in industrial off-gases, or to reduce nitrogen and phosphorus levels in wastewaters.

This review aims to outline various economically suitable microalgal sourced co-products that can be obtained along with biodiesel. We focus on three main constituents (lipids, carbohydrates, and proteins) of green microalgae, and their respective co-products. Further options to increase the economic viability of microalgae biodiesel, including the use of waste streams in a biorefinery, are also outlined.

2.3 Microalgal cell and its constituents

Whilst the term microalgae can include prokaryotic cyanobacteria (Smetana et al. 2017), phototrophic eukaryotic green microalgae have demonstrated most progress as a suitable feedstock for biofuels (Shuba and Kifle 2018). Green microalgae use 2-photosystem phototrophic mechanism (Gojkovic et al. 2015) similar to terrestrial plants, splitting water as the electron source and using the Calvin cycle for carbon fixation (Nawrocki et al. 2016). Green microalgae have 10-50 times higher cell growth rate and CO_2 fixation rates (Yen et al. 2013), fewer required process variables, and shorter generation times than terrestrial plants (Benemann 1997), improving their potential over crop-based biodiesel feedstock (Correa et al. 2017). Productivity of microalgal biomass can increase by selecting the proper culture conditions, including inoculum density (Ho et al. 2013), CO_2 concentration (Hosseini et al. 2018b), nitrogen levels, light intensity, and temperature (Chen et al. 2014).

The diversity of isolated microalgae strains is high (Hu et al. 2008; Sharma and Rai 2010), and the components contained within the cells are strongly dependent on species/strain (Diprat et al. 2017) and culture conditions (Renaud et al. 2002; Paes et al. 2017). In terms of economic biofuel production, it is better to have a higher quantity of fewer components within the cell (specifically lipids) than to have small quantities of numerous components (Soratana et al. 2014). To address this, genetic engineering has been proposed to help bypass natural selection processes; thereby reducing the number of generations it would take to create algal strains with high levels of specific high-value products (Radakovits et al. 2010; Work et al. 2010). Bajhaiya et al. (2017) outlined several proposed methodologies using transcriptional engineering that could aid in targeted genetic engineering such as modifying metabolic pathways and controlling cell quiescence.

2.3.1 Lipids

Lipids, which are fatty acids and their derivatives (Laurens et al. 2012), consist of a carboxyl group with a hydrocarbon chain. They are classified according to their carbon chain length (Yen et al. 2013), the number of double bonds present (Jung et al. 2017), and/or by their polarity (Guschina and Harwood 2013; D'Alessandro and Antoniosi Filho 2016). If the carbon chain present after the carboxyl group has no double bonds it is considered saturated, and when double bonds are present, unsaturated. When a single double bond is present it is referred to as a monounsaturated fatty acid (MUFA), whereas with two or more double bonds it is a polyunsaturated fatty acid (PUFA).

Microalgae synthesize fatty acids through esterification into phosphoglycerol-based lipids located primarily in cytoplasmic and organelle membranes, but under stressful conditions, biosynthetic pathways shift to produce triacylglycerols (TAGs), which serve primarily as energy storage (Hu et al. 2008). It is the production of TAGs that is important for the biodiesel industry, as these lipids can be extracted and undergo transesterification to produce fatty acid methyl/ethyl esters (FAMEs/FAEEs), also known as biodiesel (Arora et al. 2016; Bajhaiya et al. 2017).

Most biodiesel operations rely on stressed microalgae to enhance the desired constituents (Eibl et al. 2014). For example, when close to cell death, microalgae tend to yellow, which is an indication that the chlorophyll is becoming damaged, resulting in a loss of photosynthetic ability (Pan et al. 2011). When this occurs, energy storage becomes more accentuated, and increased amount of lipids and carbohydrates are produced (Rodolfi et al. 2009). Therefore, prior to harvesting, microalgae being used for biodiesel production are usually exposed to stressing conditions to encourage accumulation of TAGs (Markou and Nerantzis 2013; Sun et al. 2018b). Huang et al. (2010) summarized biosynthesis of the required biofuel lipids in three major steps: (i) formation of acetyl co-enzyme A (acetyl-coA) through photosynthesis in the chloroplast (to be used as the primer in the synthesis of fatty acids); (ii) elongation and subsequent desaturation of the carbon chain; and (iii) formation of TAGs.

When internal cellular focus is on cell survival, TAGs increase in the cell, and the culture exhibits little to no growth (Xiong et al. 2010). Conditions that have shown to increase TAG production in microalgae include nitrogen starvation (Pan et al. 2011; Bandopadhyay et al. 2013), mixotrophic conditions (Makarevičienė et al. 2012), and using specific phytohormones (Yu et al. 2017). However, other changes in conditions can cause a decrease in lipid production, such as temperatures above optimal (Renaud et al. 2002), and are species-specific (Rodolfi et al. 2009).

2.3.2 Carbohydrates

In microalgae, carbohydrates (or saccharides), can range between 3-70% of dry weight (Rodjaroen et al. 2007; Dragone et al. 2011) and are predominantly in the form of starch and glucose (Yamaguchi et al. 2017). These carbohydrates are classified as monosaccharide, disaccharide, or polysaccharide, depending on the number of carbon rings present in their structure. A monosaccharide is made up of one carbon ring and is the basis of di- (2 rings) and poly- (3 or more rings) saccharides (Chen et al. 2013).

In the cell, carbohydrates are similar to lipids in that their main purposes are storage and structure. Storage carbohydrates are found in starch grains, stored in the amyloplasts throughout the cell, whereas structural polysaccharides are found within the cell wall (Hu et al. 1998; Templeton et al. 2012). As the stressing conditions used to encourage lipid production can also increase carbohydrate concentrations, it has been suggested that lipid and starch synthesis may compete (Wang et al. 2009; Work et al. 2010). Lipids are, however, preferred for long-term storage and starch as a short-term reserve (Siaut et al. 2011), and, therefore, when microalgae are placed under long-term stressful conditions, starch reserves will start to be depleted before lipids.

Ho et al. (2012) found a *Scenedesmus obliquus* strain to normally have a higher carbohydrate than lipid concentration, but when put under nitrogen-starved conditions and increased light intensity, there was a significant increase in both lipids and carbohydrates. Chia et al. (2015) found *C. vulgaris* to increase in both lipids and carbohydrates when placed under nitrogen deprivation and cadmium stress. Dragone et al (2011) showed the accumulation of carbohydrates in *C. vulgaris* with both nitrogen depletion and iron depletion. For *Chlorococcum littorale*, high cell density resulted in increased cell wall thickness and as a result, numerous enlarged starch grains and two largely expanded starch sheaths occupied the chloroplast (Hu et al. 1998). Reports have also shown that different strains react differently to nitrogen stress, with some increasing in carbohydrate content, whilst others decrease (Siaut et al. 2011; Ho et al. 2013). These findings indicate that microalgal cell manipulation through stress conditions can be species or strain dependent.

2.3.3 Proteins

Proteins can range from 6-70% of microalgal dry cell weight in microalgae (Becker 2007), with the species determining if it is the dominant chemical cellular component (Renaud et al. 2002; Santana et al. 2017). Proteins, are classified according to their amino acid sequences and structures (Becker 2007). However, if nitrogen is taken as the sole factor measured as an indicator of protein, overestimation of the protein content in the cell is common due to total nitrogen also including pigments, nucleic acids and other inorganic compounds (Becker 2007; Safi et al. 2013).

Similar to the other fractions of the cell, microalgal protein content can change in response to the external environment. For example, optimizing light intensity and distribution within a bioreactor to reduce shading effects, causes increased protein content and productivity (Chen et al. 2015). Under normal conditions, nitrogen present in the external environment is consumed and converted into amino acids, then packaged as proteins. Therefore, optimized concentrations and chemical forms of nitrogen within the microalgal growing media can positively influence cell growth and protein content (Chen et al. 2015). However, as nitrogen depletion is a commonly applied stressor to increase microalgal lipid and carbohydrate content, this can lead to a decrease in overall protein and amino acid content (Ho et al. 2012; Taleb et al. 2018).

2.4 Co-products from lipid-extracted biomass (LEB)

Depending on which microalgae species and lipid extraction technique is used, the LEB will be 30-75% of the original dry biomass (Ansari et al. 2017), and contain variable amounts of different potential co-products (Figure 2.1). However, not all constituents within the microalgal cell are of high-value and a more economically beneficial approach may be to target individual components (Shurtz et al. 2017), particularly if their wholesale prices are increasing faster than the biofuel produced (Barsanti and Gualtieri 2018). Furthermore, many solvents used in lipid extraction, whether at small-scale with N,N-dimethylcyclohexylamine (DMCHA) (Samorì et al. 2013) or chloroform (Ramluckan et al. 2014), or at large-scale using hexane (Shin et al. 2018), are toxic to humans and animals; therefore, other extraction practices may need to be considered for the safety of LEB downstream processing and products. While a detailed review of safe lipid-extraction alternatives is critical to this discussion, it is outside the scope of this work.

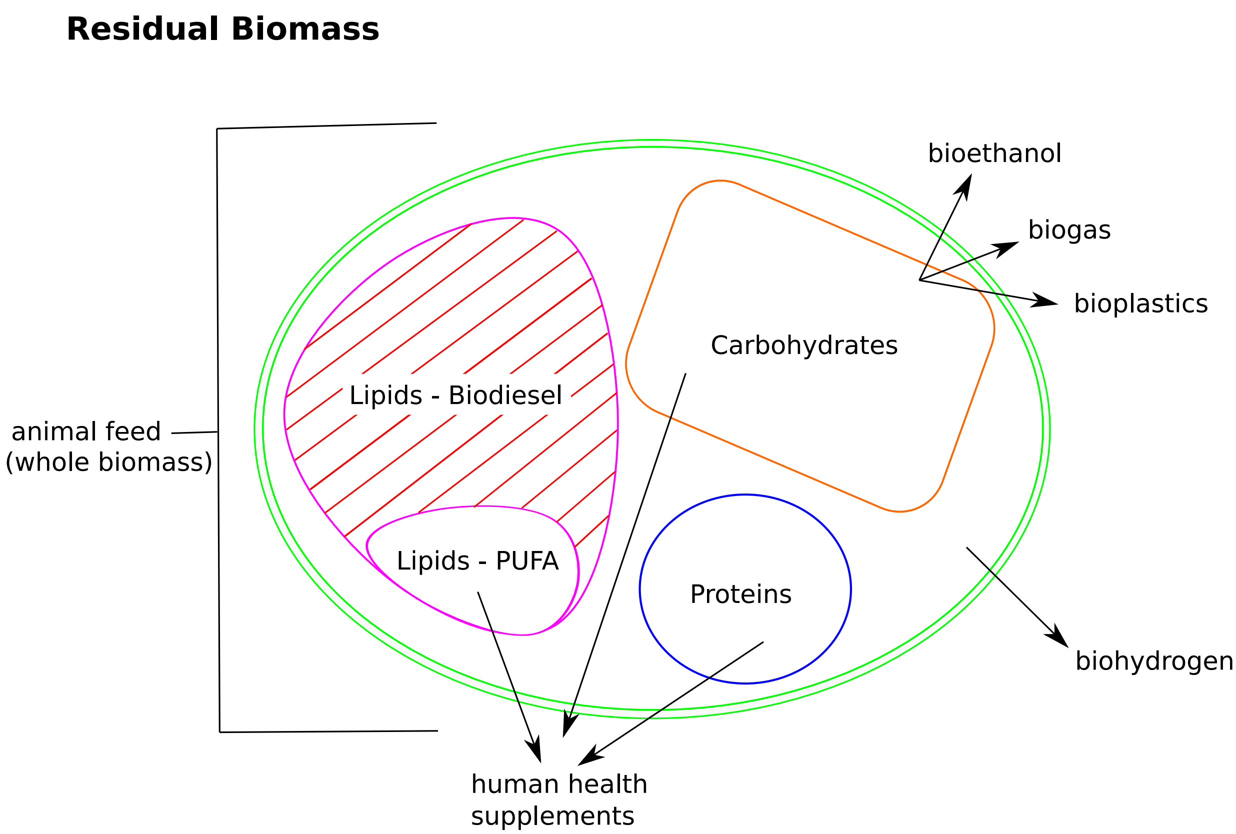

Figure 2.1: A simplified depiction of the components leftover in residual biomass. Depending on the species, 25-70% of an algal cell is made up of lipids, some of which will not be able to produce biodiesel through transesterification (i.e. polyunsaturated fatty acids, PUFAs). Meanwhile, other important components, such as carbohydrates and proteins, are leftover in the residual biomass, known as lipid extracted biomass, LEB. These components can themselves be extracted to produce various co-products such as bioethanol or human health supplements, or can remain as whole LEB and be mixed with traditional animal feeds to produce feed with a higher protein and carbohydrate content.

Carbohydrates and proteins make up the majority of LEB (Table 2.1), with for example, that of *Desmodesmus sp*. containing 41% protein and 28% carbohydrate (Souza et al. 2018). Whereas, the LEB of *Phaeodactylum tricornutum* had a 37% protein and 47% carbohydrate content

(German-Báez et al. 2017). Furthermore, in green microalgae, the chlorophyll pigments (light absorbing molecules), which do have positive human health effects, only account for 0.5-1.5% of dry matter (Krishna Koyande et al. 2019) and are, therefore, not ideal as a primary co-product.

Table 2.1: Percentages of carbohydrates and proteins leftover in lipid-extracted biomass (LEB)

Species	**% (w/w) in LEB**		**Reference**
	Carbohydrates	Proteins	
Auxenochlorella protothecoides	75	14*	(Bohutskyi et al. 2015)
Chlorella sp.	49.70	28.50	(Lee et al. 2015)[†]
	24.77 ± 2.08	55.28 ± 1.61	(Kassim et al. 2014)
Chlorella variabilis	-	36.31 ± 0.98*	(Maurya et al. 2016)
Chlorella vulgaris	12.15	38.96	(Zhao et al. 2014)
	37.30	-	(Lam et al. 2014)
Desmodesmus sp.	27.73 – 37.36	34.76 – 41.05	(Souza et al. 2018)
Nannochloropsis sp.	9.56	32.70	(Zhao et al. 2014)
Nannochloropsis gaditana	16.60 – 17.40	52.50 – 52.80	(Capson-Tojo et al. 2017)
Nannochloropsis salina	17.04	26.72	(Zhao et al. 2014)
	20 ± 1.9	27 ± 2.8	(Tran et al. 2018)
Phaeodactylum tricornutum	46.78 ± 0.47	36.67 ± 0.43	(German-Báez et al. 2017)
Scenedesmus sp.	24.70	32.40	(Yang et al. 2011)
	29.30	35.60	(Ramos-Suárez and Carreras 2014)
	40 – 48	34 – 46	(Tibbetts et al. 2015)[‡]

Scenedesmus dimorphus	54 ± 0.5 w/w	-	(Chng et al. 2016)
Scenedesmus obliquus	20.53 – 26.29	12.08 – 39.12	(Ansari et al. 2017)
Tetraselmis suecica	19.81 ± 1.32	63.04 ± 2.80	(Kassim et al. 2014)

*crude protein

† *Chlorella sp.* KR-1 strain

‡ *Scenedesmus sp.* AMDD strain

2.4.1 Animal feed

Due to the imprecise definition for the word 'algae', as it can also include bacteria and diatoms, brown and red algae are the only algae placed on the 'generally regarded as safe' database operated by the U.S Food and Drug Administration (FDA) (FDA 2015). However, the official European Union List consisting of "novel foods", as outlined in Regulation (EU) 2015/2283, has specific examples of microalgae regarded as safe for human consumption, including the green microalgae *Tetraselmis chuii* and the red microalgae *Haematococcus pluvialis* (European Commision 2017). An internet search highlights the popularity of Chlorella powder, typically made from *C. vulgaris*, as a readily obtainable food supplement for human consumption.

Research into the impact of adding microalgal biomass to both human and animal feeds has been increasing in recent years (Madeira et al. 2017). Constituents of microalgae may provide necessary added-benefits to human food, such as anti-microbial properties (Pina-Pérez et al. 2017), making LEB a candidate for human consumption. Currently however, whole microalgae cells are more commonly used in animal feeds.

Madeira et al. (2017) reviewed the growth and meat quality of livestock (ruminants, pigs, poultry, and rabbits) given whole microalgae-supplemented feed, and found various microalgae, such as *Chlorella sp*. and *Schizochytrium sp*., can successfully be used as a feed additive to enhance the growth of livestock. The addition of whole *Chlorella vulgaris* to the diet of post-partum Damascus goats (*Capra aegagrus hircus*) increased digestibility and improved feed intake by up to 12% (Kholif et al. 2017). An increase in milk yield and a positive impact on the fatty acid profile, by increasing the PUFA content, was also noted. Furthermore, microalgae LEB has been shown to have comparable or higher amounts of both proteins and carbohydrates to non-LEB (Ansari et al. 2015; Tibbetts et al. 2015), making it ideal as a food supplement.

