

LIPID PROFILING, CARBON PARTITIONING, AND INORGANIC CARBON
OPTIMIZATION TO ENHANCE GROWTH AND LIPID
ACCUMULATION IN MICROALGAE

by

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ABSTRACT

Microalgae are capable of accumulating high concentrations of lipids and other metabolites which can be used as precursor compounds for energy and valuable co-products. In order to fully exploit this resource, robust methods are needed to properly quantify and analyze the metabolites of interest. Additionally, understanding how and why these organisms synthesize these metabolites and developing optimized strategies for enhancing their metabolism is of paramount importance if algal biofuels and co-product development are to become commercially feasible. This dissertation represents the summary of work completed to develop analytical methods for quantifying lipid compounds synthesized by two Chlorophytes, *Chlamydomonas reinhardtii* sp. CC124 and *Chlorella vulgaris* UTEX 395, and the marine diatom *Phaeodactylum tricornutum* Pt-1. Additionally, *C.reinhardtii* was evaluated for factors that control and stimulate triacylglycerol (TAG) accumulation in microalgae by monitoring changes in lipid precursor compounds such as free fatty acids, mono- di- and tri-acylglycerides as well as fatty acids which were transesterified into fatty acid methyl ester (FAME); the bio-synthesized equivalent of diesel fuel. *C. vulgaris* was evaluated for optimized growth and lipid accumulation on various inorganic carbon substrates. This work resulted in a commercially applicable, two-phase growth/lipid accumulation regime which uses low grade sodium bicarbonate as the inorganic carbon substrate to enhance both growth and lipid accumulation and reduce the cost and resource overhead associated with using only carbon dioxide as the sole inorganic carbon source.

CHAPTER 1

INTRODUCTION

Background

The twentieth century ushered in an era of great technological achievement, driven in part by advancements in energy production. The discovery and refinement of petroleum as a natural resource for fuel and chemicals has enabled human innovation and led to unprecedented technological growth in industrialized nations. However, these advancements are not without cost. The current rate of producing enough crude petroleum to satisfy global demand has led to political, economic and environmental controversy (Dismukes et al., 2008). The US alone currently consumes over 18 million barrels of crude oil each day, with a significant percentage being imported from foreign sources (EIA, 2012). The US Energy Information Administration predicts that global energy demand will grow by 53 percent from 2008 to 2035, rising from 505 quadrillion Btu's in 2008 to 770 quadrillion Btu's in 2035 (EIA, 2012). China and India are expected to account for half of this global energy growth through 2035 as those countries continue to increase in GDP and bring new infrastructure online. The basic economic principle of supply and demand indicates that this increase in consumption will result in a shortage of supply, resulting in increasing cost. This trend is apparent when considering the price of crude oil over the last two decades, which has increased three-fold from ~\$33 (\$US/bbl) in 1992 to over \$90 in 2013, with drastic fluctuation occurring sporadically throughout this time period (Mabro, 2006). To add to this alarming predicament,

atmospheric concentrations of carbon dioxide have risen drastically due to the combustion of fossil fuels (NRC, 2010a). Carbon dioxide is one of the most prevalent greenhouse gases responsible for global climate change and ocean acidification (NRC, 2010b). Apart from the natural carbon cycle, industrial processes currently contribute 29 billion tonnes of carbon dioxide to the atmosphere each year (NRC, 2010b). This is more than 400x the current rate of global carbon fixation achieved by primary biota (Dukes, 2003). These numbers are staggering, and if business continues as usual, it can be expected that environmental degradation will continue as well.

Renewable sources of energy rich, biologically produced feedstocks are needed to offset this trend, and avoid serious economic and environmental consequences.

Feedstocks such as corn, soybean and sugar cane are the current primary sources for bioethanol and biodiesel, however these crops compete for arable land needed to produce food (Ferrell and Sarisky-Reed, 2010). Algal derived biofuels, especially biodiesel, have received increased attention in recent decades as a potential renewable energy resource (Dismukes et al., 2008; Hu et al., 2008; Sheehan, 1998; Yang et al., 2011). Based on life cycle analyses and conservative estimates of biodiesel yields, it has been projected that less than 5% of currently available arable land would be required to displace 100% of fossil fuel production (Chisti, 2008). This estimate is even more appealing when considering the fact that microalgae do not require arable land as they are not a conventional crop; rather, algal ponds simply need flat terrain, abundant sunlight, abundant water and nutrients. One of the key nutrients is carbon, which is necessary for cell growth as well as lipid synthesis. Biodiesel, defined as fatty acid methyl ester

(FAME) has similar chemical properties as traditional diesel, and can be produced from energy rich storage compounds such as triacylglycerol (TAG), which can be synthesized by microalgae in relatively high abundance.

Nutrient Dependent TAG Accumulation

Under optimal growth conditions, *i.e.* when the organism has the prerequisite concentration of nutrients, such as nitrogen, carbon, phosphorous and sunlight, algal biomass productivity can exceed 30 g dry weight m⁻² day⁻¹ (Gordon and Polle, 2007). However, the lipid content of this biomass is typically very low (<5% w/w) (Gordon and Polle, 2007). This is due to TAG biosynthesis being a metabolic process which is stimulated by stress inducement. Essentially, biomass synthesis and TAG biosynthesis compete for photosynthetic assimilation of inorganic carbon, and a fundamental metabolic switch is required to shift from biomass production to energy storage metabolism (Schuhmann et al., 2012). TAG is typically believed to provide a storage function within the cell that enables the organism to endure adverse environmental conditions (Sharma et al., 2012). Recent research has provided evidence that TAG may also act as a reservoir for specific fatty acids such as poly-unsaturated fatty acids (PUFAs). PUFAs play a key role in the structural components of cell membranes, and can further counteract free radical formation during photosynthesis due to their inherent antioxidant properties. As such, PUFA-rich TAG may donate specific compounds necessary to rapidly reorganize membranes through adaptive metabolic response to sudden changes in environmental conditions (Khozin-Goldberg and Cohen, 2006). In

either case, TAG is an energy rich storage compound which can be transesterified to produce FAME, the biological equivalent to diesel fuel. However, in order to maximize TAG biosynthesis, the organism must be induced through environmental stress conditions (Hu et al., 2008).

Significant work has been completed to identify and optimize stress inducement strategies which enhance TAG accumulation in microalgal species. Nutrient deprivation, specifically nitrogen depletion, is the most prevalent technique employed (Hu et al., 2008). Temperature variations, pH, salinity, light, osmotic and chemical stress inducements have all been investigated with varying success (Sharma et al., 2012). Here I will review the most successful strategies involving nutrient stress to induce TAG accumulation in a diverse set of microalgal species.

Nutrient availability is critical for cell division and intracellular metabolite cycling. Once nutrients such as nitrogen or phosphorous become deplete or limited in the medium, invariably a steady decline in cellular reproduction rates ensues. Once this occurs, the activated metabolic pathways responsible for biomass production are down-regulated and cells instead divert and deposit fatty acids into TAG (Wang et al., 2009). This may be due to two factors: (1) Lack of requisite nutrients limits the organisms' capacity to synthesize proteins necessary for biomass production (*e.g.* cellular division). In order to compensate, the organism must take advantage of alternative metabolic pathways for inorganic carbon fixation, such as fatty acid synthesis and hence store those *de novo* fatty acids as TAG (Msanne et al., 2012). (2) Photosynthesis and the electron transport chain in eukaryotic microalgae use ATP and NADPH as energy storage and

electron carrier metabolites, respectively. These metabolites are consumed during biomass production resulting in ADP and NADP^+ , which in turn become available again as acceptor molecules in photosynthesis. Under normal growth conditions, this cycle maintains a certain ratio of these metabolites; however when biomass production is impaired due to lack of requisite nutrients, the pool of NADP^+ can become depleted. This can lead to a potentially dangerous situation for the cell because photosynthesis is mainly controlled by light availability, and cannot be shut off completely. Fatty acid metabolism consumes NADPH; therefore increased fatty acid synthesis replenishes the pool of required electron acceptors in the form of NADP^+ , and *de novo* fatty acids are stored as TAG (Brown et al., 2009; Sharma et al., 2012).

Nutrient starvation to induce TAG accumulation in microalgae has been widely studied and is one of the most prevalent techniques used for biofuel production (Dismukes et al., 2008; Dukes, 2003; Hu et al., 2008). For example, when the marine diatom *Phaeodactylum tricornutum* was grown under nitrogen and phosphorous limitation, an increase in TAG accumulation was noticed in all limiting conditions (Valenzuela et al., 2012). However, cultures of *P. tricornutum* which were limited exclusively in nitrogen showed a far more significant increase in TAG than cultures which were limited solely in phosphorous. Whereas a combined limitation of both nitrogen and phosphorous resulted in the overall highest TAG concentrations in cultures of *P. tricornutum* (Valenzuela et al., 2012). When the model Chlorophyte *Chlamydomonas reinhardtii* was cultured under nitrogen limitation, an increase in TAG was also observed. Interestingly, it was specifically fully saturated C_{16} fatty acids which

were the most abundantly synthesized compounds, whereas poly-unsaturated C₁₈ fatty acids remained relatively unchanged (Gardner et al., 2013a). Reports on silicon limitation have revealed that both marine and freshwater diatoms will accumulate TAG under these nutrient limiting conditions (Sharma et al., 2012, Moll et al., 2013). Trace minerals and vitamin limitation has also been shown to increase TAG content of microalgae cultures, albeit to a much lesser extent than nitrogen or phosphorous. In fact, nitrogen appears to be the most critical nutrient affecting lipid metabolism in microalgae (Hu et al., 2008). However, one compound above all else is absolutely required for TAG biosynthesis. Fatty acids are long chain hydrocarbons, and a fundamental requirement for their synthesis is carbon availability to the organism (Palmqvist et al., 1988; Raven, 2010; Spalding, 2008). Without carbon, independent of nutrient deprivation, TAG biosynthesis is impossible. Therefore, the most successful reports of lipid induction techniques in microalgal TAG production typically involve elevated concentrations of inorganic carbon in the medium (Sharma et al., 2012). These strategies often employ a CO₂ sparge to increase dissolved CO₂ above atmospheric concentrations, or chemical addition of soluble inorganic carbon during inoculation or just prior to nutrient depletion. In either case, elevated concentrations of carbon, combined with nitrogen or other nutrient deprivation has been shown to induce TAG accumulation in virtually every microalgal species tested.

Project Rationale

Recent advancements have been made to stimulate various strains of algae to produce upwards of 50% of their dry weight as TAG by stress inducement (Chisti, 2008).

However, TAG is comprised of long chain hydrocarbons (fatty acids), and a fundamental requirement for their synthesis is carbon availability to the organism. In order to realize commercial scale development of algal derived biofuels, a fundamental understanding of how these organisms utilize carbon substrates and synthesize TAG is paramount to reducing cost and optimizing system processes. Although microalgae will grow under atmospheric concentrations of inorganic CO₂ (~400 ppm), improved biomass yields can be achieved by supplementing with additional carbon. It is often cited that this additional carbon could come from industrial waste such as power plant flue gas. However, in order to validate this concept, more research is necessary to determine the optimal concentrations and speciation of inorganic carbon supplied to microalgae cultures. Inorganic carbon can be in one of four dissolved forms in aqueous solutions; dissolved carbon dioxide (CO_{2(aq)}), carbonic acid (H₂CO₃), bicarbonate (HCO₃⁻), and carbonate (CO₃²⁻). Research has suggested that the preferential species for microalgae is CO_{2(aq)} (Raven, 2010), however at the optimal pH range for growth (pH 7 - 9) the predominant inorganic carbon species is bicarbonate. Previous research has revealed that the timely addition of sodium bicarbonate to microalgae cultures induces a significant increase in TAG accumulation (Gardner et al., 2012). This “bicarbonate trigger” has been tested and optimized on various species; including Chlorophytes and diatom species. Additionally, recent research presented in this dissertation has shown that supplementing cultures of microalgae with low doses of sodium bicarbonate can increase growth rates and stimulate the synthesis of high value metabolites such as pigments.

In order to fully exploit microalgae as a resource for biofuels and co-products, robust methods are needed for quantifying and analyzing metabolites of interest, such as the lipid precursor compounds free fatty acid (FFA) monoacylglyceride (MAG), diacylglyceride (DAG) and the energy storage compound TAG. Additionally, understanding how and why these organisms partition inorganic carbon into the various lipid fractions is of paramount importance to optimize growth and lipid accumulation for commercial applications.

Aims

This dissertation is the summary of the work completed to develop analytical methods for quantifying lipid compounds synthesized by microalgae, as well as evaluating factors that control and stimulate lipid accumulation by monitoring changes in lipid precursor compounds and fatty acids which have been transesterified into biodiesel. Additionally, an evaluation of how various inorganic carbon regimes influence growth and lipid accumulation is described. The goals of this project were the following:

- (i) Develop robust methods for quantifying and analyzing lipid precursor compounds such as FFA, MAG, DAG and TAG, as well as total cellular fatty acid transesterified into FAME.
- (ii) Verify method extraction efficacy by comparing against traditional extraction and analysis techniques using two Chlorophytes and one diatom.
- (iii) Elucidate inorganic carbon partitioning into lipid fractions and other metabolites in cultures of *Chlamydomonas reinhardtii* when grown with three unique inorganic carbon regimes.

- (iv) Determine an optimized inorganic carbon regime for enhanced growth and lipid accumulation in cultures of *Chlorella vulgaris*.

Outline of Technical Content

This dissertation documents work performed to execute the goals outlined above. Chapter 2 was published in the Journal of Microbiological Methods and focuses on method development for extracting lipid compounds from live cultures of microalgae; two Chlorophytes, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, and the marine diatom *Phaeodactylum tricornutum*. Benchmark data was collected by thoroughly testing previously reported extraction methods and comparing results to a modified extraction technique which uses microwave energy to disrupt live cells followed by solvent extraction of lipid compounds. Analytes were quantified and analyzed using gas chromatography coupled with flame ionization detection (GC-FID) to determine concentrations of FFA, MAG, DAG and TAG, based on retention time and carbon chain length. Additionally, a previously reported direct *in situ* transesterification technique (Griffiths et al., 2010) was modified to determine total cellular fatty acid content of the three live cultures. These methods were then combined to provide insight into which fraction of total FAME was derived from extractable lipids, versus which fraction was derived from membrane bound lipids.

Chapter 3 will be submitted to the Journal of Applied Phycology and describes how the methods developed in Chapter 2 can be used to elucidate inorganic carbon partitioning into lipid fractions and other metabolites. Cultures of *C. reinhardtii* were

grown under three unique inorganic carbon regimes. Extractable lipid (FFA, MAG, DAG, TAG) and total cellular fatty acid (FAME) were measured throughout the growth cycle. This approach provides a comprehensive lipid profile analysis and provides insight into how the organism changes its lipid metabolism due to nitrogen depletion and variations in inorganic carbon substrates.

Based on the results from Chapter 3 and the work of others (Gardner et al., 2012), an optimized inorganic carbon scenario was developed to enhance growth and lipid accumulation in cultures of *C. vulgaris*. Chapter 4 will be submitted to the Journal of Biotechnology for Biofuels and describes how by supplementing growth media with low doses of sodium bicarbonate and controlling the pH of the system with precisely timed sparging of CO₂, cultures of *C. vulgaris* grew faster, and accumulated significantly higher quantities of FAME.

Each technical chapter in this dissertation will include an abstract, and introductory, methodology, results, discussion, and concluding sections. Following these chapters is a project conclusion chapter, discussing future applications and related work, and a reference list of the literature cited in this dissertation. At the end of this dissertation are Appendices containing additional data and information for further studies.

CHAPTER 2

AN EFFICIENT AND SCALABLE EXTRACTION AND QUANTIFICATION METHOD FOR ALGAL DERIVED BIOFUEL

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Conceived the study, collected and analyzed data, and wrote the manuscript.

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Contributions: Collected and analyzed data, assisted with study design, discussed the results, and edited the manuscript.

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Contributions: Collected and analyzed data, assisted with study design, discussed the results, and edited the manuscript.

Co-author: Brent M. Peyton

Contributions: Discussed the results and implications, commented and edited the manuscript.

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Contributions: Assisted with study design, analyzed data, and edited the manuscript.

Manuscript Information Page

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Abstract

Microalgae are capable of synthesizing a multitude of compounds including biofuel precursors and other high value products such as omega-3-fatty acids. However, accurate analysis of the specific compounds produced by microalgae is important since slight variations in saturation and carbon chain length can affect the quality, and thus the value, of the end product. We present a method that allows for fast and reliable extraction of lipids and similar compounds from a range of algae, followed by their characterization using gas chromatographic analysis with a focus on biodiesel-relevant compounds. This method determines which range of biologically synthesized compounds is likely responsible for each fatty acid methyl ester (FAME) produced; information that is fundamental for identifying preferred microalgae candidates as a biodiesel source. Traditional methods of analyzing these precursor molecules are time intensive and prone to high degrees of variation between species and experimental conditions. Here we detail a new method which uses microwave energy as a reliable, single-step cell disruption technique to extract lipids from live cultures of microalgae. After extractable lipid characterization (including lipid type (free fatty acids, mono-, di- or tri-acylglycerides) and carbon chain length determination) by GC-FID, the same lipid extracts are transesterified into FAME and directly compared to total biodiesel potential by GC-MS. This approach provides insight into the fraction of total FAME derived from extractable lipids compared to FAME derived from the residual fraction (*i.e.* membrane bound phospholipids, sterols, etc.). This approach can also indicate which extractable lipid compound, based on chain length and relative abundance, is responsible for each

FAME. This method was tested on three species of microalgae; the marine diatom *Phaeodactylum tricornutum*, the model Chlorophyte *Chlamydomonas reinhardtii*, and the freshwater green alga *Chlorella vulgaris*. The method is shown to be robust, highly reproducible, and fast, allowing for multiple samples to be analyzed throughout the time course of culturing, thus providing time-resolved information regarding lipid quantity and quality. Total time from harvest to obtaining analytical results is less than two hours.