Another avenue for whole LEB is as feed in aquaculture, which as the world's fastest growing food sector (Fry et al. 2018) would benefit from economically efficient feeds, to increase sustainability (Camacho-Rodríguez et al. 2018). For black tiger prawns (*Penaeus monodon*), feed mixed with at least 5% whole microalgae, when compared to commercial prawn feed, resulted in no significant difference in growth rate, survival rate (Angell et al. 2018), or quantity of fatty acids or amino acids (Li et al. 2016). However, by replacing 3% of commercial fish feed with microalgae LEB as a protein substitute, Pacific white shrimp (*Litopenaeus vannamei*) had a significantly higher growth rate than those on the control diet with no LEB replacement (Ju et al. 2012). Similarly, although as yet not tested commercially on fish, many high-protein microalgae species have been researched as good candidates for fish feed supplements, such as *Chlorella protothecoides* (D'Este et al. 2017), *Scenedesmus almeriensis* (Garcia Gonzalez et al. 2018), and *Scenedesmus sp*. (Duong et al. 2015). It has been stated that improvements in microalgae production and harvesting are needed for a long-term economically feasible fish feed industry (Khan et al. 2018), but if LEB has already been recovered due to biodiesel production, and retains the microalgal protein content, it could be an excellent candidate.

2.4.2 Biogas and biohydrogen

Biogas is an anaerobic digestion product, containing 55-75% methane and 25-45% CO_2 (Harun et al. 2010b). Currently, solid state fermentation technologies are being considered in biorefineries to help produce biogas as a co-product (Abdul Manan and Webb 2017). Zhao et al. (2014) found LEB of *Nannochloropsis sp.* to have a 2% higher biogas production than its non-lipid extracted counterpart. Similarly, LEB from other species, including *Tetraselmis sp*. and *Scenedesmus sp*., were found to produce higher methane yields compared to their untreated biomass (Neves et al. 2016). These increases in methane production may be attributed to the decrease in long-chain fatty acids (LCFAs) present in the biomass, which are known as inhibitory to anaerobic microorganisms (Palatsi et al. 2009; Rasit et al. 2015). However, not all species of microalgae have shown an increase in methane yields after lipid extraction (Zhao et al. 2014), suggesting these outcomes could be species specific.

Numerous physical, chemical, or mechanical pre-treatments of the cells have been shown to increase the efficiency of biogas production (Ganesh Saratale et al. 2018). Interestingly, biological pre-treatment (introducing bacteria to the consortium) was shown to increase biogas production and also use less energy (Kavitha et al. 2017). However, it has been found that the protein content of the biomass can negatively affect the digestion process due to an increased amount of free ammonium-nitrogen (Capson-Tojo et al. 2017). While the use of microalgae with naturally low protein content for biogas production has been acknowledged (Magdalena et al. 2018), the potential to extract the protein content from LEB and then use the residual biomass, which would include cellulose-based cell wall structures, for biogas production has not been confirmed. Furthermore, using dried biomass can also decrease biogas production, possibly due to a decrease in accessibility to bacteria and archaea (Mussgnug et al. 2010; Kinnunen et al. 2014). Enhancing biogas from palm oil waste into biological compressed natural gas or by adding hydrogen gas has been proposed (Zuber et al. 2020), which could potentially increase costs. If biogas is considered an ideal co-product in a biorefinery process, there should be careful consideration of the upstream processes, particularly during harvesting and biomass pre-treatment, in order to produce the highest amount of co-product.

With processes to produce bio-hydrogen, either through the direct photosynthetic pathway of some microalgae (Hemschemeier et al. 2009) or through dark fermentation (Hnain et al. 2011), results have been mixed regarding the applicability of using LEB. In one study, the use of chloroform to extract lipids was found to decrease the bio-hydrogen yield by 50% (Yun et al. 2014). Whilst, Nobre et al. (2013) found *Nannochloropsis sp.* to be a suitable bio-feedstock for the production of bio-hydrogen after lipids and pigments had been extracted. Using the residual biomass from bio-hydrogen production has shown an increased yield in subsequent biogas production (Mussgnug et al. 2010), suggesting that LEB with good bio-hydrogen yields can also go on to generate good biogas yields. This method is known as a two-stage process (combined hydrogen and methane production) and has been reported to increase methane production by 22% in lipid-extracted Scenedesmus biomass compared to a one-stage process of anaerobic digestion (Yang et al. 2011). Work has shown that controlled pH and the addition of 2-bromoethanesulfonic acid (a chemical methanogenic inhibitor) can significantly increase bio-hydrogen production (Kumar et al. 2016), however the results were from using whole microalgae, not LEB.

2.4.3 Bioethanol

Bioethanol, produced using carbohydrates from biomass, is used as a substitute for gasoline (Hernández et al. 2015). Though bioethanol is considered a low-value product (Ho et al. 2013), pairing its production with biodiesel production could enhance the economic potential of both. Many process variables, including microalgae species and morphology, need to be considered before implementing a biorefinery approach for the production of biodiesel and bioethanol (Velazquez-Lucio et al. 2018). Biomass pre-treatment is a particularly crucial step in the process of creating bioethanol, and methods have been proposed (Phwan et al. 2018), which could be a benefit to combined production. Through acidic hydrolysis cell disruption, extracted polysaccharides can be transformed into monosaccharides, known as saccharification (Templeton et al. 2012), which are then suitable for bioethanol production (Hernández et al. 2015). This approach was found to provide a two-fold increase in ethanol production as compared to supercritical CO_2 extraction methods, while simultaneously releasing lipids (Harun and Danquah 2011).

However, while chemical hydrolysis is not the most expensive method (Simas-Rodrigues et al. 2015), other methods, such as enzymatic saccharification, are more advantageous as they have lower equipment costs (Cara et al. 2007). Enzymatic saccharification of LEB was found to have a saccharification yield of 80.9% (w/w) of the total carbohydrates, which as it is nearly the same efficiency as LEB pretreated with acid, suggests no need for any chemical pretreatment (Lee et al. 2013). Supercritical CO_2 extraction for primary lipid extraction has been also used, with the resulting LEB used as a substrate in a yeast fermentation producing ethanol without the need of a saccharification step (Harun et al. 2010a). It was found that this two-step process resulted in an increased yield of yeast biomass and 60% higher ethanol concentrations in the media compared to using non-lipid extracted cells. A similar two-step process, called combined algal processing (CAP), pretreats microalgal biomass with steam and dilute sulfuric acid, before it is used for yeast fermentation. The resulting fermentation slurry is processed to recover ethanol and lipids via thermal treatment and solvent extraction, respectively (Dong et al. 2016). This process produced 22.7 gL^{-1} of ethanol and recovered 87% of FAMEs (Dong et al. 2016), and models suggest it has the potential to reduce the minimum fuel selling price by 62% when further combined with direct hydrothermal liquefaction (Li et al. 2019).

Microalgae consist of both fermentable sugars (e.g., glucose, galactose, and mannose) and non-fermentable sugars (e.g., rhamnose, arabinose, and xylose) (Kim et al. 2014). Fermentable sugars, especially glucose, are predominant among the monosaccharides found in microalgae (Wang et al. 2017; Keris-Sen and Gurol 2017). Conversion efficiencies of biomass to ethanol are dependent on culture conditions, saccharification methods, and fermentation processes (Sanchez Rizza et al. 2017). However, culture conditions for the microalgae would have been optimized to increase lipid production, resulting in a decrease in carbohydrates and, therefore, a possible decrease in available fermentable sugars.

2.4.4 Bio-polyethylene

Traditionally, polyethylene (PE) is produced from petrochemical feedstocks (Morschbacker 2009), whereas bioethanol can be used as a precursor for the production of plant-based polyethylene (bio-PE). By using bio-PE there would be reduced anthropogenic CO_2 production and reliance on fossil fuel reserves (Zeller et al. 2013). The chemical, mechanical, and physical properties of plant-based PE is an exact match to that of petrochemical PE (Babu et al. 2013), both of which are not biodegradable (Bos et al. 2012; Iwata 2015). To create bio-PE, bioethanol is dehydrated to form ethylene, which undergoes purification and polymerization (Morschbacker 2009). The method of polymerization depends on the desirable end product, whether is low density PE (LDPE), high density PE (HDPE), or linear low density PE (LLDPE) (Shen et al. 2010). Currently, bioethanol for use in bio-PE is produced mainly from sugarcane (Shen et al. 2010), but other feedstocks including potatoes (Farajpour et al. 2020), cassava (García et al. 2009), and sugar beets (Belboom and Léonard 2016) are also used.

2.4.5 Human health products

For the production of biodiesel, saturated and monounsaturated FAMEs are predominantly used as polyunsaturated FAMEs have been shown to cause deterioration in fuel quality (Kaur et al. 2012). However, unused PUFAs separated from the biodiesel-producing fatty acids, can have positive human health benefits, such as preventing cardiovascular diseases (Calder 2015; Caporgno and Mathys 2018), inflammatory diseases (Adarme-Vega et al. 2014), and cancer

(Doughman et al. 2007). These include omega-3 fatty acids such as eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3), and the omega-6 fatty acids such as linoleic acid (LA; C18:2 n-6) and arachidonic acid (AA; C20:4 n-6).

Currently, fish oils are used extensively as EPA and DHA supplements for human health, but the origin of these fatty acids are the microalgae that are part of fish diets (Hixson et al. 2015). EPA and DHA directly from microalgae can function, therefore, as a vegetarian/vegan alternative (Doughman et al. 2007; Ryckebosch et al. 2014; Chauton et al. 2015). This approach is also arguably more beneficial to the environment by putting less pressure on fish stocks, and may reduce co-occurring pollutant levels (e.g Hg) by bypassing biomagnification up aquatic food chains.

Microalgal-produced carbohydrates have been also found to have positive human health effects. *Porphyridium sp*. polysaccharides (of unknown composition) were found to have anti-inflammatory effects on the human skin (Levy-Ontman et al. 2017), extracts from *Isochrysis galbana* and *Nannochloropsis oculata*, including the monosaccharides glucose, mannitol, and inositol, were found to have antimicrobial and anticancer properties (Hafsa et al. 2017), and a mutant of a *Thraustochytriidae sp.* type strain, which excrete high amounts of polysaccharides (unspecified composition), was able to reduce cell viability of certain ovarian, breast, and colon cancer cells (Park et al. 2017). Most industrially used polysaccharides, including xanthan, dextran, and gellan, are derived from bacteria, such as *Xanthomonas campestris*, *Leuconostoc mesenteroides*, and *Sphingomonas paucimobilis*, due to lower fermentation costs (Öner 2013). With respect to LEB, polysaccharides associated with the cell wall, including cell wall polysaccharides (CWPS) and extracellular polysaccharides (EPS), would most likely be released when the wall is broken or deteriorates during lipid-extraction, making them more available for carbohydrate-extraction. CWPS and EPS are mainly composed of the monosaccharide's glucose, galactose, xylose, and mannose (Bernaerts et al. 2018) suggesting a wide variety of possible polysaccharides may be present among the extracted carbohydrates. Therefore, carbohydrate composition studies of LEB need to be completed before any decisions are made towards choosing carbohydrates as a health-focused co-product.

Microalgae can synthesize all 20 amino acids, including the essential eight for human health (Spolaore et al. 2006; Ejike et al. 2017). Microalgae-derived proteins can be used to produce bioactive peptides, which can have positive effects on human cardiovascular health due to their anti-inflammatory and antihypertensive properties (Ejike et al. 2017). Microalgal proteins, and PUFAs, can be used as food supplements (Smetana et al. 2017), including for nutrition and health of humans (Yen et al. 2013; Lam et al. 2014), livestock (Spolaore et al. 2006; Duong et al. 2015), and aquaculture (Li et al. 2013; Chauton et al. 2015; Chandra et al. 2016). Whilst the potential of using microalgae-derived proteins as a supplement in animal (Sun et al. 2018a) and human (Kent et al. 2015) feeds have been described, their incorporation in food remains poorly developed (Batista et al. 2017). This is most likely due to the high cost associated with cultivating and processing microalgae, along with a current low demand of food products produced from microalgae (Vigani et al. 2015).

2.5 The biorefinery approach

To produce non-biofuel lipids and other potential value-adding co-products, a bio-refinery approach may be needed to systematically extract multiple factions of the cell (Gerardo et al. 2015; Chew et al. 2017). To ensure a prosperous biorefinery, the whole process from choosing the appropriate feedstock (Olguín 2012), through the separation technologies used, to the final form of the end-product must be addressed (Botero et al. 2017). Currently, the downstream process of a single bioproduct makes up approximately 30% of total production costs, whereas this increases to up to 60% for a multi-product biorefinery ('t Lam et al. 2018). Fortunately, with new emerging technologies, such as nanotechnology for cell pre-treatments or as fuel additives (Nizami and Rehan 2018), optimizations to the process can bring about lower costs. Another technique is using mild cell disruption and extraction techniques so as to not harm or denature macromolecules in the cell, but the techniques need to be ideally inexpensive and consume very little energy (Mussgnug et al. 2010; Vanthoor-Koopmans et al. 2013).

In 2015, Canada produced an estimated CAD 4.3 billion in bioproduct sales, with CAD 441.5 million attributed to the sale of co-products (Rancourt et al. 2017). This is more than a three-fold increase in sales when compared to 2009, when Canada's revenue from bioproducts was estimated at CAD 1.3 billion (Rothwell et al. 2011), showcasing the growing market for both biodiesel and co-products. Whilst we outlined various potential biodiesel production co-products, the market value of specific compound(s) after extraction will be the main factor in selecting which ones are most useful (Figure 2.2). Suggestions that microalgae could be used as a soil ameliorant have been made (Shaaban 2001; Mulbry et al. 2005; Bădescu et al. 2017), although further research is needed to confirm that LEB is a good alternative. Furthermore, if residual biomass from biodiesel production is deemed unsafe for direct consumption (e.g., either for animal feed or nutraceutical supplements), it should not be disregarded. Instead, it could be used to create heat through combustion for any upstream process requiring heat.

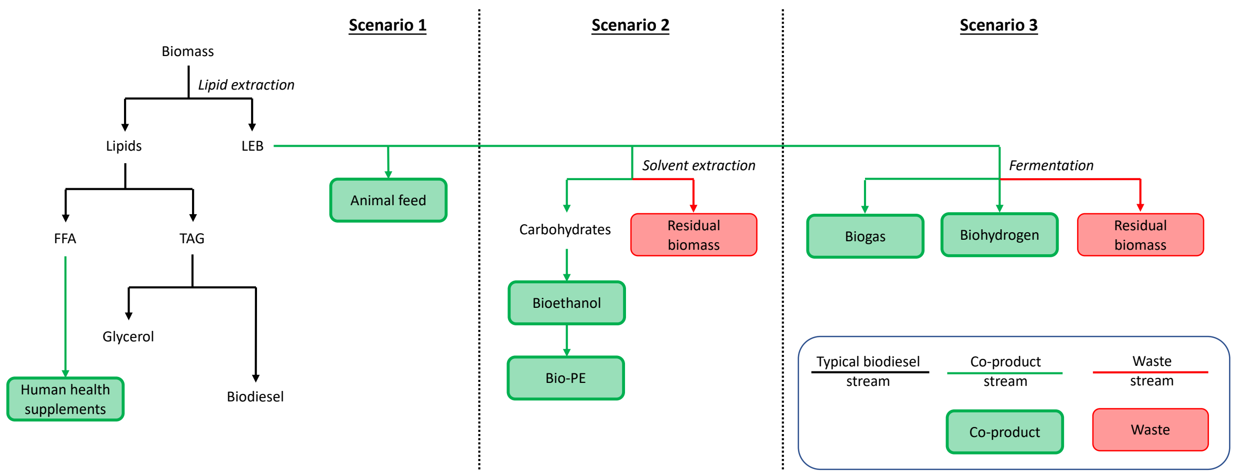

Figure 2.2: The scenarios outlined do not take into account the various processes needed for the production of the final co-products, but is instead meant as a guideline to show the various pathways LEB can be utilized. Glycerol is a natural co-product of the transesterification process, and is therefore not portrayed as a potential co-product. Scenario 1 demonstrates the typical stream of biodiesel production (bold black lines) with two end co-products that can be produced if non-toxic solvents were used for lipid extractions. Scenarios 2 and 3 demonstrate co-products from LEB that can be manufactured using different techniques (extraction vs fermentation), however culminate in residual biomass that would not be suitable for consumption. Instead, the residual biomass could be used to create heat through combustion for upstream processes, or as a possible soil ameliorant.

2.5.1 Incorporating waste streams

The diversity and adaptability of green microalgae has made them promising candidates for removal and mitigation of CO_2 in industrial off-gas emissions (Cuellar-Bermudez et al. 2015; Hosseini et al. 2018a) and for removing nutrient levels in wastewaters (Álvarez-Díaz et al. 2017) (Table 2.2). Industries producing large quantities of CO_2-rich off-gas include coal-fired power generation (Aslam et al. 2017), cement production (Lara-Gil et al. 2016), and metal smelting (Laamanen et al. 2014). With the high costs of providing additional CO_2 to microalgae production facilities (Chia et al. 2018), industrial emissions can supply “free” CO_2 as well as offer other economic advantages through greenhouse gas mitigation if carbon cap and trade programs are in place. Furthermore, waste heat in industrial off-gas can aid algal growth in regions with colder climates, helping expand the locations available for the microalgae industry (Laamanen et al. 2017).

However, there are other components in industrial off-gas to consider. Acidic gases such as SO_2 and NO_x, can be tolerated by microalgae as long as the levels do not exceed ca. 100 ppm, otherwise they can negatively affect growth (Radmann et al. 2011; Mortensen and Gislerød 2016). In simulated off-gas containing 5% CO_2 and 80 ppm NO, *Asterarcys quadricellulare* and *Chlorella sorokiniana* were able to produce high lipid contents (>40%) making them promising candidates for the production of biodiesel using industrial off-gases (Varshney et al. 2018). Nevertheless, heavy metals present in off-gases should be also considered depending on the final application of the harvested microalgae and associated biocomponents (Napan et al. 2015; Hess et al. 2017).

Table 2.2: Green microalgae used for the removal of waste CO2 from various industries and the removal of nutrient levels from various wastewater sources. Note: research conducted with artificial wastewaters or simulated flue gases are not included.

Source	Species	CO_2 fixation rate	Removal efficiency (%)			Maximum Biomass Concentration (DW mg/L)	References
			CO_2	Total Nitrogen	Total Phosphorus		
Waste CO_2							
Coal-fire power	Mixed consortia	-	12.3 – 14.9 (after 10 min.)	-	-	-	(Aslam et al. 2017)
	Nannochloropsis oculata	40.7 g/m^2/d	Up to 28.4	-	-	-	(Cheng et al. 2015)
	Chlorella fusca	360.12 ± 0.27	-	-	-	920 ± 20	(Duarte et al. 2017)
Cement	*Chlorella emersonii*	3.25 g/L	-	-	-	2000	(Borkenstein et al. 2011)
MW incinerator	*Chlorella vulgaris*	4.4 g/L/24h	-	-	-	>1300	(Douskova et al. 2009)
Steel furnace	*Chlorella sp.*	20 – 35 g/L/d	15 – 25	-	-	1872	(Kao et al. 2014)

Wastewater							
Aquaculture	*Tetraselmis suecica*	-	-	94.40 ± 0.97*	96.06 ± 2.54†	603 ± 34	(Andreotti et al. 2017)
	Isochrysis galbana	-	-	95.44 ± 0.29*	91.19 ± 2.34†	161 ± 24	(Andreotti et al. 2017)
	Dunaliella tertiolecta	-	-	66.02 ± 1.52*	91.93 ± 3.93†	380 ± 37	(Andreotti et al. 2017)
	Chlorella sp	-	-	96.6 ± 6.3	68.4 ± 5.0	896 ± 22	(Gao et al. 2018)
Dairy	*Scenedesmus quadricauda*	-	-	92.15	100[f]	360	(Daneshvar et al. 2019)
	Tetraselmis suecica	-	-	83.17	100[f]	650	(Daneshvar et al. 2019)
Piggery	*Chlorella vulgaris*	-	-	72.48 ± 10.50	86.93 ± 2.49	610 ± 40	(Sun et al. 2019)
	Neochloris aquatica	-	-	92.5 ± 1.0[a]	85.6 ± 0.3	6100	(Wang et al. 2017)
	Chlorella minutissima	-	-	82 ± 1	90 ± 2	2440[b]	(García et al. 2018)

	Acutodesmus obliquus	-	-	83 ± 3	91 ± 1	2140[b]	(García et al. 2018)
	Scenedesmus sp.	-	-	91.28	88.7	1340	(Jia et al. 2016)
Municipal	*Chlorella vulgaris*	-	-	5.03	100	470	(Álvarez-Díaz et al. 2017)
	Chlorella kessleri	-	-	4.66	100	380	(Álvarez-Díaz et al. 2017)
	Chlorella sorokiniana	-	-	6.25	100	740	(Álvarez-Díaz et al. 2017)
	Botryococcus braunii	-	-	13.71	100	1090	(Álvarez-Díaz et al. 2017)
	Neochloris oleoabundans	-	-	43.27	100	250	(Álvarez-Díaz et al. 2017)
	Scenedesmus obliquus	-	-	4.21	100	1420	(Álvarez-Díaz et al. 2017)
	Ankistrodesmus falcatus	-	-	10.73	100	590	(Álvarez-Díaz et al. 2017)
	Chlorella kessleri	-	-	>96	>99	2700 ± 80	(Caporgno et al. 2015)

	Chlorella vulgaris	-	-	~95	>98	2910 ± 20	(Caporgno et al. 2015)
	Nannochloropsis oculata	-	-	~47	>96	1050 ± 60	(Caporgno et al. 2015)
	Mixed consortia	-	-	97 ± 3[c]	47 ± 19	-	(Foladori et al. 2018)
	Asterarcys quadricellulare	-	-	61.21	80.88	433 ± 0.61	(Odjadjare et al. 2018)
	Neochloris aquatica	-	-	84.83	91.18	180 ± 0.26	(Odjadjare et al. 2018)
	Chlamydomonas sp.	-	-	100[d]	33	1500	(Wu et al. 2012)
Mixed	Mixed consortia	-	-	99.8	96.6	1470	(Chinnasamy et al. 2010)
	Scenedesmus obliquus	-	-	79 ± 0.3[d]	43 ± 1[f]	1200 ± 70	(Hernández-García et al. 2019)
	Desmodesmus sp.	-	-	82 ± 1[d]	41 ± 0.9[f]	1300 ± 100	(Hernández-García et al. 2019)

Combined							
Boiler flue gas/aquaculture	*Chlorella sp.* GD	2.333 g/L/d	Up to 53%	90[e]	99[e]	-	(Kuo et al. 2016)

*dissolved inorganic nitrogen

†dissolved inorganic phosphorus

[a] ammonium-nitrogen (NH_3-N)

[b] mg TSS/L

[c] total Kjeldahl nitrogen

[d] ammonia-nitrogen (NH_4^+-N)

[e] with 2% CO_2 aeration

[f] phosphate (PO_4^{3-})

MW = municipal waste

Nutrient requirements for the growth of algae have been determined to cost approximately 33% of total operating costs (Ventura et al. 2013). To address this, wastewaters (municipal, industrial, agricultural, or aquaculture/animal faming) with high nitrogen/phosphorus ratios, have shown promise as a growth medium substitute for microalgae (Salama et al. 2017; Guldhe et al. 2017). Reductions of nitrogen and phosphorus in wastewaters by microalgae have been shown to be around 95% and 98%, respectively (Caporgno et al. 2015), diminishing the need for costs related to supplementary nitrogen/phosphorus. In aquaculture wastewater, *Tetraselmis suecica* was shown to remove approximately 95% of dissolved inorganic nitrogen and dissolved inorganic phosphorus in as little as 2 days (Andreotti et al. 2017). On the other hand, *C. vulgaris* grown in piggery wastewater was able to remove approximately 73% total nitrogen and 87% total phosphorus (Sun et al. 2019). The integration of wastewater in a microalgae biodiesel stream could cut costs, but that alone would not make microalgae biodiesel economically feasible if the resulting microalgae have low lipid yields (<10%) (Chinnasamy et al. 2010). Thus, the addition of coproducts into a biodiesel biorefinery that utilizes waste streams such as waste CO_2 and wastewater could help further off-set overall costs.

2.5.2 Co-product feasibility

Due to the lack of co-products produced at large scale, and the ever-changing values placed on commodities in markets today, compiling genuine, present-day costs for numerous possible co-products is a challenge. The following examples provide, therefore, only estimations of potential retail value. For example, if a biorefinery could sell LEB as an animal or fish feed supplement, as a baseline the LEB mass added to the feed would generate the same amount of capital as the feed itself. With the commercial feed industry in Canada generating approximately CAD 4 billion annually (Animal Nutrition Association of Canada 2019), commercial feed enriched with 5% v/v LEB has the potential to generate an extra CAD 200 million in revenue, or approximately CAD 0.133 per kg. The farmed seafood industry in Canada spent approximately CAD 394 million on fish feed or around CAD 2.19 per kg (Canadian Aquaculture Industry Alliance 2018). Meanwhile, if omega 3 fatty acids can be fractionated from non-biodiesel lipids, a further increase in revenue could be achieved. A typical bottle of processed omega 3 tablets contains a total of 100 g of omega-3s and retails at around CAD 17.99 (jamiesonvitamins.com, 2020), which is equivalent to approximately CAD 360 per kg compared to CAD 1.39 per kg of biodiesel.

While composition and conversion efficiencies of microalgae biomass are critical factors for successful biorefineries (Laurens et al. 2017), environmental factors can also play an important role in decision-making (Romero-García et al. 2018). In order for the biorefinery approach to be environmentally sustainable, obstacles such as raw material usage (Adhikari and Pellegrino 2015), water- and land-usage, energy input, and greenhouse gas emissions from process related energy consumption need to be taken into account (Zhu et al. 2015). Low energy use and greenhouse gas emissions can be achieved by producing biodiesel using wastewater (Zhang et al. 2013) and anthropogenic CO_2 (Lara-Gil et al. 2016) when biomass yields are high. However, such outcomes vary widely depending on the feedstock used, conversion technologies, and land-use (Rathore et al. 2016).

The appropriate location of biorefineries with respect to waste streams could simultaneously cut costs and environmental impact. For example, a biorefinery placed near a wastewater facility and main product distribution centers could reduce greenhouse gas emissions (Nodooshan et al. 2018) and cost (Christenson and Sims 2011; Schoenung and Efroymson 2018), resulting from transportation of raw materials and final products.

Whilst several LCAs for biofuels, specifically for biodiesel, are to be found in the literature (Lecksiwilai et al. 2017; Collotta et al. 2017; Rajaeifar et al. 2019), it is important to recognize the varying natures and economic values of different co-products and how this will create various challenges (Wang et al. 2011). Numerous variables, including the number of co-products produced (Subhadra and Edwards 2011), biomass loading (Huang et al. 2018), co-product handling methods (Cai et al. 2018) and algal lipid content (Sander and Murthy 2010), can greatly impact the outcome of LCAs involving co-products. Additionally, LCAs that focus on the whole biorefinery system instead of being product-specific should be taken into account (Cai et al. 2018). Biorefineries for feedstocks other than microalgae, such as corn (*Zea mays*) (Shurson 2017), switchgrass (*Panicum virgatum*) (Snell and Peoples 2009), and a blended feedstock composed of corn stover and switchgrass (Davis et al. 2018a), have been proven to be economically successful as a result of co-products, proving further research to optimize this approach is worthwhile.

2.6 Conclusion

A higher cost for biodiesel compared to petrochemical sourced diesel can be attributed to several steps in the production process. Utilizing industrial waste CO_2 and nutrients in wastewaters could reduce the negative environmental impacts of other major industries, whilst simultaneously improving overall economics of biodiesel production. Furthermore, the concept of using lipid extraction to co-produce biodiesel and value-added lipid extracted biomass (LEB), or to help in the subsequent recovery of specific high-value compounds such as carbohydrates and proteins, represents another potential pathway to more economically viable production. With the bio-refinery concept, utilizing whole LEB, such as in animal feed, or extracting desired bioactive compounds, such as polyunsaturated fatty acids (PUFAs) for benefit to human health, make production more viable. To support this concept, it is recognized that further research into the effectiveness of co-product retrieval (i.e., extraction methods) and applications (i.e., optimizing economic return on production) is needed to enhance overall economic viability.

Chapter 3

3 Selection and re-acclimation of bioprospected acid-tolerant green microalgae suitable for exposure to industrial off-gas

3.1 Abstract

For mass culture of photosynthetic green microalgae, industrial flue gases can represent a low-cost resource of CO_2. However, flue gases are often avoided because they often also contain high levels of SO_2 and/or NO_2, which cause significant acidification of media to below pH 3 due to production of sulfuric and nitric acid. This creates an unsuitable environment for the neutrophilic microalgae commonly used in large-scale commercial production. To address this issue, we have looked at selecting acid-tolerant microalgae via growth at pH 2.5 carried out with samples bioprospected from an active smelter site. Of the eight wild samples collected, one consisting mainly of *Coccomyxa sp*. grew at pH 2.5 and achieved a density of 640 mg L^{-1}. Further, three previously bioprospected green microalgae from acidic waters (pH 3-4.5) near abandoned mine sites were also re-acclimated down to their *in-situ* pH environment after approximately 4 years spent at neutral pH. Of those three, an axenic culture of *Coccomyxa sp.* was the most successful at re-acclimating and achieved the highest density of 293.1 mg L^{-1} and maximum daily productivity of 38.8 mg L^{-1} day^{-1} at pH 3. Re-acclimation of acid-tolerant species is, therefore, achievable when directly placed at their original pH, but gradual reduction in pH is recommended in order to give the cells time to acclimate.

3.2 Introduction

Development of novel biotechnologies for the production of high-value, renewable bio-compounds is necessary to reduce the reliance on non-renewable resources. Photosynthetic green microalgae grown with industrial flue gas providing CO_2 as a carbon source and waste heat can be an ideal feedstock (Laamanen et al. 2017). Compared to terrestrial plants, microalgae have relatively short life-cycles (Eze et al. 2018) and much larger yields per unit area (Pankratz et al. 2019). They also have the potential to produce many bioproducts, including biofuels, and food supplements, (Khan et al. 2018), antibiotics (Senhorinho et al. 2019), biopolymers (Margesin and Schinner 2001) and bio-plastics (Kato 2019), all while using non-arable lands.

Photosynthetic green microalgae are very diverse organisms and depending on the species, are able to grow in freshwater, saltwater, or brackish water, as well as thrive at various temperatures (Darienko et al. 2015; Liu et al. 2016; Gonçalves et al. 2019; Kim et al. 2019) and light intensities (Mou et al. 2012; Deng et al. 2019). Genetically engineering these species to yield larger amounts of a desired biological compound is an attractive option (Arora et al. 2019), but there are still technical limitations as well as fundamental information lacking on wild-type microalgal strains (Fu et al. 2019).

New technologies are emerging that can use flue gases from various industries as sources of CO_2 (Laamanen et al. 2017). Unfortunately, using these CO_2 sources can create a wide range of new environmental stressors as flue gases can contain non-gaseous components (e.g., heavy metals and other particulate matter) that can discourage microalgae growth and, therefore, their ability to fix CO_2 (Santos et al. 2019). Though microalgae have been found to bioaccumulate some levels of metal contamination via intracellular mechanisms (Abinandan et al. 2019b), this depends upon temperature, pH, and metal concentration and species (Monteiro et al. 2012). Furthermore, flue gases can also contain high NO_x and SO_x concentrations (>100ppm), causing acidification when bubbled through liquid media (Zhao and Su 2014). Depending on the chemical make-up of the flue gas, pH changes of liquid media could be slight (from pH 7 to pH 6.4 using steel plant flue gas (Kao et al. 2014)) or extreme (from pH 7 to pH 2 using simulated flue gas (Jiang et al. 2013)). Due to this diverse range of stressors, in particular acidification, selecting individual species/strains tailored to particular industrial flue gas capture systems is likely required.

Acidification of the microalgal growth medium from bubbling in flue gases can cause a sharp decrease in productivity (Aslam et al. 2017), although it can be used as a stressor to induce the accumulation of targeted, valuable metabolites (Palacios et al. 2016; Soru et al. 2019). However, neutralizing the medium to accommodate commonly used neutrophilic microalgae, such as those from the genera *Chlorella* and *Chlamydomonas* (Larkum et al. 2012), will cause additional process complications, such as invasion by other species and costs from adding neutralizing chemicals, such as lime. Therefore, the use of acidophilic (typically cells with optimal growth at pH $\leq$ 4 (Johnson 1998; Hirooka et al. 2017)) or acid-tolerant cells could be more economically effective, as well as reducing the risk of undesired invasive species.