Key Words

triacylglycerol (TAG), microalgae, biodiesel, fatty acid methyl ester (FAME), Nile Red fluorescence, GC-FID/MS

Introduction

Consumption rates of traditional fossil fuels in the United States currently exceed 18 million barrels per day with a significant percentage being imported from foreign sources (EIA, 2012). The political, economic and environmental controversy over these resources has resulted in increased interest in advanced alternative fuels (Dismukes et al., 2008; Greenwell et al., 2010; Groom et al., 2008; Hill et al., 2006; Sheehan, 1998). Although algae-derived biofuels command a relatively small percentage of this emerging market, they hold great potential for truly displacing traditional sources due to their high biomass productivity, relatively small land mass requirements and high lipid yield (Chisti, 2007; Hu et al., 2008). Further, algal fuels do not directly contend in the “fuel vs. food” debate because they can grow in non-arable environments (Ferrell and Sarisky-Reed, 2010).

Biodiesel is the end result of a transesterification reaction in which lipids such as phospholipids, free fatty acids (FFA), mono, di, and tri-acylglycerides (MAG, DAG, TAG), derived from plant or animal sources, react with methanol in the presence of heat and base or acid, to produce fatty acid methyl esters (FAME) (Laurens et al., 2012a). Biodiesel is considered a “drop-in” fuel since no modifications have to be made to the current distribution infrastructure or diesel combustion engines. Biodiesel has similar combustion properties to petroleum diesel and a higher combustion efficiency than gasoline (Demirbas, 2007).

However, if biodiesel is to replace a large part of petroleum hydrocarbon based fuels, the compositional makeup of biodiesel will become highly important since biodiesel can have poorer performance than petroleum-based diesel (Hunter-Cevera et al., 2012). For example, (i) the viscosity of fully saturated hydrocarbons increases significantly at low temperatures and can lead to operational issues and (ii) short chain fatty acid methyl esters tend to be more susceptible to oxidation which can lead to corrosion, resulting in reduced engine durability (Xue et al., 2011). Therefore a comprehensive and diverse feedstock of lipid precursors along with the derived fatty acid methyl esters will be necessary to achieve the desired properties of the biodiesel fuel (Knothe, 2005).

Traditionally, microalgae species have been screened for strains that produce a high abundance of TAG using fluorometric techniques such as the Nile Red assay (Cooksey et al., 1987) and total lipid extracts have been quantified gravimetrically (Bligh and Dyer, 1959) or by gas chromatography after transesterification (e.g. Guckert and

Cooksey, 1990). However, these methods are limited by their inherent inability to identify all, or only those, compounds which may be utilized for fuel. Nile Red, for instance, roughly correlates with the amount of TAG precursors per cell (Chen et al., 2009; Cooksey et al., 1987; Elsey et al., 2007; Gardner et al., 2010, 2013). Hence, Nile Red-based measurements may underestimate the total biofuel potential of algal species that produce high fractions of FFA, MAG, DAG or membrane lipids. Additionally, the Nile Red assay is qualitative (possibly semi-quantitative) and cannot be compared between species. In contrast, gravimetric analysis can overestimate total biofuel potential by including non-fuel components (*e.g.* chlorophyll) in the total weight (Laurens et al., 2012b). Gas chromatographic analysis of FAMES has proven to be a reliable method for quantifying total biodiesel potential (Eustance et al., 2013; Laurens et al., 2012b), but fails to identify the biological precursor molecules.

Here, a new method is described which allows for high-throughput harvesting and extraction of live cultures by utilizing microwave energy for a one-step cell disruption-and-extraction of lipid precursors (FFA, MAG, DAG, TAG). Microwave energy has recently been shown to be an effective cell disruption technique, generating comparative or better lipid yields than more traditional methods such as sonication or bead beating (Lee et al., 2010; Patil et al., 2012; Prabakaran and Ravindran, 2011). Additionally, extraction of lipids from live culture significantly reduces process time and decreases the potential for degradation of intracellular lipid compounds. A portion of the lipid extract is analyzed by GC coupled with flame ionization detection (GC-FID) to identify the lipid class and carbon chain length of the fatty acid(s). In parallel, a portion of the lipid extract

is transesterified and analyzed by GC-MS to identify FAME derived from extractable lipid. Additionally, total biodiesel potential is determined by direct *in situ* transesterification of live cultures. Comparisons can be made between extractable lipid precursors, FAME derived from extractable lipids and total biodiesel potential by contrasting the carbon chain length and saturation of each molecule respectively. This approach was demonstrated for three different frequently used microalgae species; the model Chlorophyte *Chlamydomonas reinhardtii*, the marine diatom *Phaeodactylum tricornutum* and the freshwater green alga *Chlorella vulgaris*. These three organisms have been extensively studied (Gardner et al., 2012; Merchant et al., 2007; Stephenson et al., 2010; Valenzuela et al., 2012) and represent a diverse set of microalgae which are commonly used for biofuel investigations (Gardner et al., 2013; Liu et al., 2011; Spiekermann et al., 2003). The method described herein is relatively simple, fast, utilizes fairly standard equipment, and results in a comprehensive lipid profile within 2 hours of harvesting.

Materials and Methods

Microalgae Strains

Phaeodactylum tricornutum *Phaeodactylum tricornutum* strain Pt-1 (CCMP 2561) (Pt-1) was acquired from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (East Boothbay, Maine, USA) and accessions of *P. tricornutum* have previously been described (Martino et al., 2007). Pt-1 was cultured on ASP₂ medium (Provasoli et al., 1957) amended with 50 mM Tris buffer (Sigma-Aldrich, USA),

pKa 7.8, to maintain a stable pH throughout culturing. Cultures were continuously sparged with ambient air and amended with 25 mM of sodium bicarbonate (final concentration) just prior to nitrate depletion in the medium to induce TAG accumulation as demonstrated by Gardner et al. (2012).

Chlorella vulgaris *Chlorella vulgaris*, UTEX 395 (University of Texas at Austin) was cultured on Bold's basal medium (Nichols and Bold, 1965) with pH adjusted to 8.7 prior to autoclaving. Cultures were sparged with 5% CO₂ in air during the light hours.

Chlamydomonas reinhardtii *Chlamydomonas reinhardtii* CC124, obtained from the Chlamydomonas Center (Minnesota State University, Minneapolis MN), was kindly provided by John Peters, Department of Chemistry and Biochemistry at Montana State University, and was cultured on Sager's minimal medium (Harris, 1989). Cultures were sparged with 5% CO₂ (v/v) until just prior to ammonium depletion, at which time the gas sparge was switched to ambient concentrations of CO₂ (0.04%; v/v) and 50 mM of sodium bicarbonate (final concentration) were added to induce TAG accumulation (Gardner et al., 2013).

Culturing Conditions

All organisms were checked for bacterial contamination by inoculation into respective medium supplemented with 0.05% yeast extract and 0.05% glucose and incubation in the dark. Experiments were conducted in triplicate batch cultures using 70×500 mm glass tubes containing 1.2 L medium submersed in a water bath to control

temperature. Rubber stoppers, containing ports for aeration and sampling, were used to seal the tubes. Temperature was maintained at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Light ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was maintained on a 14:10 light–dark cycle using a light bank containing T5 tubes. Aeration (400 mL min^{-1}) was supplied by humidified compressed air (supplemented with 5% CO_2 (v/v) for *C. reinhardtii* and *C. vulgaris*) and controlled using individual rotameters for each bioreactor (Cole-Parmer, USA). ACS grade sodium bicarbonate was used in all experiments involving bicarbonate addition (Sigma-Aldrich, St. Louis MO).

Culture Analysis

Cell concentrations were determined using an optical hemacytometer with a minimum of 400 cells counted per sample for statistical reliability. Cell dry weights (CDW) for *C. reinhardtii* and *C. vulgaris* were determined by harvesting 30 mL of culture into a tared 50 mL Falcon tube (Fisher Scientific, Palatine, IL) followed by centrifugation at $4800 \times g$ and 4°C for 10 minutes (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H_2O (diH_2O), 18 M Ω , to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were frozen and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours. CDWs were calculated by subtracting the weight of the biomass-free Falcon tube from the weight of the Falcon tube with freeze-dried biomass.

CDWs for *P. tricornutum* were determined by filtering 10 mL of culture using 1 μm pore size glass fiber filters (Fisher Scientific) to collect the biomass. The biomass was washed with diH_2O to remove media salts and excess bicarbonate. Algal cells were dried at 70°C until the weight of the filter (plus biomass) remained constant. CDWs were

calculated by subtracting the dry weight of the clean filter from the oven-dried weight of the filter with biomass. This method was employed for the diatom due to the concern that the fragility of the organism's silicon-based frustules may result in cell disruption during the centrifugation and lyophilization steps utilized for the Chlorophytes, thereby underestimating overall CDWs. This technique was not employed for the Chlorophytes because it has been shown by us and others (Laurens et al., 2012a) that the freeze drying method is more reliable for green microalgae.

Analysis of Media Components

Medium pH was measured using a standard bench top pH meter. Ammonium and nitrate concentrations were measured using Nessler reagent (HACH, Loveland, CO) and ion chromatography (Dionex, Sunnyvale, CA), respectively, using previously described protocols (Gardner et al., 2013).

Harvesting

At the end of each experiment, cultures were transferred to a graduated cylinder and total harvest volume was recorded. Two aliquots of 45 mL were dispensed into 50-mL Falcon tubes (Fisher Scientific, Palatine, IL) and centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 5800 x g and 4°C for 10 minutes. Concentrated biomass was then transferred to Pyrex test tubes with Teflon lined screw caps (Kimble-Chase, Vineland, NJ) for microwave extraction of lipid precursors and direct *in situ* transesterification for total biodiesel potential, respectively. The remaining ~1 L of culture was centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 5800 x g and 4°C for 10 minutes. The supernatant was discarded and the remaining

concentrated biomass was combined in a 50 mL Falcon tube, washed with diH₂O, re-centrifuged, and the supernatant discarded. The washed biomass pellets were frozen at -80°C, lyophilized, and stored at -20°C for subsequent cell disruption/lipid extraction method comparisons.

Cell Disruption and Extraction of Lipids

Six different cell disruption and lipid extraction techniques were tested in biological triplicate to compare their extraction yields against yields from microwave extraction on live cultures. Unless otherwise stated, all methods were performed using approximately 30 mg of dried biomass, and the final extract was supplemented with 10 µL/mL of a 10mg/mL internal standard mix (isooctane in chloroform) to monitor instrument performance. All mixtures involving solvent and heat were carefully sealed in Pyrex test tubes with Teflon lined screw caps (Kimble-Chase, Vineland, NJ). Final extracts were analyzed for lipid precursors by gas chromatography – flame ionization detection (GC-FID).

Microwave Extraction of Lipid from Wet Biomass

45 mL of culture was transferred into a 50 mL Falcon tube and centrifuged at 4800 x g and 4°C for 10 minutes. The supernatant was discarded and the pellets were re-suspended in 3 mL diH₂O to facilitate transfer from Falcon tubes to screw-cap glass tubes for subsequent lipid extraction. Three mL of chloroform were added to the algal slurry in the test tubes, and the mixture was homogenized by vortexing for 10 s. Caps were securely fastened and samples were microwaved (Sharp Carousel, Mahwah, NJ) at 1000 watts on power level 1 (~100°C and 2450 MHz) for 1 min and then allowed to cool for 5

min. This process of vortexing for 10 s, microwaving for 1 min and cooling for 5 min was repeated until optimal extraction efficacy was established for each culture (Supplemental Data). A total of 3 min microwave exposure was required for samples of *P. tricornutum* and *C. reinhardtii*. Samples of *C. vulgaris* required a total of 10 min microwave exposure to completely disrupt cells and release lipids into the organic solvent. Transmitted light microscopy was used to verify complete disruption of cells, and epifluorescence microscopy was used to verify all extractable lipid (stained with Nile Red) had been released into the organic solvent (Nikon Eclipse E800).

It should be noted that extreme caution should be taken when applying microwave energy to an organic solvent under high heat and pressure. To address this inherent safety concern this method has since been modified by adding only 5 mL diH₂O to the algal pellet before transferring the slurry to screw cap test tubes, and test tubes were further secured by placing them in a microwaveable Tupperware container. Samples were then microwaved at 1000 watts power level 4 (2450 MHz) for a continuous 5 min and allowed to cool. Three mL of chloroform were added to each test tube when they had reached room temperature. Test tubes were vortexed for 10 s and heated at 90°C for 5 min to improve partitioning of the extracted lipids into the organic phase. Equivalent results were obtained with both versions of the method. The latter (i.e. water only-based extraction) being inherently safer, it is the recommended approach. In either case, samples were then centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the bottom of the test tube using a glass

syringe and transferred to a 2 mL GC vial (Fisher Scientific, Palatine, IL) for GC-FID analysis and subsequent transesterification.

Microwave Extraction of Lipids from Dry Biomass

Microwave extraction of lipids from dry biomass was conducted as described above except ~30 mg of dried algae biomass was used instead of live culture. Samples of *P. tricornutum* and *C. vulgaris* were extracted using the optimized microwave extraction technique with just diH₂O and no organic solvent during the microwave step. Samples of *C. reinhardtii* were collected before the optimized method was established; therefore they were extracted with diH₂O and solvent together in the microwave extraction step.

Sonication Extraction of Lipids from Dry Biomass

Dried biomass was combined with 3 mL of chloroform in a disposable 15 mL boro-silicate test tube. Samples were sonicated with a Branson S-450D sonifier equipped with a microtip probe set to 80 W (Branson, Danbury, CT) for six 20 s pulses, with each pulse followed by a 1 min cool down period in ice water. Total sonication time was 2 min. Samples were then centrifuged at 1,200x *g* for 2 min to pellet the residual biomass. One mL of the organic phase was removed from the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Bead Beating Extraction of Lipids from Dry Biomass

Dried biomass was combined with 1 mL of chloroform in a 1.5 mL stainless steel bead beating microvial with silicone cap (BioSpec Products, Bartlesville, OK). Three

types of beads (0.6 g of 0.1 mm zirconium/silica beads, 0.4 g of 1.0 mm glass beads, two 2.7 mm glass beads) were added to each vial before capping. A FastPrep bead beater (Bio101/Thermo Savant, Carlsbad, CA) was used to agitate the vials for six 20 s pulses at power level 6.5 followed by a 1 min cool down period between pulses. Total bead beating exposure time was 2 min. The mixture of solvent, residual biomass and beads was then transferred to a disposable 15 mL boro-silicate test tube, and the steel microvial was rinsed twice with 1 mL of chloroform, which was also added to the test tube, bringing the total solvent volume to 3 milliliters. Samples were then centrifuged at 1,200x *g* for 2 min to pellet the residual biomass. One mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Heat Induced Extraction of Lipids from Dry Biomass

Dried biomass was combined with 3 mL of chloroform and 3 mL diH₂O in a test tube. Samples were homogenized by vortexing for 10 s. Caps were securely fastened to test tubes and samples were heated in an oven at 95-100°C for 1 h with vortexing every 10-15 min. Samples were then centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Bligh and Dyer Extraction of Lipid from Dry Biomass

The modified Bligh and Dyer method (as described by Guckert et al. (1988), without phosphate buffer) was used without any additional physical cell disruption steps.

Briefly ~50 mg of dry biomass from each of the three organisms in triplicate was transferred to Pyrex test tubes with Teflon lined screw caps. 1.5 mL of chloroform and 3 mL of methanol were added to each test tube and samples were homogenized by vortexing for 10 s. Caps were securely fastened, samples were placed horizontally on a shaker table and allowed to mix continuously for 18 h. 1.5 mL diH₂O and 1.5 mL chloroform were then added, and samples were vortexed for 10 s before being centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the chloroform phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Direct *in situ* Transesterification for FAME Analysis

Direct *in situ* transesterification of live cultures was conducted using a previously described protocol (Griffiths et al., 2010) with modifications. FAME composition was analyzed using gas chromatography – mass spectroscopy detection (GC-MS). Briefly, 45 mL of culture were transferred into a 50 mL Falcon tube and centrifuged at 4800 x *g* and 4°C for 10 min. The supernatant was discarded and the pellets were re-suspended in 3 mL diH₂O to facilitate transfer from Falcon tubes to screw-cap glass tubes for subsequent transesterification. The tubes were centrifuged at 1,200x *g* to remove as much water from the remaining biomass as possible. One mL of toluene and 2 mL sodium methoxide (Fisher Scientific, Pittsburgh PA) were added to each test tube along with 10 µL of a 10mg/mL standard mix (C11:0 and C17:0 TAG) to monitor transesterification efficiency of the TAG into FAME. Samples were heated in an oven for 30 min at 90°C and vortexed every 10 min. Samples were allowed to cool to room temperature before 2 mL

of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) were added and samples were heated again for an additional 30 min. Samples were again allowed to cool before 10 μ L of a 10 mg/mL of C23:0 FAME were added to assess the completeness of partitioning FAME into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated salt water solution (NaCl in diH₂O) were added. Samples were heated for 10 min to facilitate FAME partitioning into the organic phase, vortexed for 10 s and centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the top layer using a glass syringe and transferred to a 2 mL GC vial for GC-MS analysis.