Acidified culture media could also discourage growth of contaminant organisms (Becker 1994) such as zooplankton, bacteria, and viruses (Day et al. 2012; Wang et al. 2013). Predation from zooplankton, such as rotifers, cladocerans, or ciliates, can annihilate dense microalgae cultures within 3 days (Moreno-Garrido and Cañavate 2001; Kumar and Sierp 2003). The use of different cultivation systems (Deruyck et al. 2019) and/or pesticides (Kim Hue et al. 2018) and other chemical treatments (Karuppasamy et al. 2018) can help alleviate some of the pressure from predators in a neutrophilic environment, but these solutions involve adding substances or modifying systems.

Acidophilic microalgae have been found to consume CO_2 equally as well as they would at neutral pH, albeit at lower bicarbonate concentrations (Piiparinen et al. 2018). Bio-fixation rates of CO_2 differ among acidophilic microalgae by being dependent on pH (Piiparinen et al. 2018), and CO_2 concentrations (Neves et al. 2019). Higher net photosynthetic rates were also observed in acidophilic *Chlamydomonas acidophila* compared to neutrophilic *Chlamydomonas reinhardtii* (Gerloff-Elias et al. 2005). Whereas, the production and accumulation of metabolites, such as carbohydrates (Tan et al. 2020) and lipids (Ruiz-Domínguez et al. 2015; Bermejo et al. 2018) in acidophilic microalgae show similar trends as neutrophilic microalgae under nutrient starvation. Whilst little is known about the primary producers in acidic environments (Hsieh et al. 2018), extremophilic microalgae, such as *Chlamydomonas acidophila*, *Euglena gracilis*, and *Coccomyxa onubensis* have been considered for industrial applications, such as recovery of metals and wastewater treatment (Malavasi et al. 2020).

This paper focuses on selection and acclimation of green microalgae to acidic conditions (pH ≤3), in order to obtain organisms that can be cultivated in low pH environments originating from the use of acidic flue gases as CO_2 sources. Growth dynamics and DNA-based phylogenetic analysis of previously bioprospected green microalgae from acidic orphan mine sites (Senhorinho et al. 2018) and recently bioprospected green microalgae from an active smelter site are examined.

3.3 Materials and methods

3.3.1 Bioprospecting of microalgae

Previous bioprospecting was completed in Northern Ontario, Canada (Eibl et al. 2014; Senhorinho et al. 2018) and forty microscopically-isolated strains were obtained. Of the forty isolated strains, three originated from low pH environments (*Coccomyxa sp.*M2 and *Chlamydomonas sp.*M23 from pH 3, and *Scenedesmus sp.*M5 from pH 4.5), which were chosen for this work. These strains had been kept at neutral pH after bioprospecting for long-term storage. As they had been stored for several years at neutral pH, their ability to acclimate back to their original environmental pH was examined.

Further bioprospecting was carried out at a metal smelter complex in Ontario, Canada. Water samples of 150 ml were gathered at eight different water bodies within 8 km of the operation, and pH was measured for each sample (Table 3.1). The samples were stored on ice while being transported to the laboratory where 100 ml of Bold's Basal Medium (BBM; pH 6.6) (Andersen 2005) was added to each sample to encourage microalgae growth. Samples with a measured pH <6.6 were modified using 1 M sulfuric acid to keep the sample at its natural pH. Unlike the previously bioprospected samples, these new samples were not grown outside of their natural pH, and microalgae were not isolated to promote the growth of a natural community. These samples would undergo low pH experimentation to understand the likelihood of selecting acidophilic or acid-tolerant microalgae from mine-impacted water bodies.

Table 3.1: Eight water samples bioprospected from an operational smelter site varied in pH due to their on-site functionality.

Sample ID	Site Description	pH
S1	Tailings from the acid plant	2.2
S2	Process water pond	7.5
S3	Process water pond	6.6
S4	Natural lake – limited smelter influence	8.4
S5	Natural pond – limited smelter influence	7.9
S6	Polishing pond - runoff from smelter	2.8
S7	Runoff from smelter	7.7
S8	Creek adjacent to smelter – limited smelter influence	6.8

3.3.2 Re-acclimation study

3.3.2.1 Cultivation at low pH

*Coccomyxa sp.*M2, *Chlamydomonas sp.*M23 and *Scenedesmus sp.*M5 were grown separately in the laboratory at room temperature (22 ± 2°C) without any constraints on pH after bioprospecting and isolating in 2014, when they were stored at neutral pH. An ability to re-adapt to their original low pH environment was essential for them to be considered for use in an acidic production environment. Each of the three strains were, therefore, independently divided and triplicated into separate, pH-controlled flasks, ranging from pH 7 to their original pH, in steps of 1 pH unit per flask. Flasks containing media with an uncontrolled pH were used as controls. Each flask began with a working volume of 450 ml of pH-modified BBM (using 1 M sulfuric acid) with a starting dry weight density of approximately 0.1 g/L. They were placed on a gyratory shaker (Model G2, New Brunswick Scientific Co.) at 125 rpm. No artificial lighting or CO_2 sources were added to aid in microalgae growth. Natural sunlight was measured adjacent to the shaker every five minutes using a silicon pyranometer (Onset Computer Corporation, Massachusetts, United States).

3.3.2.2 Cell growth analysis

For the isolates growing in the flasks, the dry weight biomass ($mg_{dw}\ L^{-1}$) was measured every three days over a growth period of three weeks. Biomass was measured by vacuum filtering 50 ml of algal culture using pre-weighed nylon hydrophilic filter paper (Fisher Scientific; GNWP0470), followed by oven drying at 60°C overnight. Once dried, the filter papers were re-weighed and the resulting dry weight biomass calculated.

Once the initial three-week period concluded, the surviving microalgae remained in their respective replicates and received new acidified BBM until the flasks were once again at 450 ml. In the following six months, pH was measured daily to ensure it would remain at the intended low pH, and the acidified media was renewed every three weeks by removing half of the volume in the flasks and adding back 100% acidified BBM into the flasks. At the end of the 6 months, a parallel three-week experiment was conducted to compare to previous growth results.

3.3.3 Selection study

3.3.3.1 Bioreactor set-up

For the selection study using the newly bioprospected microalgae, eight, 30 L, vertical gas-lift bioreactors were used. Each bioreactor, made from transparent Plexiglas (outer column diameter of 20 cm and a working depth of 100 cm), has a single sparger, located at the bottom, and attached to individual air lines. Further details of the bioreactors are outlined in Hosseini et al. (2015). In this study, only compressed air was bubbled into the system to avoid additional uncontrolled acidification by the gas.

3.3.3.2 Cultivation at low pH

After cultivating for one month, seven of the eight samples (S2-S8) showed signs of microalgae growth. Those samples were then lowered to pH 2.5 for another three weeks and only sample S6 showed good visible growth at pH 2.5 and considered for further analysis. 24 L of water from the S6 sampling site was, therefore, collected and evenly distributed across the eight bioreactors (3 L/tank; 10% starting volume). To ensure enough nutrients were supplied to the microalgae in the pond water, another 27 L of BBM was added to each bioreactor so that the resulting volume for each bioreactor was 30 L. No artificial light source was used and sunlight intensity was measured every five minutes using a silicon pyranometer. Three parameters were tested using the bioreactors, using a growth period of 18 weeks each: selection under no acidification (bioreactor control, henceforth referred to as cBR; technical duplicates), selection under acidification at pH 2.5 (aBR; technical triplicates), and selection under no acidification for eight weeks followed by acidification to pH 2.5 for the remaining experimental time (caBR; technical triplicates). The pH of the bioreactors was lowered using 1 M sulfuric acid and measured daily using a calibrated Oakton™ Waterproof Big Display pHTestr™ 30 (Fisher Scientific; CAT#13200263).

3.3.3.3 Cell growth analysis

Cell growth analysis within the bioreactors began after eight weeks of growth to ensure enough algal biomass present to be measured using dry weight biomass ($mg_{dw}\ L^{-1}$). Samples of 300 ml were centrifuged weekly at 4500 rpm for 5 minutes in pre-weighed 50 ml conical tubes. These samples were then stored at -80 °C until lyophilization at -40 °C and the tubes re-weighed. Daily productivities for the bioreactors and the isolated samples were measured based on the increase of dry weight biomass and expressed as $mg_{dw}\ L^{-1}\ day^{-1}$.

3.3.3.4 DNA extraction and sequencing

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen.com; CAT# 69104). For each sample, DNA purity and yield were determined spectrophotometrically using a 2-microliter low-volume-adapter on a Synergy HT microplate spectrophotometer (BioTek Instruments). PCR amplification of the 18S rRNA gene region was carried out on a LifePro thermocycler using the Phire™ Hot Start II DNA Polymerase kit (Fisher Scientific; CAT#F126S), forward primer 5' GCGGTAATTCCAGCTCCAATAGC 3' and reverse primer 5' GACCATACTCCCCCCGGAACC 3' (Aslam et al. 2017). DNA was denatured at 98°C for 30 minutes before completing 30 cycles of denaturing at 98°C for 5 minutes, annealing at 55°C for

30 seconds, and elongating at 72°C for 10 seconds, with a final extension of 72°C for 10 minutes and held at 4°C until amplicon size confirmation by electrophoresis could be completed. The PCR product from each sample was confirmed as a single product of ~300bp by agarose gel (1.2%) electrophoresis. Community amplicon sequencing was performed with paired-end (250bp each) Illumina MiSeq sequencing using phototrophic-specific 18S primers (Bradley et al. 2016) at Metagenom Bio Inc. (Waterloo, Canada).

3.3.4 Statistical analysis

Data presented are the averages ± standard errors (SE) within their respective technical replicates. Statistical analysis of the data was conducted in R (R Core Team 2018), and all graphical representations of the data were created using the R package ggplot2 (Wickham 2016). Community amplicon sequence data were processed using a DADA2 (Callahan et al. 2016) pipeline and taxonomic identification up to genus level was conducted using the SILVA v132 database (https://www.arb-silva.de). Unique amplicon sequence variants (ASVs) of interest were then further examined using the analysis tool BLAST and the NCBI GenBank database (NCBI Resource Coordinators 2016). The R package phyloseq (McMurdie and Holmes 2013) was used to plot relative abundances of genera in algal communities across the bioreactors, and to evaluate various indices: species richness, Pielou's evenness index, Shannon-Weaver index, and the Gini-Simpson index. The various indices were used to give a broader presentation of the trends between bioreactor types, for example the Shannon-Weaver index is more strongly influenced by species richness whereas the Gini-Simpson index gives more weight to evenness (Kim et al. 2017). Both the Shannon-Weaver index and the Gini-Simpson index were used to determine actual diversity within their respected bioreactors by calculating the effective number of species (ENS) (Jost 2006). Graphical representations of relative abundances show filtered ASVs with a mean greater than 10^{-5}.

3.4 Results

3.4.1 Re-acclimation at low pH

All three isolated microalgae were able to survive and grow at their original pH (3 - 4.5) (Figure 3.1), but dry weight biomass was lower when compared to the uncontrolled pH flasks (Figure 3.2). *Coccomyxa sp*.M2 and *Scenedesmus sp*.M5 produced the highest dry weight biomass at the uncontrolled pH (383.3 ± 16.7 mg L^{-1} and 436.7 ± 43.3 mg L^{-1}, respectively). Their highest dry weight biomass at their original pH being 280 ± 93.3 mg L^{-1} and 255.7 ± 29.4 mg L^{-1}, respectively. *Chlamydomonas sp*.M23 however produced its highest dry weight biomass at pH 7 on day 18 (379.8 ± 8.3 mg L^{-1}), whereas it only achieved 225 mg L^{-1} on day 21 at its original pH of 3. Unfortunately, two of the *Chlamydomonas sp.M23* triplicates for both pH 4 and pH 5 underwent an unknown stress approximately halfway through the growth period, drastically reducing the biomass and resulting in the lowest recorded densities. The differences in density at the different pH levels were found to be not statistically significant ($p>>0.05$, one-way ANOVA) within all three isolated microalgae.

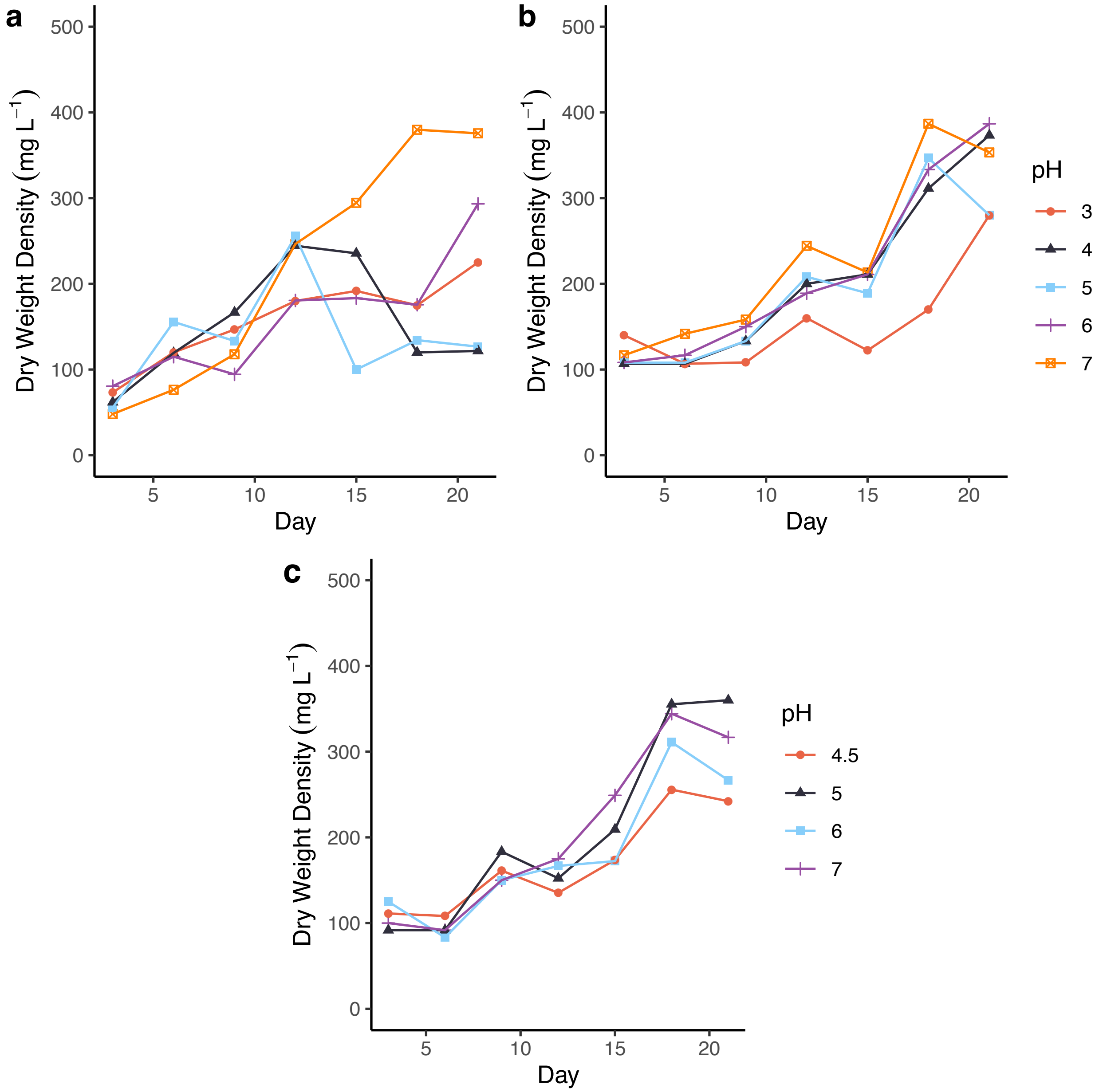

Figure 3.1: Dry weight densities (mg L^{-1}) of the isolated microalgae *Chlamydomonas sp.*M23 (a), *Coccomyxa sp.*M2 (b), and *Scenedesmus sp.*M5 (c), from pH 7 to their observed, original acidic pH. Error bars are not shown for clarity.