Transesterification of Extractable Lipids from Wet Biomass

Lipids extracted from wet biomass were transesterified for FAME analysis. Briefly, 0.5 mL of the extract was removed from the GC vial and transferred to a screw-cap glass tube. One half mL of a 5% HCl in methanol mixture was added to each test tube along with 10 μ L of a 10mg/mL standard mix (C11:0 and C17:0 TAG) to assess transesterification efficiency. Samples were capped with a Teflon lined screw cap, heated for 1 hour at 90°C and vortexed every 10 min. Samples were allowed to cool to room temperature before 0.5 mL of a 15% salt water solution (NaCl in diH₂O) was added along with 10 μ L of 10 mg/mL C23:0 FAME to assess completeness of FAME partitioning into the organic phase. Samples were heated for an additional 10 min to help partition FAME into the organic phase, vortexed for 10 s and centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the

bottom organic layer using a glass syringe and transferred to a 2 mL GC vial for GC-MS analysis.

Lipid Analysis

Nile Red Cellular TAG accumulation was monitored throughout the experiments using the Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one) (Sigma-Aldrich, St. Louis, MO) fluorescence method (Cooksey et al., 1987), which has become a generally accepted screening method for analyzing TAG in algal cultures both in academia and industry (e.g. Chen et al., 2009; Cooksey et al., 1987; da Silva et al., 2009; Elsey et al., 2007). Furthermore, Nile Red fluorescence has been correlated to TAG content using gas chromatography (GC) analysis on extracted lipid from *C. reinhardtii*, $r^2 = 0.998$ (Gardner et al., 2013). Briefly, 1 mL aliquots were removed from cultures and assayed directly with Nile Red (4 μ L from 250 μ L/mL in acetone) or by diluting 1:5 with diH₂O or salt water, for freshwater or marine cultures respectively, before staining with Nile Red (20 μ L from 250 μ L/mL in acetone). To maintain linearity of the Nile Red assay, dilution was required when population counts exceeded 1×10^7 cells/mL. Total Nile Red fluorescence was quantified on a microplate reader (Bio-Tek, USA) utilizing a monochromator set to 480/580 nm excitation/emission wavelengths. A baseline sensitivity setting of 50 was experimentally determined to maximize the signal-to-noise ratio, while accommodating fluorescent level changes over 10,000 units. To minimize fluorescence spillover, black walled 96 well plates were loaded with 200 μ L of sample. Unstained samples were used for background medium and cellular autofluorescence correction.

GC-FID GC-FID (Agilent 6890N, Santa Clara CA) analysis was conducted using 1- μ L splitless injections onto a 15 m (fused silica) RTX biodiesel column (Restek, Bellefonte PA). The initial column temperature was held at 100°C for 1 min, before being increased to 370°C at a rate of 10°C min⁻¹. The injector temperature was held constant at 320°C. Helium was used as the carrier gas and column flow was held at 1.3 mL min⁻¹ for 22 min, increased to 1.5 mL min⁻¹, held for 2 min, increased to 1.7 mL min⁻¹ and held for 12 min. All flow rate increases were set to 0.2 mL min⁻². Calibration curves were constructed for each of the following standards: C10:0, C12:0, C14:0, C16:0, C18:0, C20:0 FFA; C12:0, C14:0, C16:0, C18:0 MAG; C12:0, C14:0, C16:0, C18:0 DAG; and C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, C22:0 TAG (Sigma-Aldrich, St. Louis MO) for quantification ($r^2 > 0.99$). This GC method allows for an estimate of the amounts of FFA, MAG, DAG and TAG in a single analysis (Fig. 2.1a).

GC-MS GC-MS analysis was performed according to a previously published protocol (Bigelow et al., 2011). Briefly, 1 μ L splitless injections were performed via an autosampler into a GC-MS (Agilent 6890N GC and Agilent 5973 Networked MS) equipped with a 30 m x 0.25 mm Agilent HP-5MS column (0.25 μ m phase thickness). The injector temperature was 250°C and the detector temperature was 280°C. The initial column temperature was 80°C and was increased to 110°C at a rate of 8.0°C min⁻¹, immediately followed by a ramp at 14.0°C min⁻¹ to a final temperature of 310°C which was held for 3 min before run termination. Helium was used as the carrier gas and column flow was held at 0.5 mL min⁻¹. Quantities of FAMES were determined by quantifying each response peak with the nearest eluting calibration standard based on

retention time, using MSD ChemStation software (Ver. D.02.00.275), with additional analyses performed using a custom program. A 28-component fatty acid methyl ester standard prepared in methylene chloride (“NLEA FAME mix”; Restek, Bellefonte, PA) was used for GC-MS retention time identification and response curve generation ($r^2 > 0.99$). It should be noted that C18:1, C18:2 and C18:3 co-elute on the column used and have similar fragmentation patterns. They were therefore quantitated together as C18:1-3.

Lipid Quantification and Analysis

Lipid quantification was performed using a custom program developed specifically for this purpose (Appendix B). Chromatogram data from the GC-FID and GC-MS were exported from the instrument software (Chemstation Ver. B.02.01-SR1) as a Microsoft Excel spreadsheet. The spreadsheet was then imported into a web-based software application written in Microsoft’s ASP.NET framework using C# and MVC as the coding language and paradigm, respectively. Data were stored with unique identifiers in a Microsoft SQL database. Six point calibration curves were constructed using the LINEST function which is built into the ASP.NET framework library. Chromatogram peaks were quantified based on the closest standard, as determined by retention time of the sample peak and standard peak, respectively. All chromatograms were manually integrated and inspected prior to export to ensure accuracy and reliability. Manual calculations were performed periodically using Excel to verify software results.

Control Samples

Olive oil controls were analyzed by GC-FID to verify no thermal degradation of TAG was occurring when samples were exposed to microwave energy. Olive oil is

predominantly comprised of C₁₆ and C₁₈ TAG compounds (Kiritsakis et al., 1998). A stock solution of olive oil dissolved in chloroform (1 mg mL⁻¹) was prepared, and aliquots (n=3) were run directly on the GC-FID as a control (Fig. 2.1b). To verify thermal degradation was not occurring, 3 mL of the stock solution were transferred to a Pyrex test tube with Teflon lined screw caps and an additional 3 mL of diH₂O were added. Samples (n=3) were homogenized by vortexing for 10 s, caps were securely fastened, samples microwaved (Sharp Carousel, Mahwah, NJ) at 1000 watts on power level 1 (~100°C and 2450 MHz) for 1 min and then allowed to cool for 5 min. This process of vortexing for 10 s, microwaving for 1 min and cooling for 5 min was repeated 10 times, for a total of 10 min exposure to microwave energy. Samples were then centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial (Fisher Scientific, Palatine, IL) for GC-FID analysis (Fig. 2.1c).

Statistical Analysis

A “two one-sided tests” (TOST) procedure (Richter and Richter, 2002) was performed on the control samples to determine statistical equivalency between treated and untreated samples. Equivalence was determined for each response peak based on peak area using n=3 samples for each group. The TOST procedure requires an acceptance parameter “ θ ” to represent the hypothesized mean difference boundary. Sample means are considered equivalent if $|\mu_1 - \mu_2| \leq \theta$ with a 95% confidence interval. Statistical parameters were set at: $\theta = 0.5$, $\alpha = 0.05$.

Results and Discussion

Chromatograph Calibration, Quantification and Data Compilation

The quality of the results obtained using the methods described in this paper rely heavily on proper calibration, chromatogram quantification, quality control and quality assurance. Figure 2.1 depicts GC-FID chromatograms for a surrogate mix of calibration standards (Fig. 2.1a), two olive oil standards (to check for the potential of thermal degradation during microwave treatment; Fig. 2.1b,c), and samples from each of the three organisms used in this study (Fig. 2.1d,e,f). The surrogate mix of standards is comprised of twenty two analytical grade lipid standards; six free fatty acids (C10:0 - C20:0), four monoacylglycerides (C12:0 - C18:0), four diacylglycerides (C12:0 - C18:0), and eight triacylglycerides (C11:0 – C22:0). All of these standards are fully saturated and of even chain length, except for the C11:0 and C17:0 TAG. Six different calibration standard concentrations (0.005, 0.01, 0.05, 0.1, 0.25 and 0.5 mg/mL) were used to create the calibration curves for each compound listed above. Regression coefficients for each linear calibration curve were $r^2 > 0.99$. Excellent peak shape and resolution were achieved for each of the standards in the mix (Fig. 2.1a). The algae extract chromatograms exhibit more noise, presumably due to a range of different lipids (chain length and saturation) being produced. As an example, consider the TAG range for *C. vulgaris* (Fig. 2.1d).

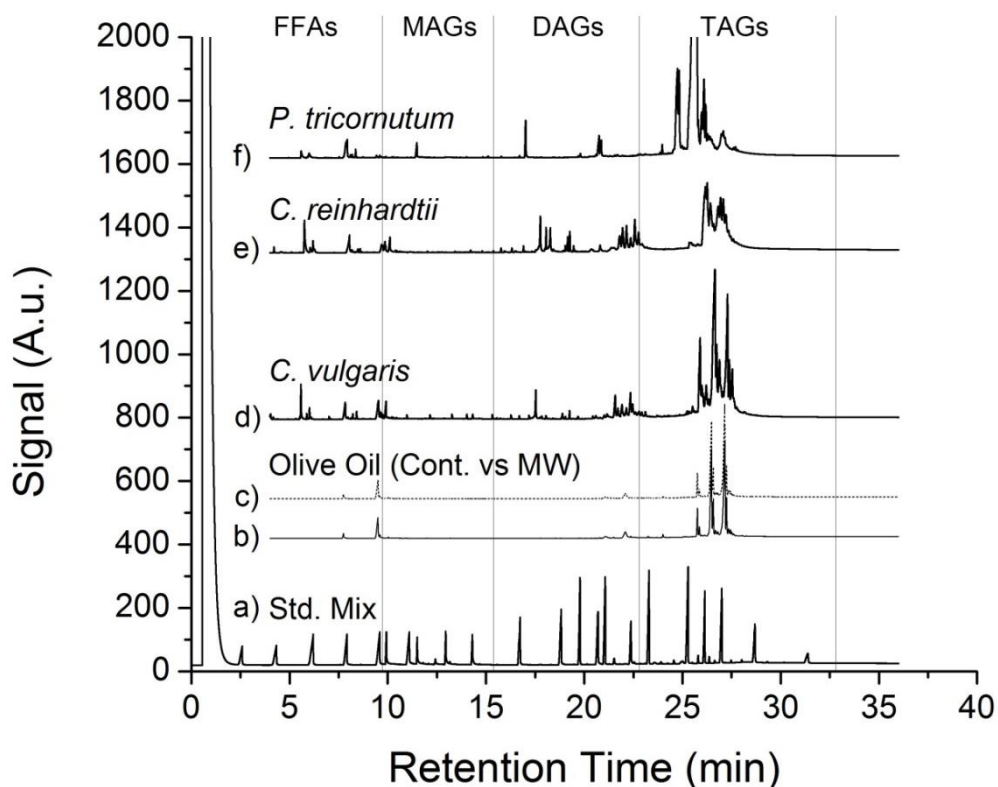


Figure 2.1: GC-FID chromatograms: (a) Standard mix of 22 analytical grade lipid standards (0.1mg/mL); from left to right – C10:0, C12:0, C14:0, C16:0, C18:0 FFA, C12:0 MAG, C20:0 FFA, C14:0, C16:0, C18:0 MAG, C12:0, C14:0 DAG, C11:0 TAG, C16:0 DAG, C12:0 TAG, C18:0 DAG, C14:0, C16:0, C17:0, C18:0, C20:0, C22:0 TAG. (b) Olive oil (1 mg/mL) was used as a control (black line) against (c) a sample of the same concentration which was microwaved for 10 min (dashed line) to assess the potential for thermal degradation of TAG. Lipid extracted via microwave energy from (d) *C. vulgaris*, (e) *C. reinhardtii* and (f) *P. tricornutum*. Chromatograms are offset to allow for alignment with standards.

The increase in baseline response during retention times of approximately 25-30 minutes is likely the result of the presence of a number of TAG with different combinations of fatty acids of different chain lengths per molecule of TAG. In comparison, the chromatogram of olive oil has a mostly flat baseline and exhibits only three groups of peaks in the TAG region. Olive oil consists predominantly of TAG with fatty acid chains consisting of 55-83% oleic acid (C18:1), 3.5-21% linoleic acid (C18:2)

and 7.5-20% palmitic acid (C16:0) (Kiritsakis et al., 1998). The three groups of peaks in Fig. 2.1b and 2.1c for the olive oil standards are TAG with various combinations of these three fatty acids at the sn-1, sn-2 and sn-3 position of the glycerol backbone. The peak at 27.15 min. (longest retention time (RT)) is a TAG with oleic acid at each position (sn-1, sn-2 and sn-3). The left-most TAG peak (ret. time 25.76) is predominantly palmitic acid, but the shift in retention time compared to the C16:0 TAG standard suggests oleic acid or linoleic acid must also be present at one position.

Microorganisms are known to produce a wider range of mono-, di- and triacylglycerides than higher plants with carbon chain length varying by more than six carbon atoms and a range of saturation states (Brown et al., 2009). However, it is uncommon to find a glycerolipid synthesized by any organism with individual fatty acids deviating by more than two carbon atoms, albeit saturation states can differ significantly (Brown et al., 2009). Therefore, the challenge of separating and quantifying all potentially possible glycerolipids was addressed by developing calibration ranges based on the residence time of each of the twenty even-chain length standards and further comparing these results to mass spectroscopy analysis of FAME derived from the extractable lipids.

The described GC-FID analysis of extractable lipid provides information regarding the lipid class and chain length (cf. Fig 2.1), however, it is not capable of chromatographically separating a number of the acylglycerides based on the degree of fatty acid chain saturation. This limitation was addressed to a large extent by transesterifying the lipid extract and comparing the FAME profile to the relative

abundance of the lipid classes, based on chain length and saturation. The details are discussed in detail below.

Comparison of Various Cell Disruption and Lipid Extraction Techniques

To test the validity of microwave energy as a suitable and rapid method for cell disruption and lipid extraction, it was necessary to compare the results of the microwave technique to those from more traditional methods (Fig. 2.2). Historically, the Bligh and Dyer (Bligh and Dyer, 1959) gravimetric method for quantifying lipid has been considered the standard for lipid extraction (Gouveia and Oliveira, 2009; Laurens et al., 2012b; Singh and Kumar, 1992; Teixeira, 2012). However, it has been shown that the Bligh and Dyer method can produce significantly lower estimates of lipid content, and this underestimation tends to increase significantly with increasing lipid content of the sample (Iverson et al., 2001). More recently, researchers have demonstrated that cell lysis by mechanical means such as sonication and bead beating aids in lipid extraction (Lee et al., 2010, 1998; Zheng et al., 2011). However, these techniques require significant processing time due to limitations of the instruments and extensive preparation of sample and materials. For example, biomass must first be frozen and lyophilized, requiring up to 48 h or more from the time of harvest until processing can commence, and often only one sample can be processed at a time. These factors can limit one's ability to acquire lipid profiles throughout the timecourse of culturing and implement adjustments as necessary.

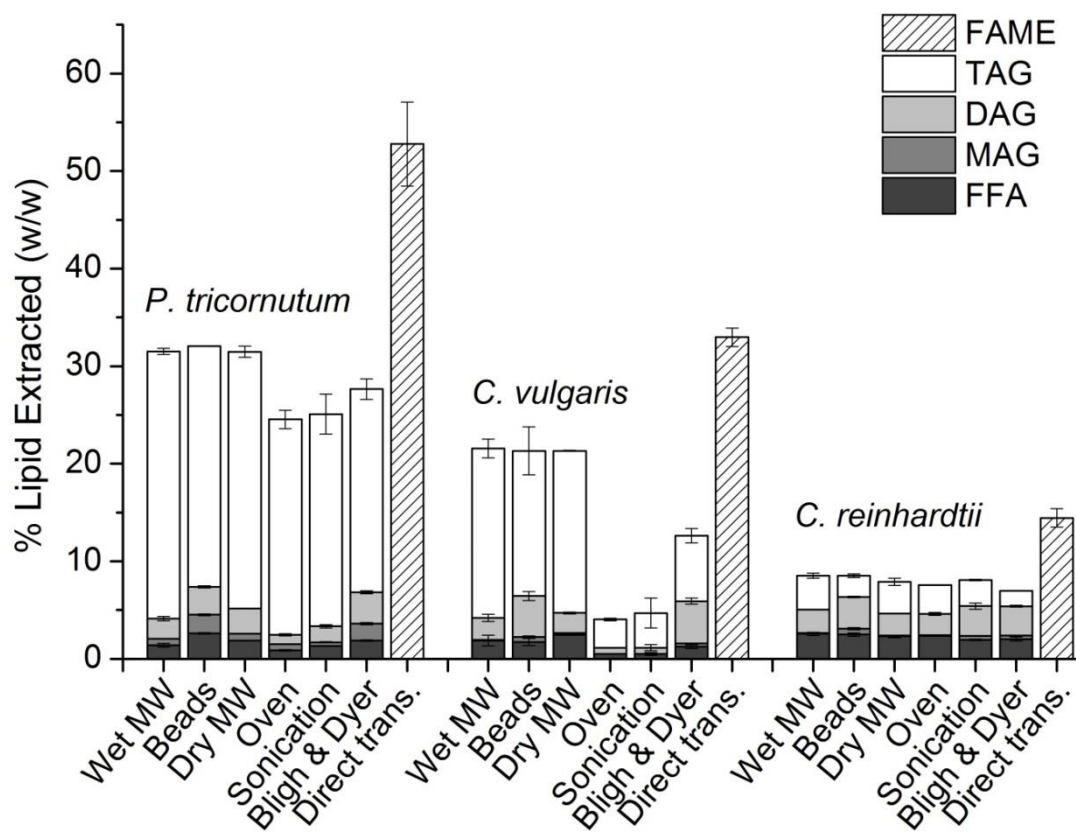


Figure 2.2: Average and standard deviation of extractable lipid % (w/w) using various cell disruption techniques on *P. tricornutum*, *C. vulgaris* and *C. reinhardtii*: Microwave extraction of wet biomass (Wet MW), bead beating (Beads), microwave extraction on dry biomass (Dry MW), heat induced extraction (Oven), sonication probe (Sonication), a modified Bligh and Dyer method and direct in situ transesterification for total biodiesel potential (Direct trans.) (n=3).

Furthermore, extensive processing times carry a greater risk of the sample composition changing between collection and analysis. For example, it has been shown that TAG can be degraded during dark hours in some algal cultures (Bigelow et al., 2011; Gardner et al., 2012) making long processing times undesirable.

Microwave extraction of lipid precursors from live culture required less than 2 hours from time-of-harvest to generation of GC data and was shown to yield as much, if not more, lipid than the other techniques tested on these three organisms (Fig. 2.2).

Further, relatively small volumes of culture (usually <50 mL) are necessary for each sample point, resulting in the ability to potentially collect multiple data points throughout even lab scale experiments.

Results from these techniques were also compared against results from direct *in situ* transesterification of live culture. Exhaustive direct *in situ* transesterification converts free and bound fatty acids into FAMES (Laurens et al., 2012b), thereby generating larger amounts of FAMES than the other techniques due to the additional conversion of fatty acids from non-extractable cellular sources (*i.e.* membrane bound lipids, chlorophyll, etc.).

It is important to assess potential losses or degradation of lipids due to the extraction technique. There was a concern that high heat generated during the microwave disruption process may result in thermal degradation of lipid compounds such as TAG. Hence, samples of olive oil were subjected to the microwave extraction process and compared to the untreated olive oil sample. Briefly, 3 mL of a 1 mg/mL (olive oil/chloroform) mixture was microwaved for 10 min according to the protocol described above. One mL of this mixture was removed and analyzed by GC-FID against a control of the same concentration which had not been exposed to microwave heat. Chromatograms for the sample and control are shown in Fig. 2.1 (b and c). Statistical equivalence was determined for each response peak as described above with 95% confidence.

Comparison of Extractable Lipid-Derived FAME to Total Biodiesel Potential

Another significant benefit of this method is the ability to determine which fraction of total biodiesel potential was derived from extractable lipid precursors (*e.g.* FFA, MAG, DAG, TAG) as opposed to residual precursors (*e.g.* membrane lipids). Additionally, the lipid class itself can be determined by the carbon chain length of the extractable lipid-derived FAME. This advancement was achieved due to the relative simplicity of extracting lipids from live cultures using microwave energy, transesterifying the extract and quantifying both the extract and transesterified extract by GC-FID and GC-MS, respectively. This approach results in two chromatograms for each sample whose comparison allows for an estimate of the lipid class and carbon chain length of the extractable lipid precursors as well as the carbon chain length and saturation of the extractable lipid-derived FAME. The comparison of the results of extractable lipid-derived FAME with direct *in situ* transesterified FAME (Griffiths et al. 2010, which was modified here to allow for the processing of small quantities of live cultures), makes it possible to assess which fraction of total biodiesel potential was likely derived from extractable lipid. The remaining difference between direct *in situ* transesterified FAME and extractable lipid-derived FAME is considered to be the residual fraction (*e.g.* membrane lipids). Further, the originating lipid class of the extractable lipid-derived FAME can be determined with relatively high confidence by comparing the carbon chain length of the molecule to the extractable lipid precursors quantified by GC-FID. This approach makes it possible to gain a comprehensive lipid assessment of a microalgae culture. It should be noted, that while the methods described here are capable of

detecting a multitude of lipid compounds, *e.g.* cyclopropane fatty acids or amide-linked fatty acids (sphingolipids), by mass-spectroscopy, such compounds were not detected in significant abundance in any of the cultures. If these compounds are present and quantifiable in samples, they would be grouped into the “Other” category in the reported values. Additionally, two different transesterification techniques were used to derive total FAME versus extractable FAME. The direct *in situ* transesterification was used for total cellular fatty acid conversion to FAME. This method uses both a base (sodium methoxide) and an acid (boron tri-fluoride in methanol) to fully transesterify all cellular fatty acid compounds into FAME. The sequential addition of a basic and acidic catalyst has been shown to improve the efficiency of direct transesterification when water is present, as is the case when using live cultures (Griffiths et al., 2010). The basic catalyst, in the presence of water, promotes the saponification of glycerolipids via alkaline hydrolysis, thereby cleaving the ester bonds between fatty acids and glycerol. The subsequent addition of acid catalyst increases the rate of esterification of liberated free fatty acids. These combined steps reduce the detrimental effects of water and results in faster and more efficient conversion of lipids to FAME (Griffiths et al., 2010).

Extractable lipid compounds were transesterified using only acid (HCl) as a catalyst. This was determined to be sufficient because there was no significant amount of water present in the extract containing the extractable lipid. Both techniques were compared using the precursor lipid extract and no yield differences were found. Since the acid only method is faster and uses less material, it was determined to be more suitable.

Phaeodactylum tricornutum *P. tricornutum* was cultured as previously described by Gardner et al. (2012) and harvested at peak TAG accumulation, as monitored by Nile Red fluorescence (Table 2.1).

Table 2.1: Comparison of mean and standard deviation of final culture growth and TAG accumulation, monitored by Nile Red fluorescence, of *P. tricornutum*, *C. vulgaris* and *C. reinhardtii* (n=3).

Organism	Dry weight (g L ⁻¹ ; DCW) ^a	Final cell density (x10 ⁶ cells mL ⁻¹)	Final total Nile Red fluorescence (x10 ³ units)	Final Nile Red specific fluorescence (units cell ⁻¹) ^b
<i>P. tricornutum</i>	0.18 ± 0.06	8.65 ± 0.49	9.71 ± 0.27	1.12 ± 0.03
<i>C. vulgaris</i>	0.88 ± 0.05	200 ± 10	4.06 ± 0.6	0.02 ± 0.003
<i>C. reinhardtii</i>	0.9 ± 0.02	7.55 ± 0.3	3.37 ± 0.16	0.45 ± 0.003

^a Dry cell weight (DCW) determined gravimetrically with lyophilized biomass

^b Calculated by fluorescence signal/cell density x 10,000 (scaling factor)

Table 2.2: Comparison of mean and standard deviation of lipid extracted with microwave energy, and total biodiesel potential from direct *in situ* transesterification of *P. tricornutum*, *C. vulgaris* and *C. reinhardtii* (n=3). All values expressed as % (lipid/CDW).

Organism	Free fatty acid (%) ^a	Mono-glyceride (%) ^a	Di-glyceride (%) ^a	Tri-glyceride (%) ^a	Sum of extracted (%) ^b	Total biodiesel potential (%) ^c
<i>P. tricornutum</i>	1.4 ± 0.2	0.65 ± 0.02	2.08 ± 0.22	27.39 ± 0.32	31.51 ± 0.33	51.19 ± 0.75
<i>C. vulgaris</i>	1.88 ± 0.54	0.06 ± 0.01	2.26 ± 0.36	17.35 ± 0.95	21.56 ± 0.06	32.96 ± 0.93
<i>C. reinhardtii</i>	2.56 ± 0.15	0.07 ± 0.01	2.43 ± 0.03	3.46 ± 0.28	8.53 ± 0.18	14.45 ± 0.95

^a From microwave extraction of wet biomass

^b Sum of neutral lipid precursors – free fatty acids, mono, di, tri-glycerides

^c Total FAMES from direct *in situ* transesterification

Lipids were extracted by the microwave extraction method. Total FAME was determined using direct *in situ* transesterification, and FAME derived from extractable lipid was determined as described above. Total FAME was 51.2 % (w/w) and the sum of all extractable lipids was 31.5 % (w/w) with TAG contributing the majority at 27.4 % (w/w) or 87 % of the extract (Table 2.2). Fig. 2.3 shows the complete FAME profile for *P. tricornutum*. Results from direct *in situ* transesterification indicate that under these

growth conditions this organism preferentially synthesized C16:1 and C16:0 lipid compounds. Total C16:1 and C16:0 FAMES were 24.8 % (w/w) and 14.7 % (w/w) respectively (Table 2.4). The fractions of total C₁₆ FAMES from extractable lipids were 54.5% for C16:1 and 51.8% for C16:0 (Table 2.4). The sum of all C₁₆ FAMES

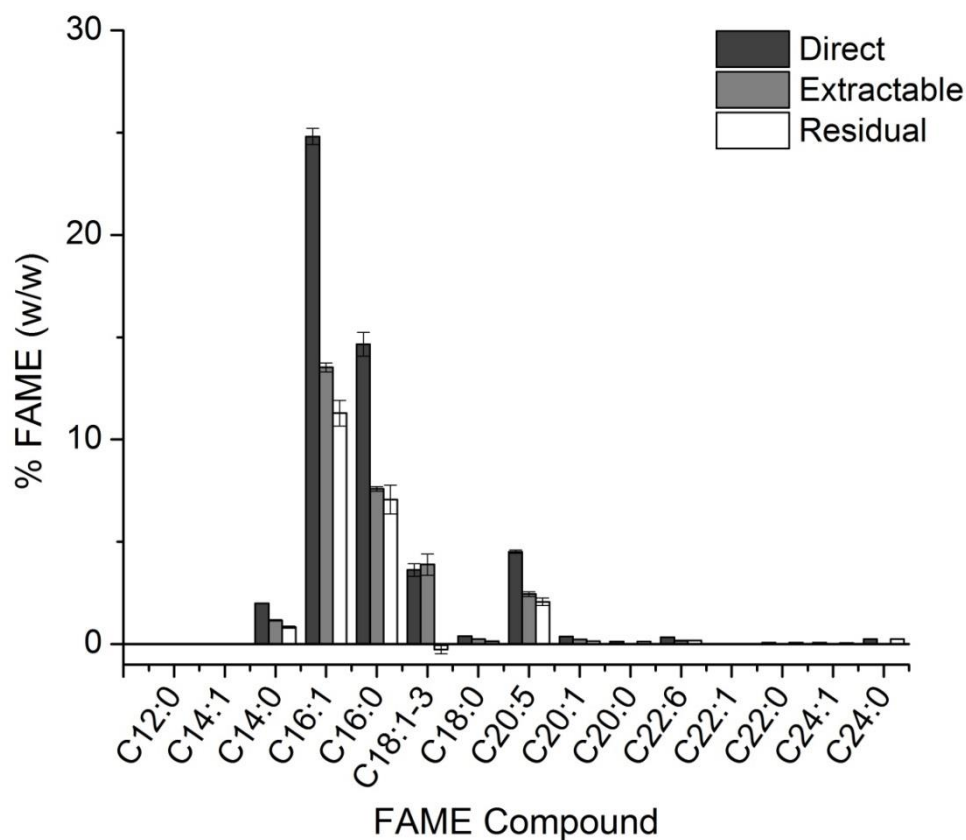


Figure 2.3: Average and standard deviation of the FAME profile for *P. tricornutum* comparing direct in situ transesterification of all fatty acids (Direct) to FAME derived from only extractable lipid precursors (Extractable). The residual fraction is the difference between direct and extractable transesterification (n=3).

derived from extractable lipid was 21.1 ± 0.3 % (w/w) (Table 2.4) and total C₁₆ TAG was 21.4 ± 0.8 % (w/w) (Table 2.3), indicating that these C₁₆ FAMES derived from extractable lipid potentially originated as C₁₆ TAG. Additionally, Fig. 2.3 shows that all

unsaturated C₁₈ FAMES were derived from extractable lipid. The sum of these unsaturated C₁₈ FAMES was 3.9 ± 0.35 % (w/w) (Table 2.4) and total C₁₈ TAG was 3.4 ± 0.04 % (w/w) (Table 2.3), indicating that the majority of unsaturated C₁₈ FAMES originated as C₁₈ TAG. Furthermore, under these conditions *P. tricornutum* produced 4.52 ± 0.07 % (w/w) eicosapentaenoic acid (EPA) (Table 2.4), which is a valuable omega-3 fatty acid marketed for its suggested beneficial effects on human health (Alonso et al., 1996). Over half of the total EPA produced was derived from extractable lipid (Table 2.4), all of which originated as C₂₀ TAG (Table 2.3). Of the 1.99 ± 0.02 % (w/w) C14:0 FAME produced by this culture, 58% were derived from extractable lipid, of which 57% originated as C₁₄ TAG and 43% originated as C₁₄ MAG.

Chlorella vulgaris Cultures of *C. vulgaris* were sparged continuously with air amended with 5% CO₂. Once peak TAG accumulation was reached, as monitored by Nile Red fluorescence (Table 2.1), the cultures were harvested for lipid extraction, total FAME determination and an estimate of FAME derived from extractable lipids. Fig. 2.4 shows the complete FAME profile for *C. vulgaris*. Interestingly, this fresh water green microalga preferentially synthesized unsaturated C₁₈ fatty acids under the culture conditions employed here, compared to *P. tricornutum* which predominantly synthesized unsaturated C₁₆ fatty acids. Total FAME content for *C. vulgaris* was 33% (w/w) and the sum of all extractable lipids was 21.6 % (w/w) with TAG contributing the majority at 17.4 % (w/w) or 80.6 % of the total extract (Table 2.2). Twenty two percent (w/w) of total FAME was unsaturated C₁₈ fatty acids, of which 66% were derived from extractable lipid (Table 2.4).

Table 2.3: Comparisons of mean and standard deviation of lipid class and carbon chain length percentages of extractable lipid constituents from *P. tricornutum*, *C. vulgaris* and *C. reinhardtii* (n=3). All values expressed as % (lipid/CDW).

Free fatty acid (%)^a						
Organism	C₁₂	C₁₄	C₁₆	C₁₈	C₂₀	C₂₂
<i>P. tricornutum</i>	N/D	0.25 ± 0.02	1 ± 0.19	0.13 ± 0	0.01 ± 0.01	N/D
<i>C. vulgaris</i>	0.11 ± 0.03	0.23 ± 0.04	0.57 ± 0.18	0.92 ± 0.28	0.05 ± 0.02	N/D
<i>C. reinhardtii</i>	N/D	N/D	1.13 ± 0.05	1.43 ± 0.11	N/D	N/D
Mono-glyceride (%)^a						
Organism	C₁₂	C₁₄	C₁₆	C₁₈	C₂₀	C₂₂
<i>P. tricornutum</i>	N/D	0.56 ± 0.04	0.04 ± 0.03	0.06 ± 0.01	N/D	N/D
<i>C. vulgaris</i>	N/D	N/D	N/D	0.06 ± 0.01	N/D	N/D
<i>C. reinhardtii</i>	N/D	N/D	0.02 ± 0.01	0.06 ± 0.03	N/D	N/D
Di-glyceride (%)^a						
Organism	C₁₂	C₁₄	C₁₆	C₁₈	C₂₀	C₂₂
<i>P. tricornutum</i>	0.39 ± 0.08	0.02 ± 0.01	1.06 ± 0.04	0.61 ± 0.19	N/D	N/D
<i>C. vulgaris</i>	0.24 ± 0.03	0.07 ± 0.01	0.48 ± 0.09	1.47 ± 0.24	N/D	N/D
<i>C. reinhardtii</i>	0.24 ± 0	0.38 ± 0.02	0.45 ± 0.01	1.36 ± 0.04	N/D	N/D
Tri-glyceride (%)^a						
Organism	C₁₂	C₁₄	C₁₆	C₁₈	C₂₀	C₂₂
<i>P. tricornutum</i>	N/D	0.73 ± 0.06	21.38 ± 0.81	3.4 ± 0.04	1.87 ± 0.47	N/D
<i>C. vulgaris</i>	N/D	0.1 ± 0.1	4.29 ± 0.5	12.81 ± 0.35	0.15 ± 0.07	N/D
<i>C. reinhardtii</i>	N/D	0.01 ± 0.01	2.05 ± 0.12	1.4 ± 0.17	N/D	N/D

^a From microwave extraction of wet biomass

N/D – not detected

The sum of these unsaturated C₁₈ FAMES derived from extractable lipids was 14.7 ± 0.2 % (w/w) (Table 2.4) and total C₁₈ TAG was 12.8 ± 0.35 % (w/w) (Table 2.3). This indicates that the majority of unsaturated C₁₈ FAMES were derived from extractable lipid and originated as C₁₈ TAG, with the remainder originating as C₁₈ DAG (1.5% w/w) and C₁₈ FFA (1% w/w) (Table 2.3).

Table 2.4: Comparisons of mean and standard deviation of percent composition of FAMES derived from *in situ* transesterification, FAMES derived from extractable lipid, and the difference being reported as the residual fraction of total FAME for *P. tricornutum*, *C. vulgaris* and *C. reinhardtii* (n=3). All values expressed as % (lipid/CDW).