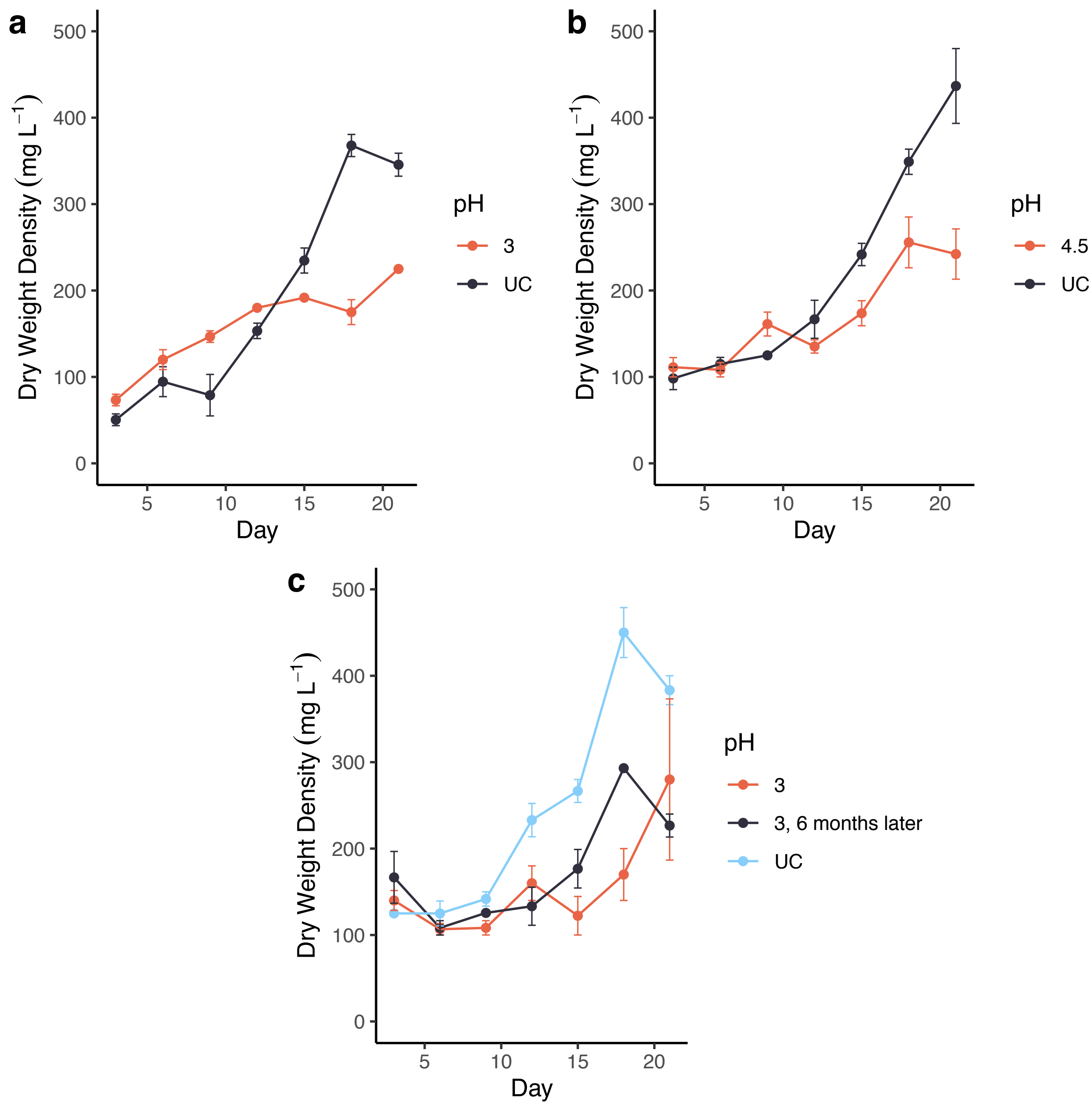

Figure 3.2: Dry weight densities ± SE (mg L^{-1}) at an uncontrolled pH and original pH of the isolated microalgae *Chlamydomonas sp*.M23 (a), *Scenedesmus sp*.M5 (b), and *Coccomyxa sp*.M2 (c). By day 21, the pH of the uncontrolled flasks had risen to an average pH of 8.7 for *Chlamydomonas sp*.M23, 9.0 for *Coccomyxa sp*.M2, and 9.1 for *Scenedesmus sp*.M5

The cell behaviour of *Chlamydomonas sp*.M23 changed at its original pH of 3. At neutral pH, *Chlamydomonas sp*.M23 cells were mostly individual and pelagic, whereas when at pH 3, they flocculated into large groups with mats forming on the bottom and sides of the flasks. This made it difficult to take non-bias samples for growth analyses and, therefore, these microalgae were not further explored. Throughout the experimental period, the pH in the uncontrolled flasks continuously rose each sampling day to a final average pH of 9.0 ± 0.21 for *Coccomyxa sp*.M2, 9.1 ± 0.12 for *Scenedesmus sp*.M5, and 8.7 ± 0.14 for *Chlamydomonas sp*.M23.

During the growing period, *Coccomyxa sp*.M2 presented the highest daily productivity of 61.1 $mgL^{-1}day^{-1}$ on day 18 at an uncontrolled pH. Whereas *Scenedesmus sp*.M5 obtained its highest daily productivity of 48.6 mg L^{-1} day^{-1} on day 18 at pH 5. At its original pH of 3 and 4.5 respectively, *Coccomyxa sp.*M2 and *Scenedesmus sp*.M5 showed highest daily productivities on day 21 (36.7 mg L^{-1} day^{-1}) and day 18 (27.3 mg L^{-1} day^{-1}), respectively.

Over the following 6 months, before measuring growth a second time, *Scenedesmus sp.*M5 at its original pH began to exhibit changes (flocculating, preferring benthic growth), similar to that of *Chlamydomonas sp.*M23. Due to this and its higher original pH (4.5), *Scenedesmus sp*.M5 was not considered for further experimental analyses. Therefore, at the 6-month point, only *Coccomyxa sp*.M2 underwent another growth analysis, during which it was able to achieve 293.1 mg L^{-1} on day 18, and a maximum daily productivity of 38.8 mg L^{-1} day^{-1}.

Throughout the experimental period, the intensity of sunlight varied (Figure 3.3) due to changes in the season and weather. Time periods were divided into 12-hour photoperiods, with average daytime intensity recorded between 7 am - 7 pm. The lowest average value measured during the night period was 0.6 W m^{-2}, which was due to moonlight and/or indoor background lighting. The higher average light values at night are due to longer summer month days, where sunrise and sunset are both included in the nighttime period of 7 pm to 7 am. The goal was to demonstrate that lighting was variable, and that subsequently no correlation was found between solar irradiance and density or productivity (p>>0.05).

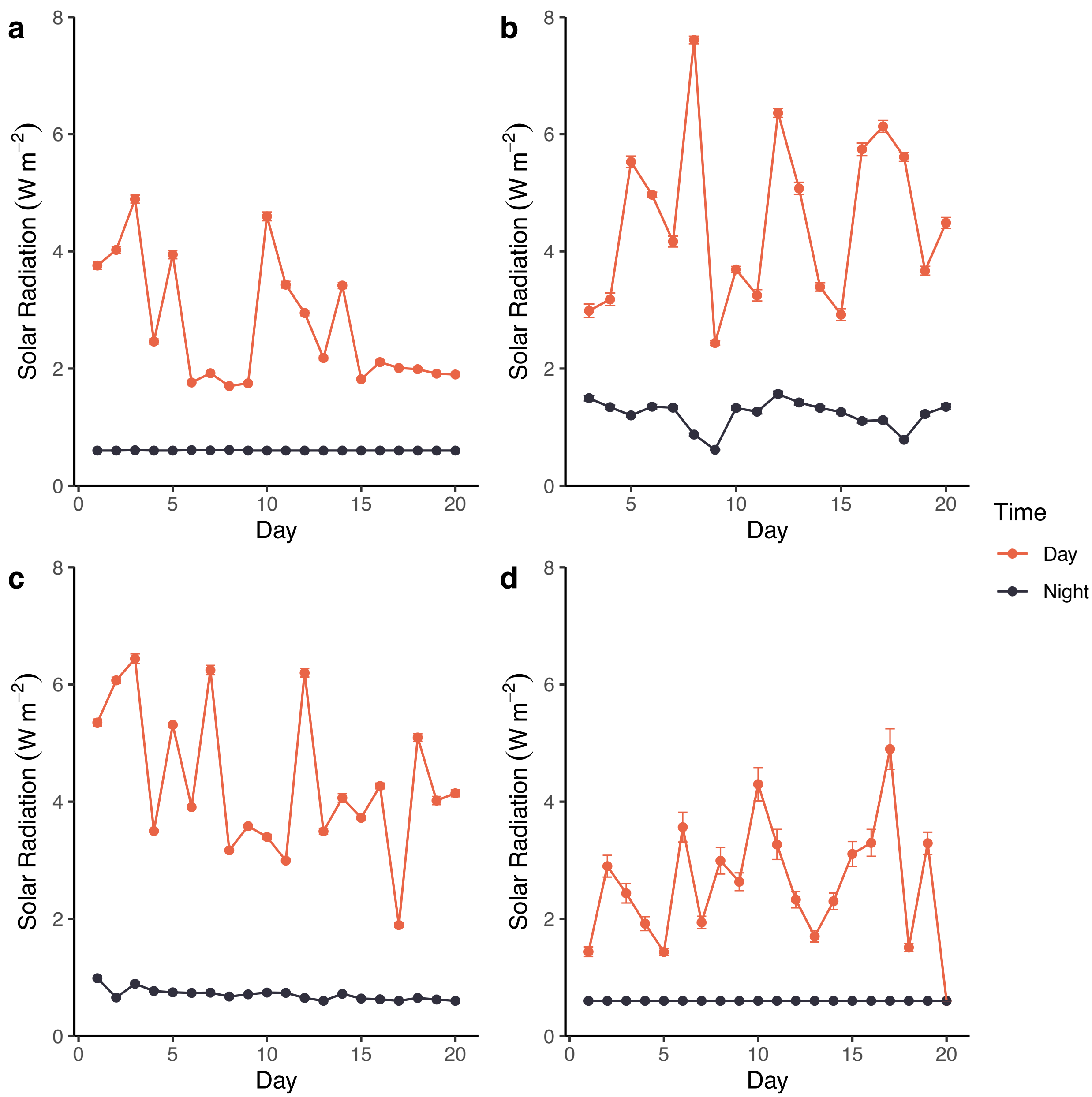

Figure 3.3: Average intensity of solar radiation for each day and night of the experimental period for a) *Chlamydomonas sp.*M23, b) *Scenedesmus sp.*M5, c) *Coccomyxa sp.*M2, and d) *Coccomyxa sp.*M2 after 6 months

3.4.2 Selection at low pH

For the first 8 weeks of the bioreactor use, only visual observations of growth were made for all the bioreactors. This was done to allow cells within the bioreactors enough time to proliferate by not taking away any cells for growth analyses. Within the first 8 weeks, growth was only noticeable in cBR and caBR and, therefore, only samples from those bioreactors were taken for growth analyses and DNA extractions. By the 13-week sampling period, enough microalgae grew in one of the replicates representing aBR to remove biomass for growth analyses and DNA extractions. Unfortunately, with the remaining 2 replicates for aBR, algal mats started to appear within the bioreactors. No samples were taken from these bioreactors until the end of the experiment when the bioreactors were emptied, as this allowed the biomass mats to be swabbed for DNA extraction. Henceforth, these bioreactors are collectively referred to as bBR.

cBR experienced no artificial pH changes and densities increased with time, exhibiting a slight exponential growth curve, and achieving the highest density on week 16 at 810 ± 2.6 mg L^{-1} (Figure 3.4). Biomass densities for aBR steadily increased once the microalgae became acclimated to the low pH (2.5) environment and reached a maximum of 640 mg L^{-1} by the end of the experimental period. The biomass in caBR reached its highest density (634.5 ± 15.3 mg L^{-1}) prior to the drop in pH at week 8, and steadily decreased over the weeks following acidification until it reached its lowest density of 440 ± 70.2 mg L^{-1} at week 16.

Figure 3.5 shows that community composition at the genus level varied by bioreactor type at the end of the experimental period. In total, there were 161 distinct ASVs (after removing chimeras) found throughout the sample period. At the end of the experimental period, similar community structures by genus were present in both the control bioreactor (cBR) and the acidified bioreactor (aBR), with a marked increase of protozoa in aBR. The green microalgae present in both the bioreactors (>99% abundance in cBR and >91% abundance in aBR) are represented by a singular taxon (i.e., one ASV). Taxonomic identification at the species level using BLAST identified this ASV as *Coccomyxa sp*., but further species identification was complicated due to its similarity to various strains of *Coccomyxa sp.,* including *Coccomyxa elongate*, *Coccomyxa simplex*, and *Coccomyxa subellipsoidea*. The community structure of caBR was populated almost entirely (>99% abundance) by a single ASV in the green microalgae genus *Chlorella*. The taxonomic identification at the species level using BLAST identified it as *Chlorellaceae sp*. (Sequence ID MF070534.1) with a 99% identity, 0% gaps, and an e-value 4e-166, which is an unclassified, bioprospected green microalga. Interestingly, the bioreactors that exhibited different growing behaviour (benthic growth; bBR) displayed higher fungal and protozoan contributions to the community compared to the other three bioreactor types. Meanwhile, there were no significant differences in diversity (Table 3.2) among bioreactors and time ($p>>0.05$, Kruskal-Wallis). Gini-Simpson diversities and Shannon-Weaver diversities were strongly and positively correlated with each other (rho = 0.991, $p<<0.05$, Spearman's rank correlation) with no significant differences among reactors ($p>>0.05$, Kruskal-Wallis). Richness (operationally defined as ASV richness) increased over time for all three experimental bioreactors, of which aBR had the highest increase of 6 species. The variations between the diversity values and evenness suggests a degree of dominance within all four communities.

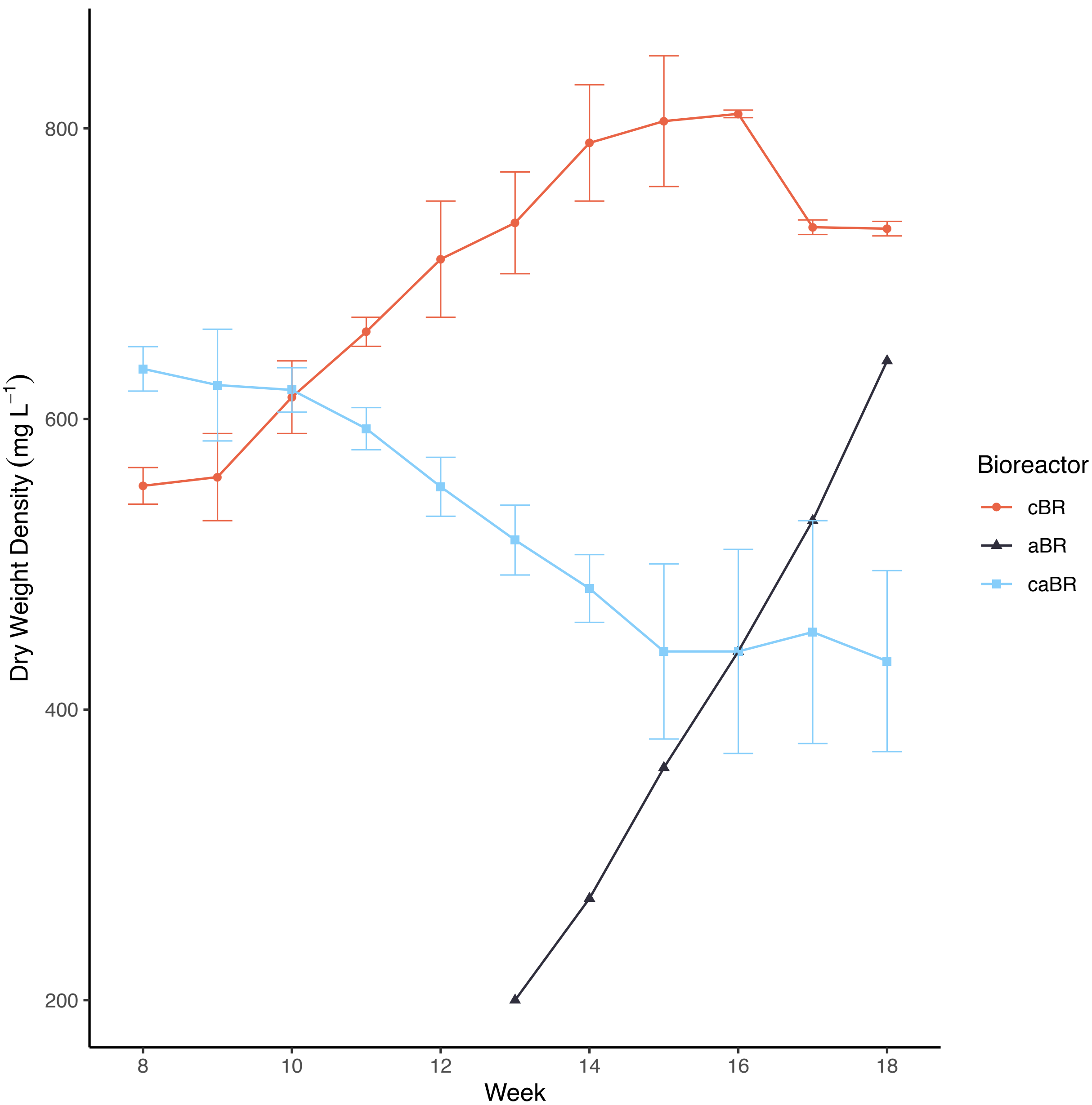

Figure 3.4: Biomass densities (mg L-1) for each experimental bioreactor; results shown are the means ± standard error of their respective replicates. Bioreactor aBR was originally triplicated, however only one produced pelagic microalgae, which is the suitable growing parameter for the bioreactors used. Therefore, data was measured solely from that bioreactor.