Direct Trans-esterification % Lipid Content (w/w)											
Organism	C14:0	C16:1	C16:0	C18:1-3 ^b	C18:0	C20:5	C20:1	C20:0	C22:6	C24:0	Other ^c
<i>P. tricornutum</i>	1.99 ± 0.02	24.81 ± 0.4	14.66 ± 0.59	3.62 ± 0.32	0.39 ± 0.01	4.52 ± 0.07	0.37 ± 0.01	0.12 ± 0	0.34 ± 0.01	0.25 ± 0	0.13 ± 0.02
<i>C. vulgaris</i>	0.04 ± 0	3.07 ± 0.07	6.88 ± 0.17	22.18 ± 0.72	0.67 ± 0.02	N/D	0.05 ± 0.03	0.04 ± 0	N/D	0.05 ± 0	0.03 ± 0.02
<i>C. reinhardtii</i>	0.04 ± 0	2.01 ± 0.1	3.35 ± 0.22	8.64 ± 0.53	0.33 ± 0.03	N/D	0.07 ± 0.04	0.05 ± 0.03	N/D	N/D	0.02 ± 0.0
% Extractable Lipid Fraction (w/w) ^a											
Organism	C14:0	C16:1	C16:0	C18:1-3 ^b	C18:0	C20:5	C20:1	C20:0	C22:6	C24:0	Other ^c
<i>P. tricornutum</i>	1.16 ± 0.04	13.52 ± 0.22	7.59 ± 0.12	3.89 ± 0.52	0.25 ± 0.02	2.45 ± 0.12	0.23 ± 0.01	N/D	0.16 ± 0.02	N/D	N/D
<i>C. vulgaris</i>	N/D	2.57 ± 0.16	4.04 ± 1.08	14.72 ± 0.19	0.65 ± 0.05	N/D	N/D	N/D	N/D	N/D	N/D
<i>C. reinhardtii</i>	0.03 ± 0	1.99 ± 0.31	1.7 ± 0.14	4.28 ± 0.27	0.03 ± 0	N/D	0.04 ± 0.02	0.03 ± 0.02	N/D	N/D	N/D
% Residual Lipid Fraction (w/w)											
Organism	C14:0	C16:1	C16:0	C18:1-3 ^b	C18:0	C20:5	C20:1	C20:0	C22:6	C24:0	Other ^c
<i>P. tricornutum</i>	0.83 ± 0.06	11.29 ± 0.62	7.07 ± 0.7	-0.27 ± 0.2	0.14 ± 0.03	2.07 ± 0.19	0.14 ± 0.01	0.12 ± 0	0.18 ± 0.02	0.25 ± 0	0.13 ± 0.02
<i>C. vulgaris</i>	0.04 ± 0	0.49 ± 0.05	2.84 ± 0.42	7.46 ± 1.1	0.02 ± 0.02	N/D	0.05 ± 0.03	0.04 ± 0	N/D	0.05 ± 0	0.03 ± 0.02
<i>C. reinhardtii</i>	0.02 ± 0	0.02 ± 0.71	1.65 ± 0.83	4.36 ± 0.21	0.3 ± 0.03	N/D	0.04 ± 0.06	0.01 ± 0.01	N/D	N/D	0.02 ± 0.0

^a From microwave extraction of wet biomass, followed by transesterification of extracted lipid

^b C18:1, C18:2, and C18:3 taken together

^c Sum of other compounds detected

N/D – not detected

Furthermore, 10% (w/w) of total FAMES for this culture of *C. vulgaris* were C₁₆ fatty acid methyl ester, with 6.6% (w/w) derived from extractable lipid. Table 2.3 shows

that 65% of the C₁₆ FAMES derived from extractable lipid were originally C₁₆ TAG with the remainder originating as C₁₆ DAG and C₁₆ FFA.

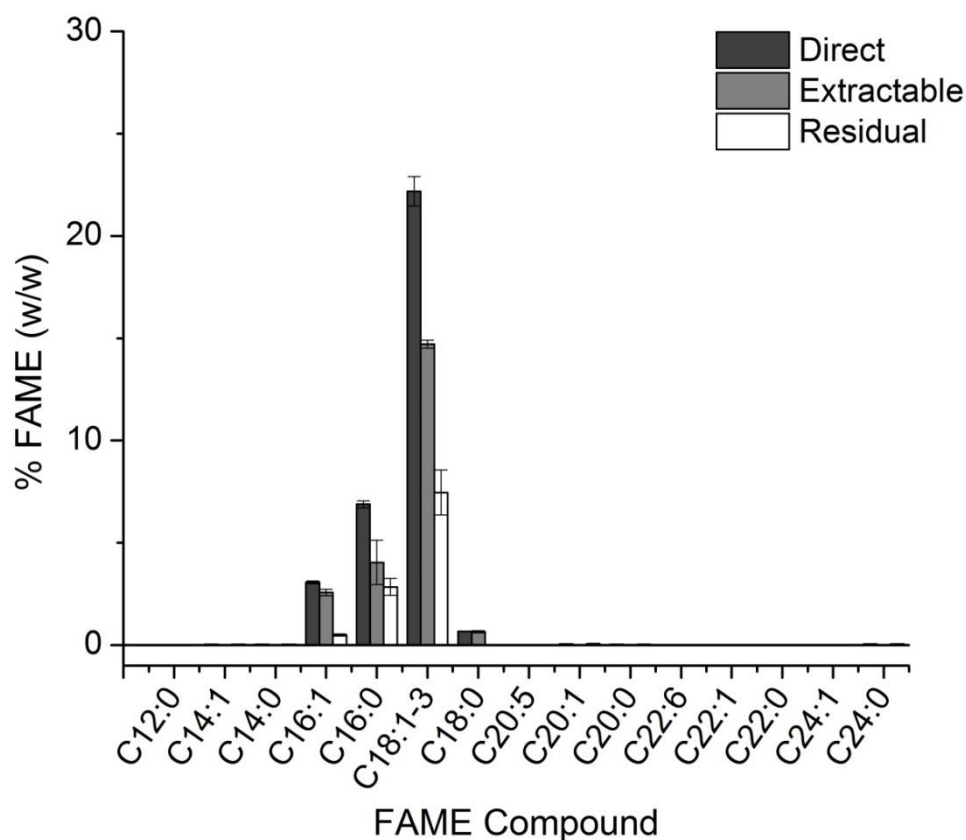


Figure 2.4: Average and standard deviation of the FAME profile for *C. vulgaris* comparing direct *in situ* transesterification of all fatty acids (Direct) to FAME derived from only extractable lipid precursors (Extractable). The residual fraction is the difference between direct and extractable transesterification (n=3).

Chlamydomonas reinhardtii The model Chlorophyte, *C. reinhardtii*, was cultured as previously reported by Gardner et al. (2013) until peak TAG accumulation was achieved as monitored by Nile Red fluorescence (Table 2.1). The cultures were harvested and lipid was extracted using the microwave extraction method. Total FAMES were determined using direct *in situ* transesterification, and FAMES derived from

extractable lipid were determined as described above. Total FAME content was determined to be 14.5 % (w/w) and the sum of all extractable lipids was 8.5 % (w/w) with TAG contributing the majority at 3.5% (w/w) or 41.2 % of the extract and DAG accounting for 2.4% (w/w) or 28.2 % of the extract (Table 2.2). *C. reinhardtii* is not known as an organism capable of producing a significant amount of TAG, and therefore is not typically regarded as a contender for industrial production of algal biofuel (Hu et al., 2008). However, it is widely studied and one of the first microalgae to have its genome fully sequenced. Thus, it is still a reasonable candidate for future research regarding lipid profile and metabolite analysis. Figure 2.5 shows the complete FAME profile for *C. reinhardtii*.

Similar to *C. vulgaris*, *C. reinhardtii* predominantly synthesized unsaturated C₁₈ lipid compounds. Total unsaturated C₁₈ FAMES were 8.6 % (w/w), of which ~50% were derived from extractable lipid. Unlike *C. vulgaris*, in this culture of *C. reinhardtii*, not all of the C₁₈ FAMES derived from extractable lipid came from C₁₈ TAG. Instead, ~33% were originally C₁₈ FFA, ~33% C₁₈ DAG and ~33% C₁₈ TAG (Table 2.3). All unsaturated C₁₆ FAME compounds were derived from extractable lipid. However, the original source of these compounds is ambiguous. Total unsaturated C₁₆ FAMES derived from extractable lipid were 1.99 ± 0.31 % (w/w), total C₁₆ FFA were 1.0 ± 0.19 % (w/w), total C₁₆ DAG were 0.45 ± 0.01 % (w/w) and total C₁₆ TAG were 2.05 ± 0.12 % (w/w). Unambiguously discerning which fraction of these extractable lipid precursors were responsible for the unsaturated C₁₆ FAMES is not possible with this method and will require additional method development.

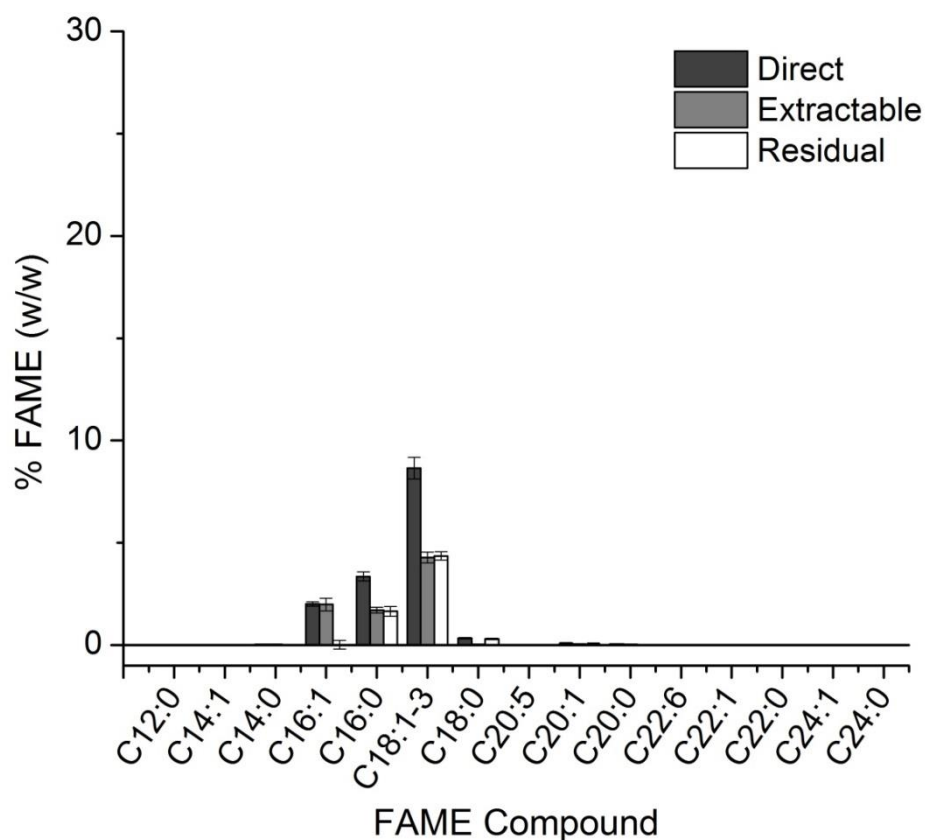


Figure 2.5: Average and standard deviation of the FAME profile for *C. reinhardtii* comparing direct in situ transesterification of all fatty acids (Direct) to FAME derived from only extractable lipid precursors (Extractable). The residual fraction is the difference between direct and extractable transesterification (n=3).

Conclusion

We describe a new rapid method for the efficient extraction of lipids from live cultures of microalgae for the purpose of quantifying the lipid content and characterizing the lipid carbon chain length and saturation. Additionally, we developed methods to determine the fraction of total biodiesel potential (as FAME) derived either from extractable lipid or the residual fraction. Furthermore, the method can frequently determine from which lipid class (FFA, MAG, DAG, TAG) a particular FAME was

derived. Since properties of biodiesel depend heavily on the length and degree of saturation of the FAME, the methods described here can assist in ‘fine-tuning’ the final product, be it biodiesel or a higher value product, through the choice of certain algae or growth conditions.

Specifically, here we demonstrate the usefulness of this method by demonstrating differences in lipid profiles for three frequently studied microalgae. It is demonstrated that the marine diatom *P. tricornutum* preferentially produced unsaturated C₁₆ lipid compounds, whereas the two freshwater green microalgae, *C. vulgaris* and *C. reinhardtii*, predominantly synthesized unsaturated C₁₈ compounds. From an industrial standpoint, it will be beneficial to choose the appropriate algae and culture conditions to produce the largest possible amount of biodiesel with the appropriate FAME profile or to screen for algal products with higher value than biodiesel. For instance *P. tricornutum* was shown to produce an omega-3 fatty acid, specifically eicosapentaenoic acid (C20:5), which has been suggested to benefit human health. This method has determined that these compounds were primarily derived from triacylglyceride storage molecules.

In order to apply the methods described here to other algae, plants or different culturing conditions, verification of extraction and transesterification efficacy will have to be conducted. Such checks are fairly quick and are described in detail for the algae and culturing conditions employed here.

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Supplemental Material

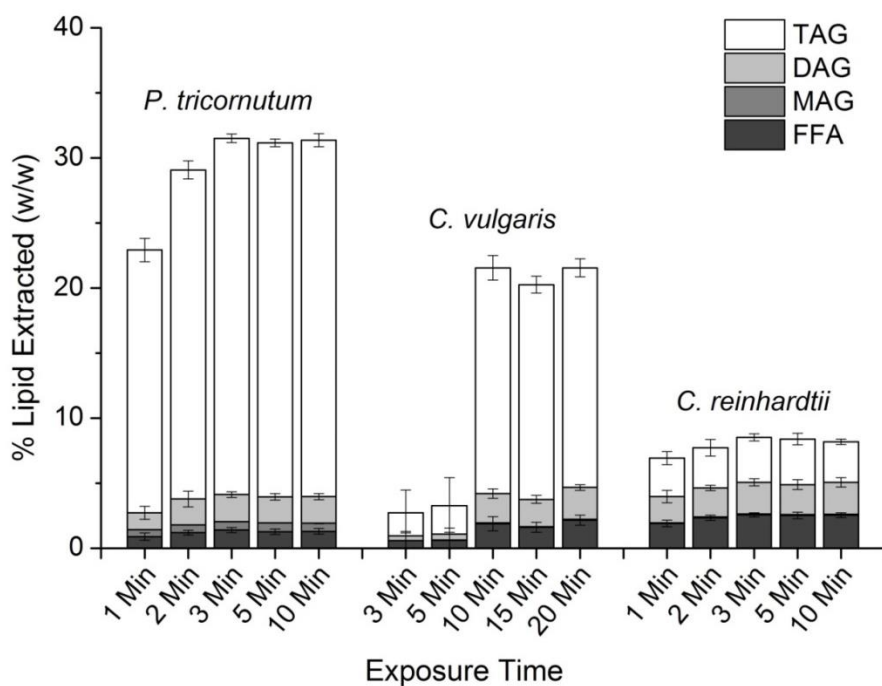


Figure 2.S1: Average and standard deviation of extractable lipid % (w/w) using microwave energy on live cultures of *P. tricornutum*, *C. vulgaris* and *C. reinhardtii*. Samples were exposed to microwave energy for various lengths of time to test the efficacy of extraction and determine the optimal time of exposure to microwave energy (n=3). These parameters were determined for each organism by comparing the percent of extractable lipid (w/w) released for each exposure time length. The “shortest” time length resulting in maximum lipid recovered was chosen as the optimal exposure time.

CHAPTER 3

CARBON PARTITIONING IN LIPIDS SYNTHESIZED BY *CHLAMYDOMONAS*
REINHARDTII WHEN CULTURED UNDER THREE UNIQUE
INORGANIC CARBON REGIMES

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Conceived the study, collected and analyzed data, and wrote the manuscript.

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Contributions: Collected and analyzed data, assisted with study design, discussed the results, and edited the manuscript.

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Contributions: Discussed the results and implications, commented and edited the manuscript.

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Contributions: Assisted with study design, analyzed data, and edited the manuscript.

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Title: Carbon Partitioning in Lipids Synthesized by *Chlamydomonas reinhardtii* when Cultured Under Three Unique Inorganic Carbon Regimes

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Abstract

Using relatively straight forward methods for quantifying free fatty acids (FFA), mono- (MAG), di- (DAG), tri-acylglycerides (TAG), and total cellular fatty acids (FAME), lipid profiles over time were established for *Chlamydomonas reinhardtii* when grown under three unique inorganic carbon regimes. Prior to nitrogen depletion in the medium, FFA and MAG were the predominant lipid metabolites, but were quickly reallocated into DAG and subsequently TAG after nitrogen depletion. C₁₆ DAG did not accumulate significantly in any of the treatments, whereas the C₁₈ DAG content increased throughout both exponential and stationary growth. C₁₆ and C₁₈ TAG began to accumulate after nitrogen depletion, with C₁₆ TAG contributing the most to overall TAG content. C₁₆ fatty acids exhibited a shift towards saturated C₁₆ fatty acids after nitrogen depletion. Results provide insight into inorganic carbon partitioning into lipid compounds and how the organism's lipid metabolism changes due to N-deplete culturing and inorganic carbon source availability.