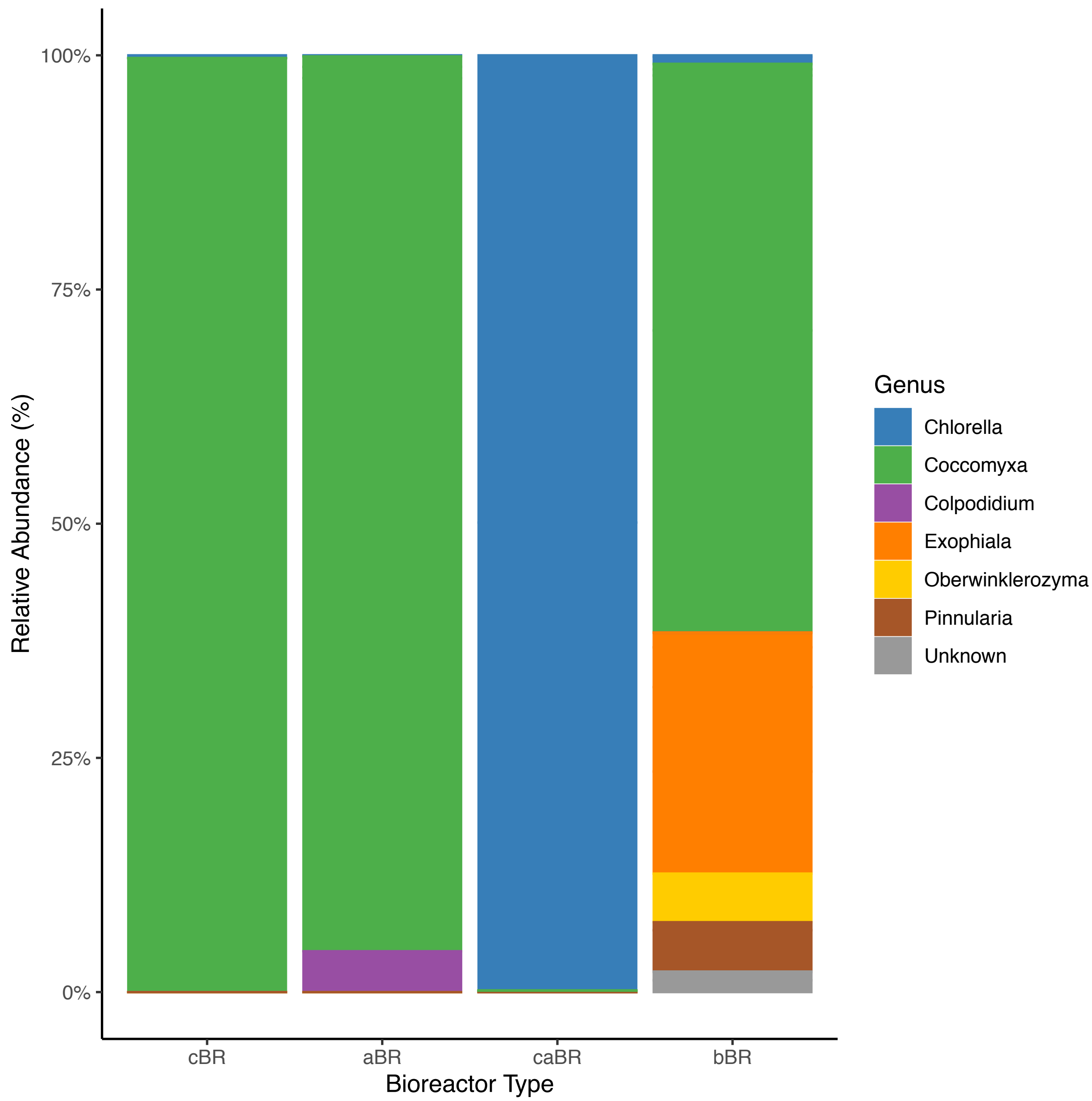

Figure 3.5: Relative abundances. (based on ASVs with a mean greater than 10-5) for each experimental bioreactor type (control, cBR; acidified, aBR; control + acidified, caBR) at the end of the experimental period (week 18), including the bioreactors that exhibited benthic growth (bBR).

Table 3.2: Diversity indices (Shannon-Weaver, Gini-Simpson, species richness, and evenness) and actual diversity (calculated as ENS; Shannon-Weaver and Gini-Simpson) for each bioreactor type at the beginning of their respective sampling periods and at the end of the sampling period. Bioreactors bBR were only swabbed once at the end of the experimental period and therefore have only one result. No statistically significant differences were found.

Bioreactor Type	Experimental Week	Richness	Evenness	Shannon-Weaver Index	Gini-Simpson Index	Shannon-Weaver Diversity	Gini-Simpson Diversity
cBR	8	6	0.045	0.099	0.027	1.104	1.028
	18	9	0.027	0.059	0.016	1.061	1.017
aBR	13	7	0.164	0.318	0.122	1.375	1.139
	18	13	0.171	0.438	0.163	1.550	1.194
caBR	8	13	0.041	0.101	0.028	1.109	1.029
	18	17	0.078	0.257	0.101	1.359	1.139
bBR	18	30	0.420	1.429	0.618	4.194	2.658

3.5 Discussion

3.5.1 Re-acclimation at low pH

While growth was reduced when all three isolated microalgae were exposed to the low pH of the waterbody they were bioprospected from, an increase in density over time was still obtained. Interestingly, the maximum daily productivity for *Coccomyxa sp.*M2 at pH 3 for 6 months was higher and achieved 3 days earlier (38.8 mg L^{-1} day^{-1} on day 18) than those grown directly at pH 3 (36.7 mg L^{-1} day^{-1} on day 21), indicating the need for an acclimation period. Borowitzka (2018) describes the acclimation period as a time when microorganisms allow for changes in gene expression in response to an environmental change. If these changes are tolerated, then the microorganism can adapt to the new environment, but if not homeostasis will not be restored, resulting in cell death or, in some cases, stress-avoidance life-cycle changes (Borowitzka 2018).

Furthermore, the microalgae were grown in a more natural environment than typical lab strains (i.e., no artificial light or additional CO_2 sources) and the lack of CO_2 and lighting at a variable intensity most likely resulted in lower growth rates (Jiang et al. 2013; Mandotra et al. 2016). Microalgae grown at low pH with a CO_2 source such as *Scenedesmus parvus* (pH 3, 15% CO_2; 78 mg L^{-1} day^{-1}) (Tan et al. 2020), and *Coccomyxa onubensis* (pH 2.5, 2.5% CO_2; 90 mg L^{-1} day^{-1}) (Fuentes et al. 2020), demonstrate that the maximum daily productivities shown in this study have the potential to increase under non-natural environmental conditions.

Similar to our results, it has been found that microalgae that grow ideally at neutral conditions have been also shown to grow at low pH (≤ 3), albeit at lower densities (Abinandan et al. 2019a). Acidophilic microalgae strains have been reported to be closely related to their neutrophilic relatives, suggesting a rather fast adaptation from neutral to acidic environments (Zettler et al. 2002). Since our microalgae grow ideally at neutral conditions, but survive at low pH, they can be considered acid-tolerant. While the three bioprospected microalgae appeared to acclimate when placed directly into their original, lower pH, it would most likely be beneficial to allow them to progressively acclimate through step-wise pH changes down to the target pH. This could potentially allow the strains to more easily adapt into becoming acidophilic, rather than acclimating to become acid-tolerant.

During our acclimation experiment, the pH of the media was constantly increasing and needed to be altered daily to keep it at the desired experimental pH. External alkalization by microalgae has been attributed as a homeostatic response to acidic conditions (Fukuda et al. 2011) or due to growth by consuming HCO_3^- as their primary carbon source (Beltrán-Rocha et al. 2017), and can be associated with an increase in phosphate and 2,6-diaminopimelate metabolites (Guo et al. 2017). An increase in pH was noted at all levels of our experimental conditions, regardless of the starting pH and this alkalization of the medium may be also attributed to an increase in nitrate uptake, which in turn will release ammonium cations into the medium (Ullrich et al. 1998). Future considerations of operational conditions could be the addition of CO_2 gas when the microalgae are actively photosynthesizing, which would help decrease the amount of HCO_3^- consumed by the microalgae and therefore reduce the amount of alkalization of the medium.

Interestingly, a steep decline in medium pH led to flocculation of *Chlamydomonas sp.*M23 and *Scenedesmus sp.*M5, though as they were affected at different times, this suggests a species-specific stress response. Various summarized reports (González-Fernández and Ballesteros 2013) indicated strain specific flocculation due to a high external pH of 8-12. A small decrease in pH (~1 unit) was also found to induce flocculation of *Pleurochrysis carterae* and *Emiliania huxleyi* (Moheimani and Borowitzka 2011). Reducing the pH of the growth media has also had an effect on microalgae harvesting, with reports of flocculation after reducing pH from neutral to 3 (Aljuboori et al. 2016) and improved cell removal at pH 2 (Laamanen and Scott 2017). At these low pH values, the cell wall's negatively charged surface begins accepting more protons, reducing its charge and forming flocs (Aljuboori et al. 2016). Flocculation efficiency at low pH increases as biomass concentrations increase (Liu et al. 2013), but as seen in this work, and also described by Pérez et al. (2017), induced flocculation appears to be species specific.

3.5.2 Selection at low pH

Using acidophilic, or acid-tolerant, microalgae could aid the production of microalgal products utilizing the CO_2 in acidic industrial waste gases. We recommend looking at microalgae growing on the industrial site, and select for acidophilic/acid-tolerant species and lowering media pH to the expected pH of media infused with flue gas. For example, we found that microalgae taken from a polishing pond on an active smelter site (approximately pH 3 at the time of sampling) are acid-tolerant, as their densities at neutral pH exceeded those at pH 3. This is an outcome similar to the microalgae that underwent re-acclimation from pH 7 down to pH 3. This was expected as the pH in the polishing pond was found to be constantly changing between pH 2.6 to pH 6.1 throughout the year. However, it is worth noting that the densities of microalgae found in bioreactor aBR continued to increase after the cells became acclimated to their acidic environment (during week 13 of the experimental period), and, therefore, have the potential to be acidophilic.

Interestingly, *Coccomyxa sp*. was identified as a good acid-tolerant candidate for growth at low pH during the re-acclimation and selection experiments. Hirooka et al. (2017) found numerous genetic adaptations, such as lost fermentation pathways, increased arsenic detoxification genes, and increased heat-shock proteins, that were present in the acidophilic *Chlamydomonas eustigma* but not in their neutrophilic relative *Chlamydomonas reinhardtii.* While it remains unclear if both species are identical or if they are unique, given that they come from similar environments, it is plausible to assume that these acid-tolerant species were once neutrophilic species before mining activity started in the area. Darienko et al. (2015) concludes the use of numerous barcode regions (in particular, V4, V9, and ITS2) are necessary for identifying Coccomyxa species, and, therefore, a more expansive genetic analysis than18S is required for a more definitive species confirmation of bioprospected samples.

The differences among diversities and species richness/evenness indicates a strong dominance of one effective species within the experimental bioreactors, which increased when the environment suited benthic growth (shown in bioreactor bBR). The community structure of the bioreactor bBR demonstrated the highest richness, which was mainly attributed to the increased presence of fungal and protozoan taxa. Manipulation of light, particularly blue light (460nm) (Hultberg et al. 2014), was found to activate the formation of a *C. vulgaris* biofilm. However, as all of the bioreactors in our work were subject to the same natural lighting, the biofilm present in bBR were most likely produced via competitive interactions between species. Similar results of a biofilm occurring were found with various bacteria and diatoms present during the growth of *Tetraselmis sp*. strains (Isdepsky and Borowitzka 2019) and, therefore, the increased presence of the taxa found in bBR may have initiated benthic growth. Nutrient supplementation and herbivore presence have also been shown to significantly affect microalgae assemblages when taken from the same pool of species (Rodrigo et al. 2009). Since our bioreactors began with the same amount of nutrients and no predator species were found by DNA analysis, our small changes in diversity could be attributed to pH; however, since our starting samples were from a natural environment, albeit from the same pond, changes in diversities could also be attributed to how the samples were acquired (i.e., individual samples could have different concentrations of the same microorganisms).

The communities found in the bioreactors cBR (control), aBR (acidic), and caBR (control + acidic) shared a common structure in which one microalgae genus and species remained the dominant microorganism (>91% abundance) over various fungal and protozoan microorganisms. Unlike cBR and caBR, the acidic bioreactor, aBR, showed a slight increased abundance (>5% abundance) of protozoan from the genus *Colpodidium*. As these protozoans originate from the same environment as the microalgae, and since our bioreactors are in a controlled, indoor environment, it is likely they co-evolved with the microalgae. Therefore, the increase in protozoan abundance at low pH may not be a contamination, and that this predatory pressure may actually help increase microalgae growth. Nevertheless, an acidic environment will most likely avoid or reduce other contaminant species that are not acid-tolerant.

The community structure found in bBR consists of a similar structure to that found in acid mine drainage (AMD) ponds, which consist of microalgae, fungi, and protozoans (Dean et al. 2019). Biofilms tested in and around AMD ponds (pH 2.8-2.9) have been found to have higher dominance of protozoans, compared to samples taken from a flowing stream that had a higher dominance of filamentous green microalgae (Prasanna et al. 2011). Mutualistic interactions between bacterium and green microalgae species has proven to be more beneficial in biotechnological processes involving high CO_2 capture (Choix et al. 2018). Warmer water temperature (~25°C) has been also shown to positively influence predator development within a microalgae system (Galès et al. 2020), but this is less likely with this work as the bioreactors were indoors and at a constant temperature (22°C).

3.6 Conclusion

Whilst it may be preferable to maintain bioprospected microalgae at the pH of the environment they were obtained, re-acclimation of those stored at neutral pH can nevertheless successfully develop acid-tolerant microalgae. These are of particular interest for use in acidic media caused by sparging in industrial flue gases containing CO_2, but also contain other acid gasses, such as SO_2 nd NO_x that can reduce media pH <4. Re-acclimation of one algal species, *Coccomyxa sp.M2*, down to pH 3 from long-term storage at neutral pH showed lower, but continual growth when compared to growth at neutral pH. Whereas, two species, *Scenedesmus sp.M5* and *Chlamydomonas sp.M23*, demonstrated operationally challenging behaviour (i.e., establishment of benthic growth) when returned from neutral pH to their bioprospected pH of 3 during a three-week growing period. Longer acclimation periods and/or a stepwise drop in pH are suggested for future testing.

Of the eight bioprospected samples taken from waters at an active smelter site, microalgae from one water body (S6) grew successfully at pH 2.5. However, differences in community structures and species diversity were observed when S6 was placed in different pH environments. Overall, *Coccomyxa sp.* was the dominant species found when sampled at pH 2.5 and at neutral pH. Whereas, *Chlorellaceae sp.* was the most abundant in an environment that was lowered to pH 2.5 after 8 weeks growing at neutral pH. Due to the differences in community structure shown in bioreactor caBR (control + acidified) compared to bioreactor aBR (acidifed), it is recommended to grow wild samples only at the target pH, to avoid contamination from other microorganisms.

Chapter 4

4 Dark stress as means to increase lipid content and improve fatty acid profiles of an acid-tolerant microalgae exposed to gas containing 6% CO2

4.1 Abstract

The use of wastewaters and flue gasses as low-cost and environmentally-friendly sources of nutrients and CO_2 for the cultivation of microalgae for lipids has become an increasingly researched option. However, these approaches present a challenge, as whilst flue gasses typically contain 6-10% CO_2, excessive medium acidification can be caused by the presence of NO_x and SO_2 in the gas. The use of acidophilic or acid-tolerant species is a possible solution, but little is known about these microalgae. In this study, we investigate the growth of a bioprospected acid-tolerant mixed photosynthetic green microalgae culture (91% *Coccomyxa sp.*) at pH 2.5 and fed with gas containing 6% CO_2. At the end of exponential phase, lipid accumulation and its profile, along with the elemental composition of the biomass was analysed over one week during which biomass was exposed to either continued light-dark cycle conditions or continual dark conditions, the latter as an inexpensive method for applying stress. After three days of dark stress, the biomass consisted of approximately 28% of lipids, which was 42% higher than at the end of the exponential phase. Oleic acid (C18:1), pentadecanoic acid (C15:0), and palmitic acid (C16:0) were the dominant fatty acids at the end of the exponential phase, and light-dark and dark-treated biomass, respectively. Overall, dark conditions favoured polyunsaturated fatty acid

production and showed an increase in nitrogen content. This suggests the use of dark stress to stimulate production of desirable high-value lipids as a low-cost alternative to other commonly used stressors.