Key Words

Triacylglycerol (TAG), microalgae, biodiesel, fatty acid methyl ester (FAME), Nile Red fluorescence, *Chlamydomonas reinhardtii*

Introduction

The political, economic, and environmental uncertainties facing traditional production of petroleum is driving research and investment towards renewable sources of

biofuel (Dismukes et al., 2008; Greenwell et al., 2010; Groom et al., 2008; Hill et al., 2006; Sheehan, 1998). Feedstocks such as corn, soybean, and sugar cane are the current primary sources for bioethanol and biodiesel, however these crops compete for arable land needed to produce food (Ferrell and Sarisky-Reed, 2010). Microalgae may provide a sustainable source for renewable biofuels due to their high biomass productivity, relatively small land mass requirements, and high lipid yield (Chisti, 2007; Hu et al., 2008).

Commercialization of algae derived biofuels has been limited in part due to downstream processes such as harvesting, extraction, and refinement. However, overcoming cultivation challenges involving contamination of growth reactors, microalgae strain selection, and techniques to stimulate higher lipid yield may potentially increase production in the future (Hunter-Cevera et al., 2012). In fact, maximizing culture lipid content has been identified as having the greatest potential for cost reduction and should be a near-term research focus (Davis et al., 2011).

Biomass produced from healthy cultures of microalgae consists mainly of proteins, carbohydrates, inorganic salts, moisture, and lipids (Laurens et al., 2012a). Accumulation of energy-rich storage compounds such as starch and triacylglycerides (TAG) occurs when cultures are exposed to nutrient stress, osmotic stress, radiation, pH fluctuations, temperature fluctuations, and/or chemical additions (Gardner et al., 2010, 2012; Sharma et al., 2012; Skrupski et al., 2012). However, these stress effects can greatly reduce the overall productivity of the system (Chisti, 2007; Hu et al., 2008). Strain performance of microalgae cultures, in terms of lipid productivity, is assessed by

total fatty acid content at time of harvest (Greenwell et al., 2010). Fatty acid methyl esters (FAMES) can be derived from energy-rich storage compounds such as TAG by chemical transesterification of the extracted lipids. Although much attention is focused on increasing TAG content in a strain, other lipid compounds such as free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG) and residual lipids (*e.g.* membrane bound phospholipids, sterols, etc.) also contribute to total FAME content. Improving strain performance requires a better understanding of how and when each of these compounds is synthesized (Greenwell et al., 2010).

To overcome some of these challenges we utilize a new method previously reported (Lohman et al., 2013) to track changes in lipids synthesized by the model Chlorophyte *Chlamydomonas reinhardtii* CC124 when grown under three unique inorganic carbon regimes. *C. reinhardtii* is a widely studied microalga with a fully sequenced genome and extensive library of peer reviewed literature, making it an ideal candidate to further our investigations into the mechanisms and regulations of lipid synthesis, carbon fixation, carbon allocation between biosynthetic pathways and induction by stress. Although the wild type of *C. reinhardtii* is not typically regarded as an oleaginous microalga, this study of lipid biosynthesis may provide knowledge that can be transferred to other species or mutants that are more suited for large scale biofuel production. To our knowledge, there have been no previous reports which provide such a comprehensive lipid profile for this organism. Not only are total FAMES measured over time, but the lipid class (FFA, MAG, DAG, TAG) and carbon chain length of the precursor lipid compounds are measured as well. This work investigates how various

inorganic carbon substrates affect growth, inorganic carbon partitioning into lipids and lipid biosynthesis in *C. reinhardtii*.

Materials and Methods

Strain and Culturing Conditions

Chlamydomonas reinhardtii CC124, obtained from the Chlamydomonas Center (Minnesota State University, Minneapolis MN), was kindly provided by John Peters, Department of Chemistry and Biochemistry at Montana State University, and was cultured on Sager's minimal medium in order to minimize heterotrophic influence (Harris, 1989). Cultures were grown under three different inorganic carbon regimes as detailed below.

Low CO₂

Cultures were sparged with atmospheric air, containing ambient concentrations of CO₂ (0.04%; v/v) continuously.

High CO₂

Cultures were sparged with atmospheric air amended with 5% CO₂ (v/v) during the light hours and switched to ambient concentrations of CO₂ (0.04%; v/v) during the dark hours.

Bicarbonate

Cultures were sparged with atmospheric air amended with 5% CO₂ (v/v) during the light hours and switched to ambient concentrations of CO₂ (0.04%; v/v) during the dark hours until just prior to ammonium depletion. At which time, the gas sparge was

switched to ambient concentrations of CO₂ (0.04%; v/v), and 50 mM of sodium bicarbonate (final concentration) was added to induce TAG accumulation, as done in previous studies with *C. reinhardtii* (Gardner et al., 2013a).

Culture Analysis

Cultures were checked for bacterial contamination by inoculation into Sager's minimal medium supplemented with 0.05% yeast extract and 0.05% glucose and incubated in the dark. Experiments were conducted in triplicate batch cultures using 70×500 mm glass tubes containing 1.2 L medium submersed in a water bath to control temperature at 24°C ± 1°C. Rubber stoppers, containing ports for aeration and sampling, were used to seal the tubes. Light (400 μmol photons m⁻² s⁻¹) was maintained on a 14:10 light–dark cycle using a vertical light bank containing T5 fluorescent bulbs. Aeration (400 mL min⁻¹) was supplied by humidified atmospheric air (amended with 5% CO₂ (v/v) for High CO₂) and controlled using individual rotameters for each bioreactor (Cole-Parmer, USA). ACS grade sodium bicarbonate was used in all experiments involving bicarbonate addition (Sigma-Aldrich, St. Louis MO).

Cell concentrations were determined using an optical hemacytometer with a minimum of 400 cells counted per sample for statistical reliability. Dry cell weights (DCWs) were determined by harvesting 30 mL of culture into a tared 50 mL centrifuge tube (Fisher Scientific, Palatine, IL) followed by centrifugation at 4800x *g* at 4°C for 10 minutes (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H₂O (diH₂O), 18 MΩ, to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were then frozen

and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours. DCWs were calculated by subtracting the weight of the biomass-free centrifuge tube from the weight of the centrifuge tube with lyophilized biomass.

Analysis of Media Components

Medium pH was measured using a standard bench top pH meter. Ammonium and nitrate concentrations were measured using Nessler reagent (HACH, Loveland, CO) and ion chromatography (Dionex, Sunnyvale, CA), respectively, using previously described protocols (Gardner et al., 2013a).

Total Protein and Chlorophyll Measurements

Total protein content of algal cultures were determined as follows: approximately 5 mg lyophilized biomass were suspended in 250 mL lysis buffer (50 mM Tris 7.8, 2% SDS, 10 mM EDTA, Protease Inhibitor (Sigma-Aldrich, St. Louis MO)) and incubated at room temperature for 1 h. Cellular debris was concentrated by centrifugation at 12,000x *g* for 10 min. Total protein in the supernatant was determined by Coomassie Plus assay kit (Thermo Scientific, USA) using the manufacturer's protocol.

Chlorophyll a, b, and total were determined using 95% ethanol extraction and absorption correlation. One mL of culture was centrifuged at 6,000x *g* for 5 min, after which the supernatant was discarded. One mL 95% ethanol was added to the centrifuged pellet, which was then vortexed and lightly sonicated to disperse the pellet. The cellular debris was pelleted by centrifugation at 14,000x *g* for 3 min and absorption was read at

649 and 665 nm. Calculations of chlorophyll ($\mu\text{g mL}^{-1}$) were conducted as previously described (Harris 1989).

Harvesting

Cultures were harvested once per day throughout the experiments. Two aliquots of 45 mL were dispensed into 50-mL centrifuge tubes (Fisher Scientific, Palatine, IL) and centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 4800x *g* at 4°C for 10 minutes. Concentrated biomass was then transferred to Pyrex test tubes with Teflon lined screw caps (Kimble-Chase, Vineland, NJ) for microwave extraction of lipid precursors and direct *in situ* transesterification for total FAME analysis, respectively.

Microwave Extraction of Lipid from Wet Biomass

Extraction of lipid using microwave energy was conducted according to previously described procedures (Lohman et al., 2013). Briefly, the biomass pellet, obtained from 45 mL of culture via centrifugation and decanting in a 50 mL centrifuge tube, was re-suspended in 3 mL diH₂O to facilitate transfer into screw-cap glass tubes for subsequent lipid extraction. Caps were securely fastened and samples were microwaved (Sharp Carousel, Mahwah, NJ) at 1000 watts on power level 1 (~100°C and 2450 MHz) for 1 min and then allowed to cool for 5 min. This process of vortexing for 10 s, microwaving for 1 min and cooling for 5 min was repeated until optimal extraction efficacy was established (Lohman et al., 2013). Three mL of chloroform was added to the algal slurry in the test tubes, and the mixture was homogenized by vortexing for 10 s. Samples were then centrifuged at 1,200x *g* for 2 min to enhance phase separation. One

mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial (Fisher Scientific, Palatine, IL) for gas chromatography – flame ionization detection (GC-FID) analysis.

Direct *in situ* Transesterification for FAME Analysis

Direct *in situ* transesterification of live cultures was conducted using a previously described protocol (Griffiths et al., 2010) with modifications. FAME composition was analyzed using gas chromatography – mass spectroscopy detection (GC-MS). Briefly, the biomass pellet, obtained from 45 mL of culture via centrifugation and decanting in a 50 mL centrifuge tube, was re-suspended in 3 mL diH₂O to facilitate transfer from the centrifuge tubes to screw-cap glass tubes. Test tubes were centrifuged at 1,200x *g* for 5 min and decanted to remove as much water from the remaining biomass as possible. One mL of toluene and 2 mL sodium methoxide (Fisher Scientific, Pittsburgh PA) were added to each test tube along with 10 µL of a 10mg/mL standard mix (C11:0 and C17:0 TAG) to monitor transesterification efficiency of the TAG into FAME. Samples were heated in an oven for 30 min at 90°C and vortexed every 10 min. Samples were allowed to cool to room temperature before 2 mL of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) were added and samples were heated again for an additional 30 min. Samples were again allowed to cool before 10 µL of a 10 mg/mL of C23:0 FAME was added to assess the completeness of partitioning FAME into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated salt water solution (NaCl in diH₂O) were added. Samples were heated for 10 min at 90°C to facilitate FAME partitioning into the organic phase, vortexed for 10 s and centrifuged at 1,200x *g* for 2

min to enhance phase separation. One mL of the organic phase was removed from the top layer using a glass syringe and transferred to a 2 mL GC vial for GC-MS analysis.

Lipid Analysis

Nile Red Cellular TAG accumulation was monitored throughout the experiments using the Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one) (Sigma-Aldrich, St. Louis, MO) fluorescence method (Cooksey et al., 1987), which has become a generally accepted screening method for analyzing TAG in algal cultures both in academia and industry (*e.g.* Chen et al., 2009; Cooksey et al., 1987; da Silva et al., 2009; Elsey et al., 2007). Furthermore, Nile Red fluorescence has been correlated to TAG content using gas chromatography (GC) analysis on extracted lipid from *C. reinhardtii*, $r^2 = 0.998$ (Gardner et al., 2013a). Briefly, 1 mL aliquots were removed from cultures and assayed directly with Nile Red (4 μ L from 250 μ L/mL in acetone) or by diluting 1:5 with diH₂O before staining with Nile Red (20 μ L from 250 μ L/mL in acetone). To maintain linearity of the Nile Red assay, dilution was required when population counts exceeded 1×10^7 cells/mL. Total Nile Red fluorescence was quantified on a microplate reader (Bio-Tek, USA) utilizing a monochromator set to 480/580 nm excitation/emission wavelengths. A baseline sensitivity setting of 75 was experimentally determined to maximize the signal-to-noise ratio, while accommodating fluorescent level changes over 10,000 units. To minimize fluorescence spillover, black walled 96 well plates were loaded with 200 μ L of sample. Unstained samples were used for background medium and cellular autofluorescence correction.

GC-FID GC-FID analysis was performed according to our previously published protocol (Lohman et al., 2013). Briefly, a 1 μL splitless injection was performed via an autosampler into a GC-FID (Agilent 6890N, Santa Clara CA) equipped with a 15 m (fused silica) RTX biodiesel column (Restek, Bellefonte PA). The initial column temperature was held at 100°C for 1 min, before being increased to 370°C at a rate of 10°C min⁻¹. The injector temperature was held constant at 320°C. Helium was used as the carrier gas and column flow was held at 1.3 mL min⁻¹ for 22 min, increased to 1.5 mL min⁻¹, held for 2 min, increased to 1.7 mL min⁻¹ and held for 12 min. All flow rate increases were set to 0.2 mL min⁻². Calibration curves were constructed for each of the following standards: C12:0, C14:0, C16:0, C18:0, and C20:0 FFAs; C12:0, C14:0, C16:0, and C18:0 MAGs; C12:0, C14:0, C16:0, and C18:0 DAGs; along with C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, and C20:0 TAGs (Sigma-Aldrich, St. Louis MO) for quantification ($r^2 > 0.99$). This GC method allows for an estimate of the amounts of FFA, MAG, DAG and TAGs in a single analysis as detailed in Lohman et al., (2013).

GC-MS GC-MS analysis was performed according to a previously published protocol (Bigelow et al., 2011). Briefly, 1 μL splitless injections were performed via an autosampler into a GC-MS (Agilent 6890N GC and Agilent 5973 Networked MS) equipped with a 30 m x 0.25 mm Agilent HP-5MS column (0.25 μm phase thickness). The injector temperature was 250°C and the detector temperature was 280°C. The initial column temperature was 80°C and was increased to 110°C at a rate of 8.0°C min⁻¹, immediately followed by a ramp at 14.0°C min⁻¹ to a final temperature of 310°C which was held for 3 min before run termination. Helium was used as the carrier gas and

column flow was held at 0.5 mL min⁻¹. Quantities of FAMES were determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275), with additional analyses performed using a custom program described below. A 28-component fatty acid methyl ester standard in methylene chloride (“NLEA FAME mix”; Restek, Bellefonte, PA) was used for GC-MS retention time identification and response curve generation ($r^2 > 0.99$). It should be noted that C18:1, C18:2 and C18:3 co-elute on the column used and have similar fragmentation patterns. They were therefore quantitated together as C18:1-3.

Lipid Quantification and Analysis

Lipid quantification was performed using a custom program developed specifically for this purpose (Appendix B). Chromatogram data from the GC-FID and GC-MS were exported from the instrument software (Chemstation Ver. B.02.01-SR1) as a Microsoft Excel spreadsheet. The spreadsheet was then imported into a web-based software application written in Microsoft’s ASP.NET framework using C# and MVC as the coding language and paradigm, respectively. Data were stored with unique identifiers in a Microsoft SQL database. Six point calibration curves were constructed using the LINEST function which is built into the ASP.NET framework library. Chromatogram peaks were quantified based on the closest standard, as determined by retention time of the sample peak and standard peak, respectively. All chromatograms were manually integrated and inspected prior to export to ensure accuracy and reliability. Manual calculations were performed periodically using Excel to verify software results.

Results and Discussion

Lipid content of microalgal cultures are traditionally analyzed at time-of-harvest by extraction and subsequent gravimetric analysis or by transesterification and subsequent quantification of the resultant FAME content. Many analyses include direct or *in situ* transesterification of dry biomass and subsequent FAME analysis. These approaches, however, are unable to identify partitioning of inorganic carbon into fatty acid or from where a specific compound was derived. Further, these approaches do not provide a comprehensive evaluation of lipid profiles over time. By using relatively straight-forward methods for quantifying extractable lipid classes, including FFA, MAG, DAG and TAG via GC-FID, and total FAME via GC-MS, lipid profiles over time were established for *C. reinhardtii* when grown under three unique inorganic carbon regimes. Extractable lipids were characterized to include lipid class (FFA, MAG, DAG, TAG) and carbon chain length for comparison against FAMEs. Data points were collected each day throughout the growth cycle to provide comprehensive lipid profile analyses.

Culturing Characteristics

To further the current state of knowledge of lipid biosynthesis in *C. reinhardtii*, the organism was cultured with atmospheric concentrations of CO₂ (0.04% v/v) or elevated CO₂ (5% v/v) until near nitrogen depletion in the culturing medium. At which time, the 5% CO₂ systems were either left at 5% CO₂ or switched to atmospheric CO₂ and amended with 50 mM NaHCO₃. This was done to evaluate three different inorganic carbon regimes during the end of the exponential growth phase and during TAG

accumulation (*i.e.*, high CO₂, low CO₂, or low CO₂ with high bicarbonate concentrations). Cell concentrations and CDW are evaluated over time (Figure 3.1a) and experimental results are tabulated for two time points in Table 3.1; directly before nitrogen depletion in the bulk medium (2.8 d) and at the end of the experiments (7 d).

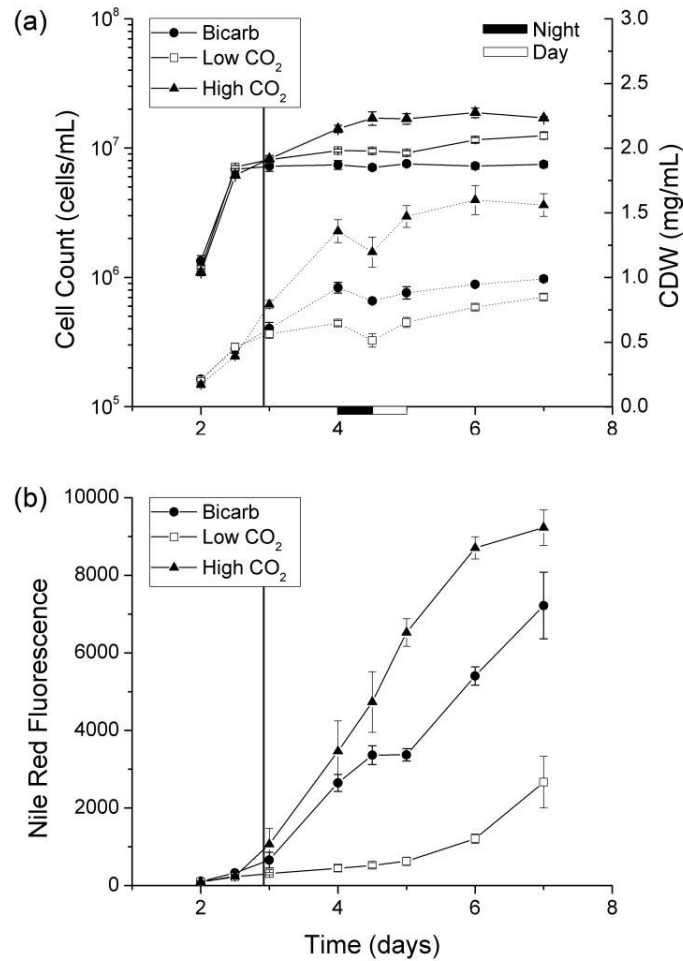


Figure 3.1: Mean and standard deviation of (a) cell counts (solid lines) and cell dry weights (dotted lines), (b) Nile Red fluorescence intensity for cultures of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻ (n=3). Line indicates time of nitrogen depletion of the culture medium.

Growth, as monitored by cell counts and DCWs, was similar between experimental conditions before nitrogen depletion (note – the low CO₂ condition was slightly higher due to an initially higher inoculum) (Figure 3.1a). However, significant differences were observed after extended culturing in nitrogen deplete media.

Table 3.1: Comparison of mean and standard deviation of culture growth, protein and chlorophyll content, and TAG accumulation, monitored by Nile Red fluorescence, of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻. Values are reported for just prior to nitrogen depletion (2.8 d) and for the completion of the experiment (7 d) (n=3).

at nitrogen depletion (2.8 d)						
Treatment	Dry weight (g L⁻¹; DCW)^a	Cell density (x10⁶ cells mL⁻¹)	Nile Red fluorescence (x10³ units)	Nile Red specific fluorescence (units cell⁻¹)^b	% protein (w/w)	Chlorophyll (a + b) (µg/mL)
50 mM HCO ₃ ⁻	0.45 ± 0.04	6.83 ± 0.45	0.327 ± 0.03	0.48 ± 0.02	38.35 ± 2.16	15.2 ± 0.55
Ambient Air	0.46 ± 0.03	7.15 ± 0.19	0.228 ± 0.06	0.32 ± 0.08	39.42 ± 2.24	15.85 ± 0.07
5% CO ₂	0.4 ± 0.03	6.16 ± 0.05	0.243 ± 0.03	0.39 ± 0.05	37.21 ± 2.43	15.25 ± 0.43
Final harvest (7 d)						
Treatment	Dry weight (g L⁻¹; DCW)^a	Cell density (x10⁶ cells mL⁻¹)	Nile Red fluorescence (x10³ units)	Nile Red specific fluorescence (units cell⁻¹)^b	% protein (w/w)	Chlorophyll (a + b) (µg/mL)
50 mM HCO ₃ ⁻	0.99 ± 0.02	7.48 ± 0.39	7.22 ± 0.86	9.69 ± 1.51	30.71 ± 0.98	14.71 ± 0.34
Ambient Air	0.84 ± 0.02	12.5 ± 0.08	2.67 ± 0.67	2.16 ± 0.62	35.52 ± 1.28	17.16 ± 0.27
5% CO ₂	1.56 ± 0.09	17.1 ± 0.04	9.23 ± 0.46	5.4 ± 0.38	30.84 ± 1.32	15.21 ± 0.32

^a Dry cell weight (DCW) determined gravimetrically with lyophilized biomass

^b Calculated by fluorescence signal/cell density x 10,000 (scaling factor)

The cultures supplemented with NaHCO₃ only doubled in cell concentration once, whereas the culture growing on ambient air doubled approximately two times and the culture growing on 5% CO₂ doubled approximately three times (Table 3.1). The

cessation or delayed cell cycle in cultures supplemented with NaHCO_3 is a phenomenon that has been observed and documented for other species of green microalgae (Gardner et al., 2013a, 2012; White et al., 2012). Interestingly, the final DCW of cultures supplemented with NaHCO_3 exceeded those of cultures grown under ambient air ($0.99 \text{ g} \cdot \text{L}^{-1}$ vs. $0.84 \text{ g} \cdot \text{L}^{-1}$) due to higher TAG content. However, cultures grown on 5% CO_2 achieved the overall highest DCW of $1.56 \text{ g} \cdot \text{L}^{-1}$ (Table 3.1). Dry cell weights decreased during the dark cycle for all cultures (Figure 3.1a) due to a decrease in protein concentration (Supplemental Data, Figure 3.S1 d 4.5) which rebounded during the light hours, indicating a metabolic shift towards catabolism of protein during the dark hours after medium nitrogen depletion, as discussed below. These data provide further evidence that although *C. reinhardtii* is capable of growth under CO_2 “limited” conditions, significant increases in cell number and dry weight can be achieved by providing cultures with elevated concentrations of inorganic carbon.

Protein and total chlorophyll (a + b) content was also monitored throughout each experiment. Similar to growth, protein and chlorophyll concentrations were initially equivalent between treatments and increased throughout the exponential growth phase before nitrogen depletion. After nitrogen depletion lipids began accumulating and the protein content per cell (calculated as g of protein per g of CDW or % w/w) was reduced in all treatments (Table 3.1). The concentration of protein (g protein per liter of culture) remained roughly the same in the cultures after nitrogen depletion (Supplemental Data, Figure 3.S1) but due to the accumulation of lipids the fraction of protein per cell decreased.

Total chlorophyll content also changed after nitrogen depletion and varied significantly between treatments. Cultures supplemented with NaHCO_3 reduced their total chlorophyll content by 3%, whereas cultures grown on ambient air exhibited an 8% increase in chlorophyll content. Cultures grown on 5% CO_2 remained relatively constant in chlorophyll content (Table 3.1). Nitrogen deprivation has been shown to down-regulate protein and chlorophyll biosynthesis (Miller et al., 2010) and can result in the catabolism of protein to regenerate ammonia availability in *C. reinhardtii* (Cullimore and Sims, 1980; Msanne et al., 2012) as observed during the dark hours of culturing for all treatments (Supplemental Data, Figure 3.S1 d 4.5).

TAG Screening by Nile Red Fluorescence

The Nile Red fluorescence assay (Cooksey et al., 1987) has become a generally accepted screening method for analyzing TAG in algal cultures both in academia and industry (e.g. Chen et al., 2009; Cooksey et al., 1987; da Silva et al., 2009; Elsey et al., 2007). Cellular TAG accumulation was monitored throughout the experiments using Nile Red fluorescence as an indicator of neutral lipid content. Furthermore, the Nile Red fluorescence data presented in Figure 3.1b has been correlated to extracted TAG, as quantified by GC-FID analysis for all cultures in this experiment ($r^2 = 0.96$, Supplemental Data, Figure 3.S2). As expected, cellular TAG was below detection before nitrogen depletion, but increased in cultures supplemented with additional inorganic carbon after nitrogen depletion (Figure 3.1b). Cultures grown on ambient air accumulated TAG after nitrogen depletion, albeit at a much slower rate and to a significantly lower final concentration. Table 3.1 presents Nile Red fluorescence results for all three treatments at

two time points; directly before nitrogen depletion in the bulk medium (2.8 d) and at the end of each experiment (7 d). Data are presented as total fluorescence and specific fluorescence, where values are normalized to cell density by dividing the total fluorescence signal by cell count and multiplying by 10,000 (scaling factor). On the final day of the experiments, the cultures grown on 5% CO₂ had achieved the highest total fluorescence; however, cultures supplemented with NaHCO₃ had a 1.8 x higher specific fluorescence than cultures grown on 5% CO₂, indicating a higher TAG content per cell (Table 3.1), which was further verified by GC-FID analysis and is discussed below.

Extractable Lipid and Precursor Classification

Extractable lipid is defined here as intracellular lipids which have either been incorporated into energy storage compounds (triacylglycerides) or are precursor compounds such as free fatty acids, monoacylglycerides or diacylglycerides, which can be further processed into TAG or membrane bound lipid compounds. Extractable lipid can be liberated from a lysed cell using non-polar solvents. Previous work has shown that microwave energy is an efficient cell disruption technique for *C. reinhardtii* and can be combined with solvent extraction followed by GC-FID analysis for classification and carbon chain length identification of extractable lipid from microalgae cultures (Lohman et al., 2013).

Extractable lipid profiles were determined over time for cultures of *C. reinhardtii* and are shown in Figure 3.2. The extractable lipid profiles are based on lipid class (FFA, MAG, DAG, TAG) and are separated based on inorganic carbon treatment; a) low CO₂ and amended with 50 mM NaHCO₃, b) low CO₂, and c) high CO₂ (5% v/v). In addition,

results are tabulated for two time points in Table 3.2; directly before nitrogen depletion in the bulk medium (2.8 d) and at the end of the experiments (7 d). All three inorganic carbon treatments exhibited similar lipid profiles prior to nitrogen depletion, with a relatively higher abundance of FFA (~2.3% w/w) and MAG (~0.4% w/w), which was decreasing as DAG was increasing, with no discernible TAG (Figure 3.2, Table 3.2). However, after nitrogen depletion, a significant shift in carbon partitioning between lipid classes occurred in all three treatments. FFA and MAG content gradually declined, whereas the DAG content continued to increase until reaching a pseudo steady-state around 2.5% (w/w) for all three treatments (Figure 3.2, Table 3.2). TAGs increased directly after nitrogen depletion in cultures grown on 5% CO₂ (Figure 3.2c) and cultures amended with 50 mM NaHCO₃ (Figure 3.2a). Cultures grown on ambient air also began accumulating TAG, albeit at a slower rate, and to a significantly lower final concentration (0.76% w/w). Additionally, TAG accumulation in cultures grown on ambient air was delayed a full day after nitrogen depletion (Figure 3.2b). These data suggest a reallocation of free fatty acid into TAG biosynthesis once cultures undergo nutrient stress, and further indicate that elevated levels of inorganic carbon promote significant TAG accumulation. Therefore, this work offers additional evidence for previously reported *de novo* TAG biosynthesis and inorganic carbon supplementation hypotheses (Fan et al., 2011; Gardner et al., 2013a; Msanne et al., 2012; Sharma et al., 2012; White et al., 2012).

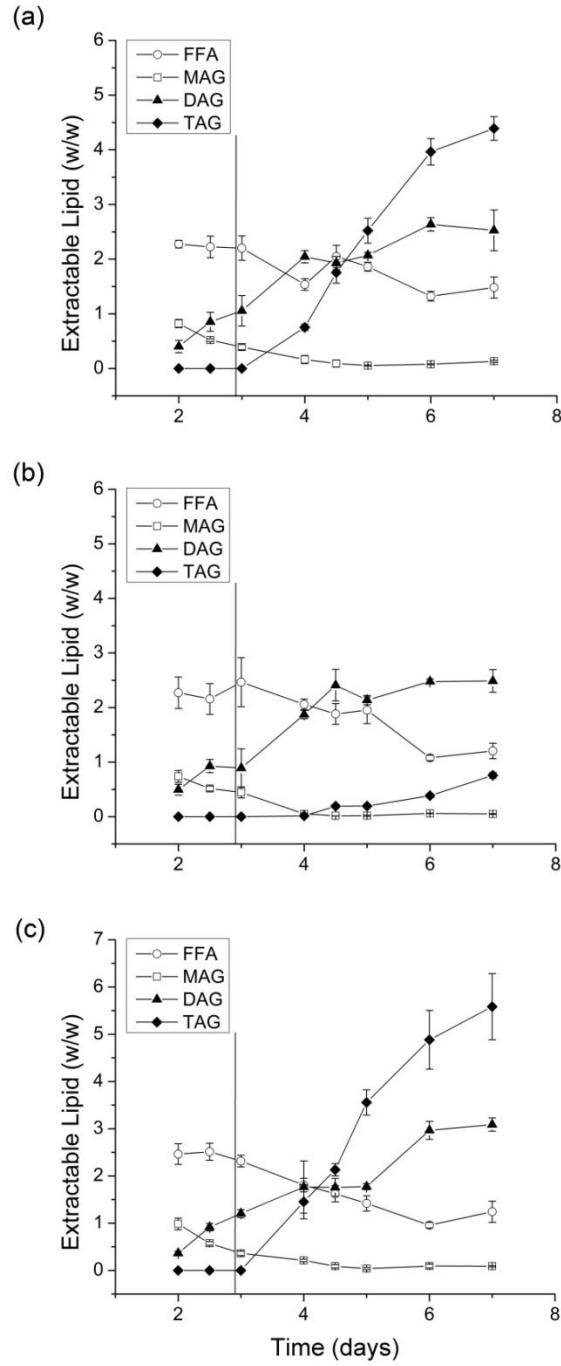


Figure 3.2: Mean and standard deviation of extractable FFA, MAG, DAG, and TAG weight percent (%) (weight extractable/weight biomass) from cultures of *C. reinhardtii* when cultured with (a) low CO₂ with 50 mM HCO₃⁻, (b) low CO₂ (ambient air), and (c) high CO₂ (5% v/v) (n=3). Line indicates time of nitrogen depletion of the culture medium.

Table 3.2: Comparison of mean and standard deviation of lipid class extracted with microwave energy, and total biodiesel potential from direct *in situ* transesterification from cultures of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻. Values are reported for just prior to nitrogen depletion (2.8 d) and for the completion of the experiment (7 d) (n=3). All values expressed as weight percent (%) (weight extractable or FAME/weight biomass).

At nitrogen depletion (2.8 d)						
Treatment	Free fatty acid (%)^a	Mono-glyceride (%)^a	Di-glyceride (%)^a	Tri-glyceride (%)^a	Sum of extracted (%)^b	Total biodiesel potential (%)^c
50 mM HCO ₃ ⁻	2.2 ± 0.22	0.39 ± 0.05	1.06 ± 0.28	N/D	3.65 ± 0.55	10.11 ± 0.98
Ambient Air	2.46 ± 0.45	0.44 ± 0.1	0.89 ± 0.35	N/D	3.8 ± 0.9	9.82 ± 0.54
5% CO ₂	2.32 ± 0.12	0.36 ± 0.05	1.2 ± 0.08	N/D	3.88 ± 0.25	8.66 ± 0.55
Final harvest (7 d)						
Treatment	Free fatty acid (%)^a	Mono-glyceride (%)^a	Di-glyceride (%)^a	Tri-glyceride (%)^a	Sum of extracted (%)^b	Total biodiesel potential (%)^c
50 mM HCO ₃ ⁻	1.48 ± 0.19	0.13 ± 0.02	2.53 ± 0.37	4.39 ± 0.22	8.53 ± 0.8	11.52 ± 0.73
Ambient Air	1.21 ± 0.14	0.05 ± 0.01	2.49 ± 0.21	0.76 ± 0.05	4.5 ± 0.41	9.81 ± 0.53
5% CO ₂	1.24 ± 0.22	0.09 ± 0.01	3.09 ± 0.14	5.58 ± 0.7	10 ± 1.08	11.09 ± 0.61

^a From microwave extraction of wet biomass

^b Sum of extractable lipid precursors – free fatty acids, mono-, di-, tri-glycerides

^c Total FAMES from direct *in situ* transesterification

N/D – not detected

To our knowledge, this is the first reported work that monitors FFA, MAG, DAG, and TAG concentrations over time. Generally, whole biomass or a biomass extract is transesterified into FAME, which is then assumed to be derived from TAG (Guckert and Cooksey, 1990; Laurens et al., 2012b; McNichol et al., 2012). Thus most, if not all, work reported previously does not provide the temporal resolution to demonstrate how

inorganic carbon is partitioned between FFA, MAG, DAG and TAG, or how lipid synthesis changes during nutrient deplete culturing.

To further investigate lipid biosynthesis in *C. reinhardtii*, extractable lipid profiles for each treatment based on lipid class (FFA, MAG, DAG, TAG) as well as carbon chain length are shown in Figure 3.3. Only C₁₆ and C₁₈ compounds are presented for clarity, as these are the predominant fatty acid chain lengths synthesized by *C. reinhardtii* (James et al., 2011); end of experiment time point values for all fatty acids are reported in Table 3.3. As discussed earlier, a similar trend in lipid metabolism was observed in all three inorganic carbon treatments, albeit with variations in overall concentrations. Before nitrogen depletion, the cells had a higher content of FFA and MAGs (both C₁₆ and C₁₈) relative to DAGs, and TAGs were not detectable. However, only the C₁₈ DAGs increased throughout the exponential growth phase, and continued to increase during stationary phase until reaching a pseudo steady-state around 1.8% (w/w) for each treatment (Figure 3.3d, e, and f). C₁₆ DAGs only began to accumulate after nitrogen depletion and to a much lower concentration (~0.5% w/w, Table 3.3) than C₁₈ DAGs (~1.5% w/w, Table 3.3) (Figure 3.3a, b, and c). Additionally, although C₁₈ TAGs began to accumulate after nitrogen depletion in cultures grown on 5% CO₂ and cultures supplemented with NaHCO₃, it is the C₁₆ TAGs that contribute the most to overall cellular TAG content (Figure 3.3, Table 3.3).