4.2 Introduction

Photosynthetic green microalgae are a diverse group of microorganisms often used to produce lipids for biofuels (Gozmen Sanli et al. 2020) or human health products (Lupette and Benning 2020). While microalgae biomass has the advantage of large yields per unit area (Saranya and Shanthakumar 2020) and the ability to grow on non-arable land, their high production costs put them at a disadvantage when compared to other renewable and non-renewable resources, such as terrestrial plants or petrochemical oils. Various solutions to reducing the costs of cultivating microalgae at large scale include utilizing a biorefinery approach (Bhattacharya and Goswami 2020; Desjardins et al. 2020), developing low-cost photobioreactors (Erbland et al. 2020), and using industrial wastes, such as wastewater (Kotoula et al. 2020) and flue gas (Laamanen et al. 2017; Cheng et al. 2019) as sources of nutrients and CO_2. In particular, the use of industrial flue gases as sources of CO_2 has been shown to be effective, including from coal-fired boilers from a beef processing plant (Aslam et al. 2019), soda boilers at a pulp and paper mill (Ekendahl et al. 2018), and flue gas from a combined heat and power plant (Gentili 2014).

However, the presence of NO_x and SO_2 in flue gases can cause acidification of the growth media, and therefore use of acidophilic or acid-tolerant microalgae species could be crucial for successful cultivation in these systems. In recent years, there has been increasing research towards understanding the biology and ecology of acidophilic and acid-tolerant species, with species diversity at low pH being higher than previously assumed (Hsieh et al. 2018). As acidophilic green microalgae species evolved from their neutrophilic relatives (Hirooka et al. 2017), they have been found to comparably acquire and accumulate metabolites, such as carbohydrates and lipids (Ruiz-Domínguez et al. 2015; Tan et al. 2020). Although acidic conditions are not widely used in current, non-flue gas using, commercial large-scale microalgae cultivation, a reduced pH environment could possess the ability to reduce contamination by non-target species (Hirooka et al. 2020).

Accumulation of high-target bioactive compounds have consistently been shown to increase under nutrient starvation (Srinuanpan et al. 2018), increased irradiance (Solovchenko et al. 2013), and increased salinity (Pancha et al. 2015). However, each of these stress-inducing environmental variables have a common disadvantage – high cost at large-scale (Sui et al. 2020). Generally, there are two techniques to induce the accumulation of bioactive compounds. The first uses a two-stage cultivation process, where microalgae are grown to late exponential or early stationary phase under suitable growing conditions and then are put under environmental stress for a short duration. This encourages the cells to increase certain secondary metabolites before harvesting. The second approach is to use a stress/growth process where the microalgae are grown in a stressed environment promoting cells to increase target metabolite production throughout their growth. Whether adding a variable or taking one way, each stressor represents an additional process cost, including through an impact on subsequent downstream processing (Ananthi et al. 2021).

One approach to inducing the accumulation of target metabolites through a low-cost environmental stressor that does not affect downstream processes could be dark stress. Interestingly, many studies explore light stress as either differing LED wavelengths (Jung et al. 2019), or as differing light intensities (Iasimone et al. 2018), but very few explore dark stress. Efficiently utilizing light sources without increasing energy has been found to increase lipid content by 16% (Hosseini et al. 2018c), whereas a 2-fold increase in total lipids was found when microalgae were placed in constant dark (Bai et al. 2016).

In this study, we focussed on the impact of applying dark stress on the lipid profile of a mixed bioprospected microalgae culture dominated (91%) by the photosynthetic, acid-tolerant green microalgae *Coccomyxa sp*. Growth curves under a normal growing environment (pH 2.5, 12-hour photoperiod, 6% CO_2) were obtained over exponential growth, and lipid accumulation and profiles then studied between light-treated cells and dark-treated cells.

4.3 Materials and methods

4.3.1 Microalgae cultivation

A microalgae consortium was bioprospected from an active mine site and genetically identified to be predominantly (91%) acid-tolerant *Coccomyxa sp*. while grown at pH 2.5. The remaining 9% is made up of other green microalgae, protozoa, and fungi species. The microalgae were inoculated in 2 L flasks (triplicated) containing 1.2 L of acidified (pH 2.5) Bold's basal media (BBM; (Andersen 2005)) with a starting density of approximately 100 mg L^{-1} dry weight biomass. The pH of the flasks was lowered from neutral pH using 1 M sulfuric acid and measured daily using a calibrated Oakton™ pHTestr™ 30 (Fisher Scientific; CAT#13200263). The flasks were placed on a gyratory shaker (Model G2, New Brunswick Scientific Co.) at 125 rpm and maintained under red and blue LEDs (approximately 22 μmol s^{-1} m^{-2}) with a 12-hour light/dark cycle. A simulated flue gas mixture of 6% CO_2 and 94% N_2 was continuously bubbled through the flasks for eight hours, starting at the beginning of the 12-hour light photoperiod.

Daily biomass productivity was measured based on the daily increase of dry weight biomass throughout the growth period and expressed as mg_{dw} L^{-1} day^{-1}. Average biomass productivity (mg_{dw} L^{-1} day^{-1}) was measured based on the increase of dry weight biomass from the beginning of the growth period to the end of exponential phase, and specific growth rate (day^{-1}) calculated using Eq. (1):

$$\mu = \frac{\ln^{X_2}/_{X_1}}{t_2 - t_1} \qquad (1)$$

where X_1 and X_2 are the biomass densities at the beginning and end of the exponential phase, respectively, t_1 and t_2 are the time in days at which exponential phase begins and ends, respectively.

Individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed from the flasks once the highest density was achieved (end of exponential phase), to be used as controls. The CO_2 biofixation rate was calculated for the control sample using Eq. (2):

$$CO_2\ biofixation\ rate\ (mg\ L^{-1} day^{-1}) = PC_{carbon} \left({M_{CO_2}}/{M_C} \right) \qquad (2)$$

where P is the average biomass productivity, C_{carbon} the carbon content, which was determined by elemental analysis (described in section 2.4), M_{CO2} the molecular weight of CO_2, and M_C the molecular weight of carbon.

4.3.2 Microalgae under light-dark and dark conditions

After exponential phase had been reached under the same growing conditions as previously mentioned (12-hour light/dark cycle, pH 2.5, 6% CO_2), the biomass experienced either light-dark treatments or dark treatments. During light-dark treatments, the biomass remained under 12-hour light/dark cycle with 6% CO_2 for a subsequent 7 days. Individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed on days 1, 2, 3, 4, and 7. These samples represent ordinary conditions befitting cells during stationary and/or decline phase, and were used for comparison against the dark-treated cells. All samples were stored at -80°C until the appropriate analysis was conducted.

During dark treatment, the cells were placed on a gyratory shaker at 125 rpm under constant darkness for a subsequent 7 days. Unlike the flasks exposed to the normal 12-hour light/dark cycle, these flasks did not receive any CO_2 since photosynthesis does not occur in the dark. After the flasks were placed in the dark, individual samples for lipid analysis (duplicated) and elemental analysis (duplicated) were removed from the flasks on days 1-4, and 7. All samples were stored at -80°C until the appropriate analysis was conducted.

4.3.3 Lipid and elemental analysis

For lipid analysis, frozen, wet samples were sent to Lipid Analytical Laboratories Inc. at Guelph University, Ontario, Canada. Lipids were extracted based on the Bligh and Dyer method (Bligh and Dyer 1959), which were then quantified via transmethylation (Morrison and Smith 1964). Analysis of the fatty acid methyl esters was completed using an Agilent 7890B gas-liquid chromatograph with a 60 m DB-23 capillary column.

For elemental analysis, samples were lyophilized and sent to the Perdue Central Analytical Facility at Laurentian University, Ontario, Canada, who used a Thermo Scientific^{TM} FlashSmart^{TM} elemental analyzer with a 2m PTFE column.

4.3.4 Statistical analysis

Data presented are the averages ± standard errors within their respective technical replicates. Statistical analysis of the data was conducted in R (R Core Team 2018), and all graphical representations of the data were created using the R package ggplot2 (Wickham 2016). The statistical significance threshold was placed at 0.05.

4.4 Results

4.4.1 Microalgae growth

The *Coccomyxa sp*. consortium showed atypical exponential growth (Figure 4.1), where the lag phase included two reductions in density and where the exponential phase included varying slopes until it reached the highest biomass density (550 ± 36.1 mg L^{-1}), which was observed on day 19. The largest increase in biomass was observed between day 17 and 18, with a daily productivity of 130 mg L^{-1} day^{-1}. The average biomass productivity was 25.71 mg L^{-1} day^{-1} and the average CO_2 biofixation rate was 44.64 mg L^{-1} day^{-1}. The exponential phase was determined to be between days 9 and 19, with a specific growth rate of 0.136 ± 0.004 day^{-1}.

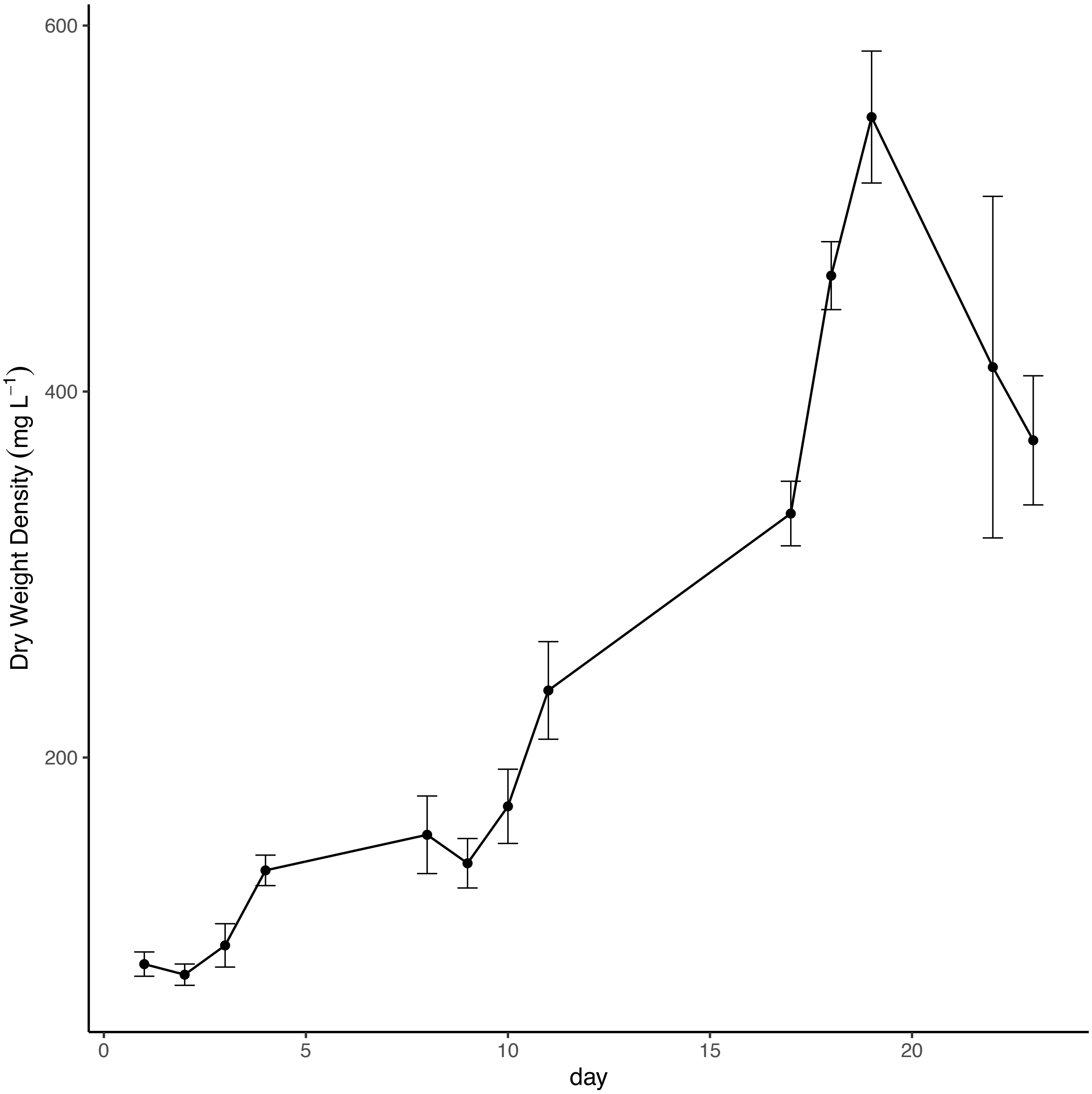

Figure 4.1: Growth curve (mean ± standard error) of the *Coccomyxa sp*. consortium under a 12-hour light/dark cycle and 6% CO_2.

4.4.2 Lipid accumulation after exponential phase

After the exponential phase, the highest total lipid content accumulated by light-dark treated cells occurred after 7 days of exposure (17.81 ± 1.04%) whereas continual dark-treated cells accumulated more total lipids after only 3 days of exposure (27.67 ± 9.92%) (Figure 4.2).

Interestingly, dark treatment resulted in a larger ratio of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs; 19.47% and 21.04%, respectively) whereas 7-days of light-dark treatment had a reduction in the ratio of omega-3s and omega-6s (1.94% and 9.53%, respectively). The main fatty acids found in both treatments are outlined in Table 4.1. While there were no significant differences between the treatments and the types of fatty acids (two-way ANOVA, $p = 0.98$), there were significant differences between saturated fatty acids (SFAs) and the monounsaturated (MUFAs) and polyunsaturated fatty acids within the samples (two-way ANOVA, $p \ll 0.05$).

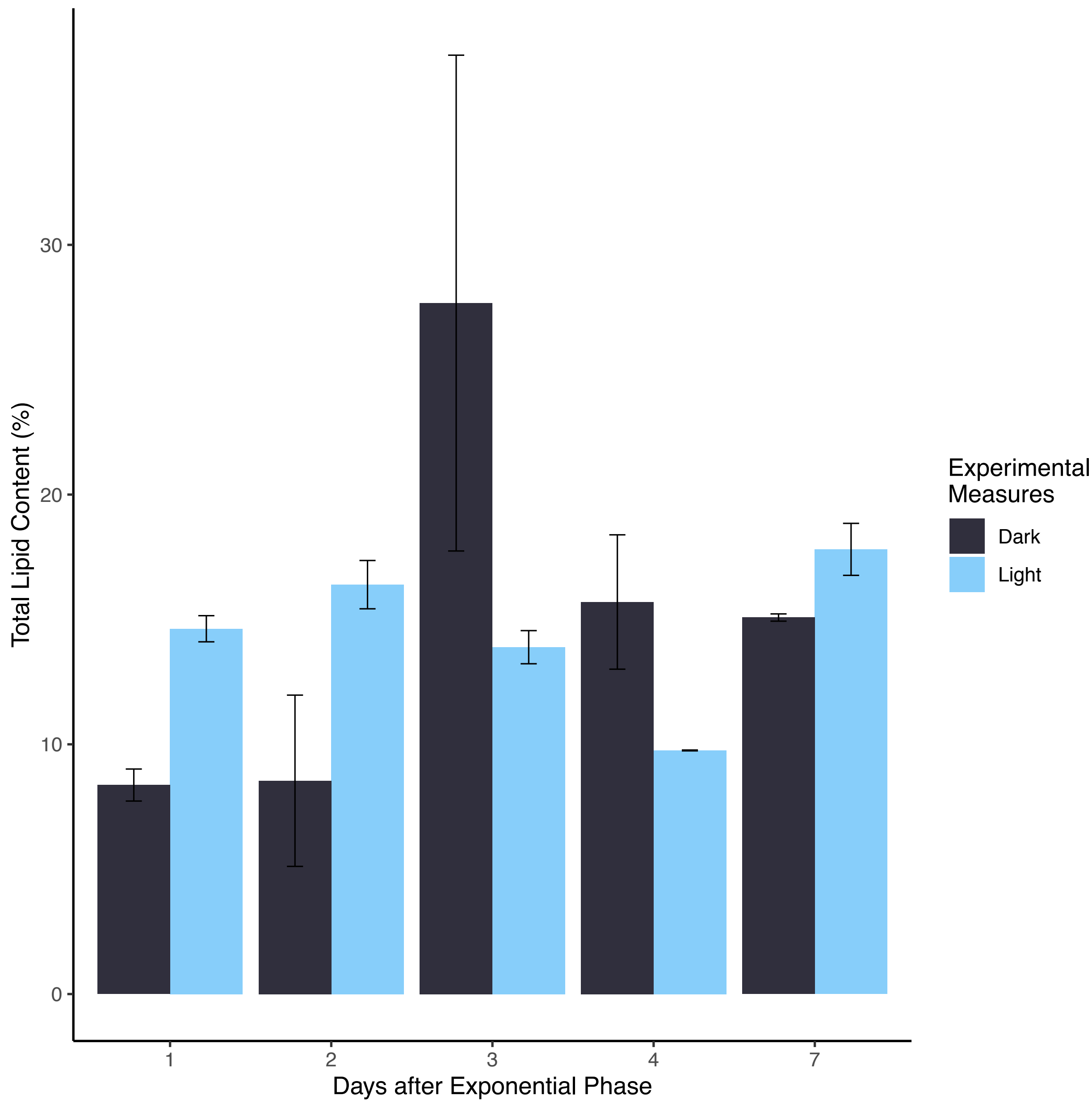

Figure 4.2: The total lipid content (%, mean ± standard error) of the dried biomass after exponential phase.

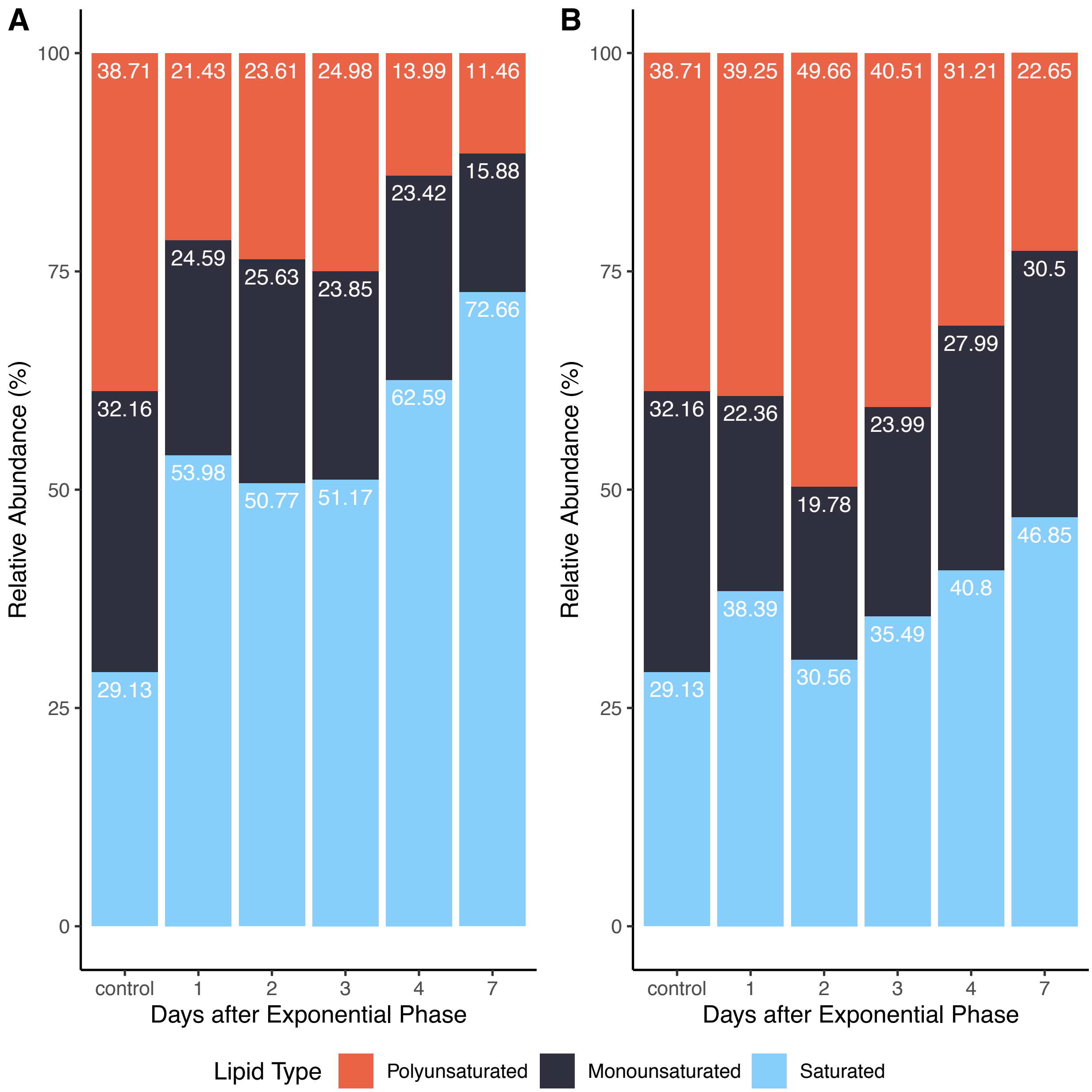

Figure 4.3: The lipid profile of *Coccomyxa sp*. consortium during A) dark-light growing conditions (12-hour light/dark cycle, 6% CO_2) and B) under continual dark conditions. The control is the lipid profile at the end of the exponential phase.

Table 4.1: Fatty acid profile of the treatment biomass', outlining the main fatty acids present during each day after exponential phase ended (day 0 being the last day of exponential phase).

	Saturated (%)			Monounsaturated (%)		Polyunsaturated (%)		
	C15:0	C16:0	C18:0	C16:1	C18:1	C18:2n6	C18:3n3	C18:3n6
Light-dark								
Day 1	29.53	2.16	18.79	2.62	20.96	0.00	0.31	19.87
Day 2	27.48	1.55	18.92	2.90	21.78	0.00	0.35	22.13
Day 3	28.84	2.27	16.48	2.70	19.88	0.00	0.55	21.89
Day 4	35.54	3.93	16.04	5.04	16.55	0.31	0.86	8.59
Day 7	30.69	1.15	37.55	6.44	8.72	0.03	0.18	8.31
Dark								
Day 1	13.17	14.10	8.19	1.55	19.53	11.03	13.28	13.48
Day 2	0.99	26.53	1.15	2.10	15.94	23.08	24.67	0.00
Day 3	1.05	31.64	1.03	2.34	18.91	20.04	18.46	0.01
Day 4	1.07	36.77	1.26	2.56	21.28	15.68	14.07	0.04
Day 7	1.10	42.90	1.31	2.77	23.90	14.10	7.22	0.10

4.4.3 Elemental analysis after exponential phase

Carbon, hydrogen, nitrogen, and sulphur levels in the biomass were analysed, and show a statistically significant difference between the elemental groups for all treatments (Kruskal-Wallis rank sum, $p<<0.05$), except for hydrogen and nitrogen (Wilcoxon rank sum, $p>0.05$) (Table 4.2). Slight increases in nitrogen and sulfur content was observed in the treatments, when compared to the control.

Table 4.2: Elemental composition of the algal biomass at the end of exponential phase (control) and on the day of the highest lipid content obtained for each treatment. Light-dark treated cells achieved their highest lipid content 7 days after the exponential phase, whereas dark-treated cells achieved it 3 days after the exponential phase.

Treatment	Highest lipid content (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)
Control	19.48 ± 1.1	47.39	6.93	3.99	0.11
Light	17.81 ± 1.04	47.76	7.02	4.02	0.11
Dark	27.67 ± 9.92	46.25	6.53	7.27	0.28

4.5 Discussion

Under standard growing conditions, the maximum daily productivity of 130 mg L^{-1} day^{-1} of the acid-tolerant bioprospected *Coccomyxa sp*. consortium is on par with results shown by Fuentes et al. (2020) and Vaquero et al. (2012) for bioprospected acidophilic *Coccomyxa onubensis*. However, higher productivities presented by Vaquero et al. (2014) and Casal et al. (2011) for *C. onubensis* (410 mg L^{-1} day^{-1}) and *Coccomyxa acidophila* (130-250 mg L^{-1} day^{-1}), suggest that further improvements can be made to enhance biomass productivity at low pH. Increases in biomass productivity could occur by means of utilizing a different growing media (Xie et al. 2019) or by reducing phosphorus levels (Maltsev et al. 2019). Furthermore, the CO_2 biofixation rate during the cultivation period (44.64 mg L^{-1} day^{-1}) was approximately half of the biofixation rate of the acidophilic species *Chlamydomonas acidophila*, as reported by Neves et al. (2019). It is, however, worth noting that their reported specific growth rate was considerably larger (0.92 day^{-1}) than our results (0.136 day^{-1}). According to Deamici et al. (2018), the neutrophilic *Chlorella fusca*, with a growth rate of approximately 0.14 day^{-1}, also had an increased biofixation rate of 171.7 mg L^{-1} day^{-1}, suggesting the *Coccomyxa sp*. consortia would not be an ideal candidate in systems with increased CO_2 levels.

However, when cells undergo three days of dark stress after experiencing exponential phase, total lipid content in the cells increase. Interestingly, the most abundant fatty acids are different for each treatment. Lipid from biomass sampled at the end of the exponential phase was 28.74% oleic acid (C18:1), whereas dark-light-treated cells favoured pentadecanoic acid (C15:0) at 28-35%, and dark treated cells, palmitic acid (C16:0) at 27-43%. Typically, biomass with high SFA and MUFA contents are favoured for biodiesel production, as these fatty acids are easily transesterified (Sajjadi et al. 2018). However, C15:0 has been found to have positive outcomes on human health by having anti-inflammatory, antifibrotic and red blood cell-stabilizing properties (Venn-Watson et al. 2020).

Overall, dark-treated cells had a higher variance of PUFAs (23-50%) compared to the light-dark treated cells (12-25%) and the control (38%), whereas the light-dark treated cells favoured SFA production (51-73%) and would be more suitable for biodiesel production. A possible explanation for the increase in PUFAs in dark-treated cells is the absence of photosynthesis (Spijkerman and Wacker 2011) which was found to correlate with fatty acid composition, in particular a significant increase in PUFAs as photosynthesis rates decline. Therefore, the higher PUFA content in dark-treated cells suggest they would be an ideal dietary supplement as these fatty acids are known for their heath beneficial properties (Caporgno and Mathys 2018; Krishna Koyande et al. 2019). In particular, dark-treated cells had a high abundance of omega-6 fatty acids (15-26%) including linoleic acid (C18:2n6), which has the potential to reduce the risk of cardiovascular disease and type-2 diabetes (Belury et al. 2018; Marangoni et al. 2020).

An increase in nitrogen in the biomass of the two treatments was reported over the 7 days of exposure. This could be an indication of an increase in amino acid synthesis (Huang et al. 2019), which could have had a positive outcome in total proteins for the biomass and, therefore, also supports the use of this algae as a food enricher. The increase of nitrogen content in the dark-treated cells remains unclear, as conflicting reports of nitrification occurring in the dark have been made (Delgadillo-Mirquez et al. 2016; Petrini et al. 2020). However, increased nitrogen could make lipid extracted biomass (LEB) a possible land ameliorant (Chu et al. 2020; Arun et al. 2020). The microalgae or LEB could be used as a liquid fertilizer (Deepika and MubarakAli 2020) or dry fertilizer in the form of biochar (Santos and Pires 2018), and increase yield and quality of crops (Nayak et al. 2019).

4.6 Conclusion

The acid-tolerant microalgae consortia were found to grow on par with other Coccomyxa strains at low pH (pH 2.5), with a specific growth rate of 0.136 ± 0.004 day^{-1} and a maximum density of 550 ± 36.1 mg L^{-1}. During the experiments, the dominant fatty acids were oleic acid (end of the exponential phase), and post exponential phase, pentadecanoic acid (light-dark treated biomass), and palmitic acid (dark-treated biomass). From our results, the mixed microalgae culture accumulated the maximum level of lipids after three to four days of dark treatment after exponential phase of growth. Dark stress predominantly favoured the production of polyunsaturated fatty acids (PUFAs), making dark conditions an ideal stressor for photosynthetic microalgae designated for dietary supplements as an end-product. During dark stress conditions, no stressors were physically added to the media and no extra energy was used in order to increase total lipids and the production of PUFAs. Therefore, unlike commonly used stressors (i.e. increased salinity and/or illumination), dark stress has no impacts on downstream processes and no impacts on overall energy consumption. An increase in biomass nitrogen content from dark stress further supports the use of lipid extracted biomass as a soil ameliorant.

Chapter 5

5 Conclusion

5.1 Summary of thesis

Photosynthetic green microalgae are highly recognized in the literature as a suitable bio-feedstock for fuels, health products, and food supplements; however, the economic viability of cultivating microalgae at large-scale has been proven difficult to overcome. The use of a bio-refinery approach was reviewed, with an emphasis on creating co-products from lipid-extracted biomass (LEB). LEB was shown to be a promising bio-feedstock for various products such as animal/fish feed, bio-hydrogen, biogas, and human health products. Meanwhile, the use of industrial flue gas as an alternative, low-cost source of CO_2 was explored in further detail.

Flue gas, with its potential for high SO_2 and NO_x concentrations, causes acidification when bubbled through the liquid growing media. The effects of this acidification were explored using bioprospected microalgae. These microalgae were discovered in mine-impacted sites, and therefore showed promise at growing in acidified environments. In particular, a wild sample from an active mine site, a consortium made up of >91% of a single *Coccomyxa sp.*, was found to grow ideally at pH 2.5. This consortium achieved the highest density at 550 mg L^{-1} at lab scale, with an average total lipid content of 13%. While investigating the accumulation of lipids, dark stress became an ideal low-cost alternative to commonly-used stressors. Dark stress was found to increase lipid content by 42% and, due to the lack of photosynthesis occurring in the dark, accumulated a higher ratio of polyunsaturated fatty acids (PUFAs), which are beneficial towards human health. Since the use of dark stress did not impact downstream processes or increase energy use, this low-cost alternative became an ideal candidate for inducing microalgae lipids designated for dietary supplements.

5.2 Contributions to research literature

Sabrina M. Desjardins, Corey A. Laamanen, Nathan Basiliko, and John A. Scott (2020). Utilization of lipid-extracted biomass (LEB) to improve the economic feasibility of biodiesel production from green microalgae. Environmental Reviews. 28(3): 325-338. https://doi.org/10.1139/er-2020-0004

Sabrina M. Desjardins, Corey A. Laamanen, Nathan Basiliko, and John A. Scott (submitted). Selection and re-acclimation of bioprospected acid-tolerant green microalgae suitable for growth at low pH. Extremophiles.

Sabrina M. Desjardins, Corey A. Laamanen, Nathan Basiliko, and John A. Scott (in progress). Dark stress as means to increase lipid content and improve fatty acid profiles of an acid-tolerant microalgae exposed to gas containing 6% CO2. Applied Environmental Microbiology.

Corey A. Laamanen, Kyle Moreau, **Sabrina M. Desjardins**, Shannon McLean, John A. Scott (in progress). Life cycle assessment of regional microalgal biodiesel production for use in underground mines.

Corey A. Laamanen, **Sabrina M. Desjardins**, Gerusa N.A. Senhorinho, John A. Scott (submitted). Harvesting microalgae for health beneficial dietary supplements. Algal Research.

Miranda R. Gauthier, Gerusa N.A. Senhorinho, Nathan Basiliko, **Sabrina M. Desjardins**, John A. Scott (submitted). Green photosynthetic microalgae from low pH environments as a potential source of antioxidants. European Journal of Phycology.

5.3 Future work

This work focuses primarily on novel solutions towards the economic feasibility of microalgae cultivation at large-scale, and therefore various important areas of research are still significant to consider such as exploring the use of non-simulated industrial flue gas as a source of CO_2 (i.e., at high C concentrations) and at outdoor light levels, and comparing those results with other locally and non-locally bioprospected acid-tolerant strains. Furthermore, while acidic environments are a result of using industrial gases high in SO_2 and NO_2, other factors, such as possible metal contamination, that could affect microalgae growth or downstream processes need to be considered. Meanwhile, integrity analysis of the cultivation tanks, and other materials that will come into contact with the acidified media, to assess any potential damage the acidic environment could cause.

Furthermore, while this thesis focuses on the use industrial flue gas as a low-cost source of CO_2 for operational algae cultivation, future work should also consider the use of wastewater as an alternative and low-cost source of nutrients essential for microalgae growth. Current research suggests wastewater as invaluable resource for microalgae growth, and has a higher pH than typical growing medias. Therefore, the combined use of flue gas and wastewater on overall pH and growth would provide insight into the growth dynamics of acid-tolerant/acidophilic microalgae, including the influence of nitrogen of phosphate uptake, tracking of stress genes, the biology of the cell throughout each growth phase, and the bio-fixation rates of varying CO_2 concentrations.

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