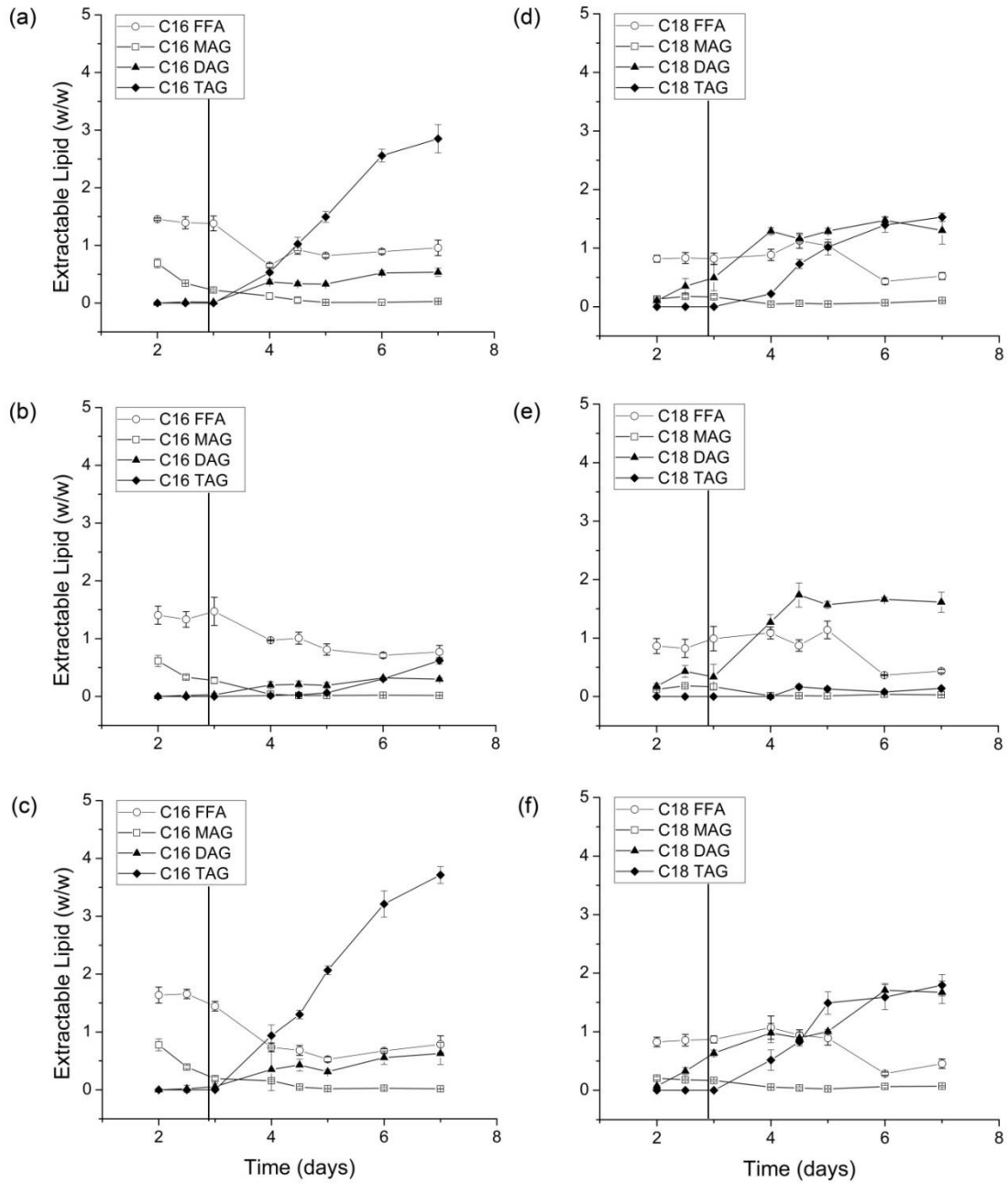


Figure 3.3: Mean and standard deviation of extractable C₁₆ and C₁₈ FFA, MAG, DAG, and TAG weight percent (%) (weight extractable/weight biomass) from cultures of *C. reinhardtii* when cultured with (a, d) low CO₂ with 50 mM HCO₃⁻, (b, e) low CO₂ (ambient air), and (c, f) high CO₂ (5% v/v) (n=3). Lines indicates time of nitrogen depletion of the culture medium.

These observations support the theory that C₁₆ FFAs and C₁₆ MAGs were being rapidly reallocated into C₁₆ DAG and then immediately into C₁₆ TAG for energy storage (Fan et al., 2012, 2011; James et al., 2011). In contrast, the majority of C₁₈ DAG compounds were being stored, perhaps for further integration into membrane bound lipids via phosphorylation (Bolling and Fiehn, 2005; Fan et al., 2012).

Table 3.3: Comparison of mean and standard deviation of lipids (class and chain length) extracted with microwave energy from cultures of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻. Values are reported for completion of the experiment (7 d) (n=3). All values expressed as weight percent (%) (weight extractable/weight biomass).

Free fatty acid (%)^a				
Treatment	C₁₂	C₁₄	C₁₆	C₁₈
50 mM HCO ₃ ⁻	N/D	N/D	0.96 ± 0.13	0.52 ± 0.06
Ambient Air	N/D	N/D	0.77 ± 0.11	0.43 ± 0.03
5% CO ₂	N/D	N/D	0.79 ± 0.15	0.46 ± 0.08
Mono-glyceride (%)^a				
Treatment	C₁₂	C₁₄	C₁₆	C₁₈
50 mM HCO ₃ ⁻	N/D	N/D	0.03 ± 0.01	0.1 ± 0.01
Ambient Air	N/D	N/D	0.02 ± 0	0.03 ± 0.01
5% CO ₂	N/D	N/D	0.02 ± 0	0.07 ± 0.01
Di-glyceride (%)^a				
Treatment	C₁₂	C₁₄	C₁₆	C₁₈
50 mM HCO ₃ ⁻	0.24 ± 0.03	0.45 ± 0.04	0.54 ± 0.07	1.3 ± 0.23
Ambient Air	0.27 ± 0.01	0.31 ± 0.01	0.3 ± 0.02	1.61 ± 0.17
5% CO ₂	0.3 ± 0.12	0.49 ± 0.16	0.63 ± 0.17	1.67 ± 0.69
Tri-glyceride (%)^a				
Treatment	C₁₂	C₁₄	C₁₆	C₁₈
50 mM HCO ₃ ⁻	N/D	0.01 ± 0	2.85 ± 0.25	1.53 ± 0.07
Ambient Air	N/D	N/D	0.62 ± 0.06	0.14 ± 0.01
5% CO ₂	N/D	0.08 ± 0.07	3.71 ± 1.15	1.79 ± 0.48

^a From microwave extraction of wet biomass

N/D – not detected

FAME Profiling

Total FAME profiles were determined for *C. reinhardtii* when cultured under the three unique inorganic carbon regimes by direct *in situ* transesterification of the biomass (Griffiths et al., 2010). Figure 3.4 presents profiles of saturated and unsaturated C₁₆ and C₁₈ FAME for cultures grown with a) low CO₂ and amended with 50 mM NaHCO₃, b) low CO₂, and c) high CO₂ (5%) as the inorganic carbon source. Only C₁₆ and C₁₈ FAMES are presented for clarity, as these are the predominant fatty acid chain lengths synthesized (James et al., 2011). Data for all fatty acid values at the end of the experiments are reported in Table 3.4.

All three conditions exhibited similar fatty acid profiles prior to nitrogen depletion, with significantly higher concentrations of unsaturated C₁₈ FAMES, and an equal abundance of saturated and unsaturated C₁₆ fatty acids. Unsaturated C₁₈ compounds remained the most prevalent form of fatty acid throughout the experiments; however after nitrogen depletion all three conditions shifted from synthesizing equivalent concentrations of unsaturated and saturated C₁₆ fatty acids towards a preferential synthesis of fully saturated C₁₆ fatty acids. As discussed above, C₁₆ TAG contributes the majority to overall TAG in all three conditions. Although this trend towards synthesis of fully saturated C₁₆ fatty acids occurred in all three inorganic carbon regimes, it was most pronounced in cultures grown on 5% CO₂ and cultures supplemented with NaHCO₃, indicating this response was increased due to inorganic carbon availability.

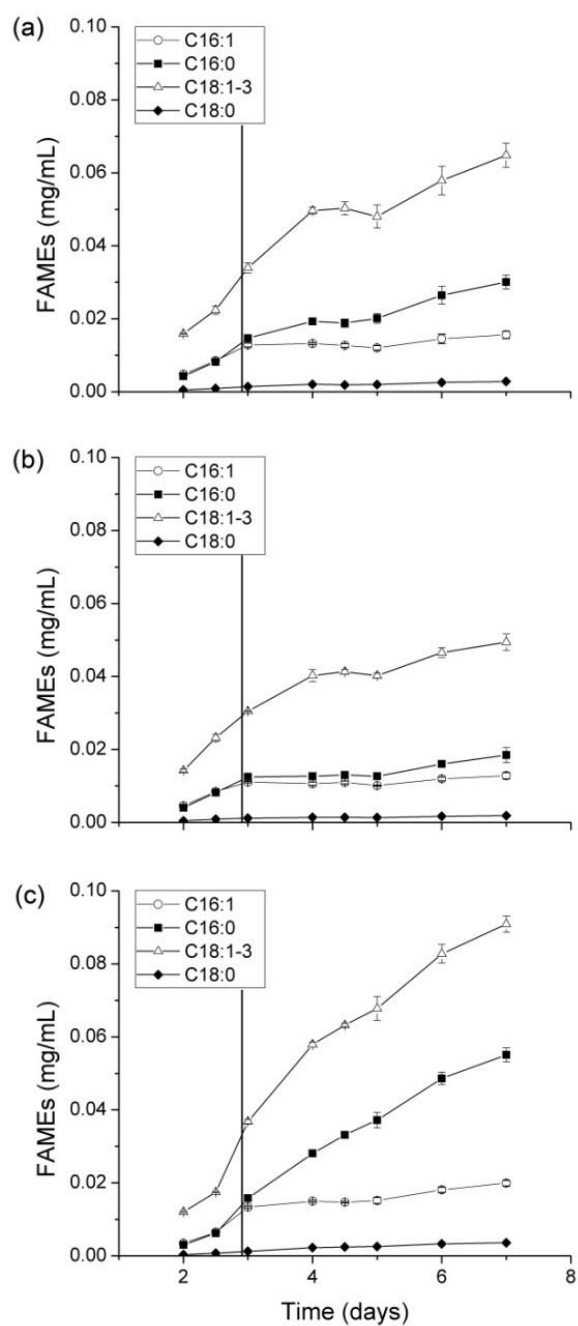


Figure 3.4: Mean and standard deviation of the C₁₆ and C₁₈ FAME profiles (mg FAME/mL culture) from cultures of *C. reinhardtii* when cultured with (a, d) low CO₂ with 50 mM HCO₃⁻, (b, e) low CO₂ (ambient air), and (c, f) high CO₂ (5% v/v) (n=3). Line indicates time of nitrogen depletion of the culture medium.

Table 3.4: Comparisons of mean and standard deviation of percent composition of FAMES derived from *in situ* transesterification for cultures of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻. Values are reported for just prior to nitrogen depletion and for the completion of the experiment (n=3). All values expressed as weight percent (%) (weight FAME/weight biomass).

At nitrogen depletion (2.8 d)								
Treatment	C14:0	C16:1	C16:0	C18:1-3^a	C18:0	C20:1	C20:0	Other^b
50 mM HCO ₃ ⁻	0.03 ± 0	2.03 ± 0.21	2.33 ± 0.25	5.38 ± 0.5	0.22 ± 0.02	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0
Ambient Air	0.02 ± 0	1.94 ± 0.14	2.19 ± 0.1	5.34 ± 0.29	0.21 ± 0.01	0.03 ± 0.01	0.07 ± 0.02	0.03 ± 0
5% CO ₂	0.01 ± 0	1.7 ± 0.12	2.01 ± 0.12	4.68 ± 0.29	0.16 ± 0.01	0.04 ± 0	0.04 ± 0.01	0.02 ± 0
Final harvest (7 d)								
Treatment	C14:0	C16:1	C16:0	C18:1-3^a	C18:0	C20:1	C20:0	Other^b
50 mM HCO ₃ ⁻	0.03 ± 0	1.57 ± 0.11	3.03 ± 0.21	6.53 ± 0.38	0.29 ± 0.02	0.02 ± 0	0.03 ± 0	0.02 ± 0
Ambient Air	0.01 ± 0	1.52 ± 0.09	2.18 ± 0.21	5.84 ± 0.23	0.22 ± 0.02	N/D	N/D	0.02 ± 0
5% CO ₂	0.02 ± 0	1.29 ± 0.05	3.56 ± 0.17	5.88 ± 0.38	0.23 ± 0.01	0.04 ± 0	0.06 ± 0	0.01 ± 0

^a C18:1, C18:2, and C18:3 taken together

^b Sum of other compounds detected

N/D – not detected

Most interesting is the change in total FAME over time. Figure 3.5 presents total FAME values for each sample point and for each culture condition. Values are reported as a) mg/mL, b) % w/w, and c) pg/1000 cells. When total FAME is calculated as a concentration (*i.e.* mg FAME/mL culture), an obvious increase was observed in all treatments, with cultures grown with 5% CO₂ achieving the highest final concentration. However, when total FAME is calculated as a percent of DCW, no statistically significant change was observed over time, or between culture conditions except at day 4.5 (Figure 3.5b) when the FAME content was assessed once at the end of the dark cycle.

FAME (w/w) reported at day 4.5 is statistically higher (2 sample T Test $p < 0.05$) for all treatments due to a decrease in DCW (Figure 3.1a) caused by a decrease in protein concentration (Supplemental Data, Figure 3.S1 d 4.5), as discussed earlier. Finally, when total FAME is calculated as a weight per cell, increases in FAMES were observed, with the highest FAME productivity obtained in cultures supplemented with 50 mM NaHCO_3 . These results highlight the importance of monitoring FAME throughout the growth cycle.

From an industrial standpoint, measuring FAME as a fraction of dry biomass (mass of FAMES per mass of DCW) may only be pertinent if cultures can achieve high biomass concentrations, whereas measuring FAME mass per volume of culture harvested or as a productivity rate (mass of FAMES per volume per time) may be more relevant for production systems. On the other hand, measuring and reporting the amount of FAMES per cell (mass FAME per cell) is valuable from a research perspective to identify physiological changes due to different culturing conditions.

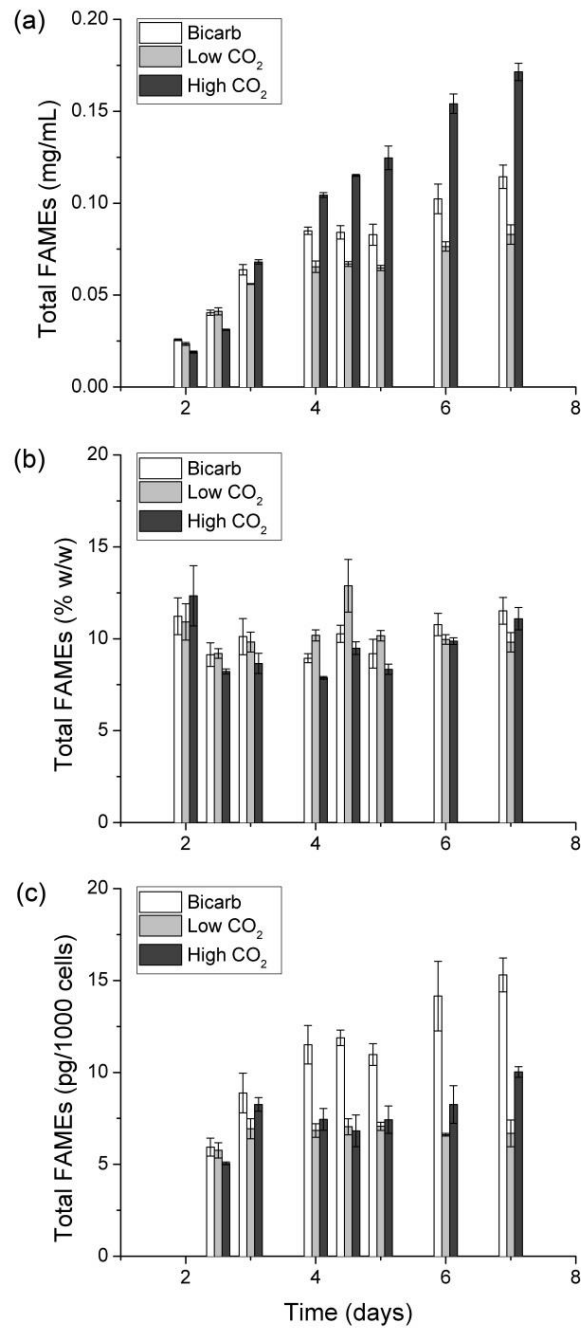


Figure 3.5: Comparison of total FAME values for *C. reinhardtii* when reporting data as (a) concentration (mg FAME/mL culture), (b) weight percent (mg FAME/mg biomass), and (c) FAME per cell (pg FAME/1000 cells) (n=3).

Conclusion

Results presented herein provide insight into which lipid compounds *C. reinhardtii* synthesizes and how the organism's metabolism changes due to nitrogen depletion and inorganic carbon source availability. These results help elucidate how inorganic carbon is being partitioned between lipid classes (*i.e.* FFAs, MAGs, DAGs and TAGs) and may provide knowledge that can be transferred to other species or mutants that are better suited for large scale biofuel production. Additionally, this approach can be utilized for determining the optimal time of harvest during biodiesel production as well as during screening for high value product accumulation (such as Ω -3 fatty acids).

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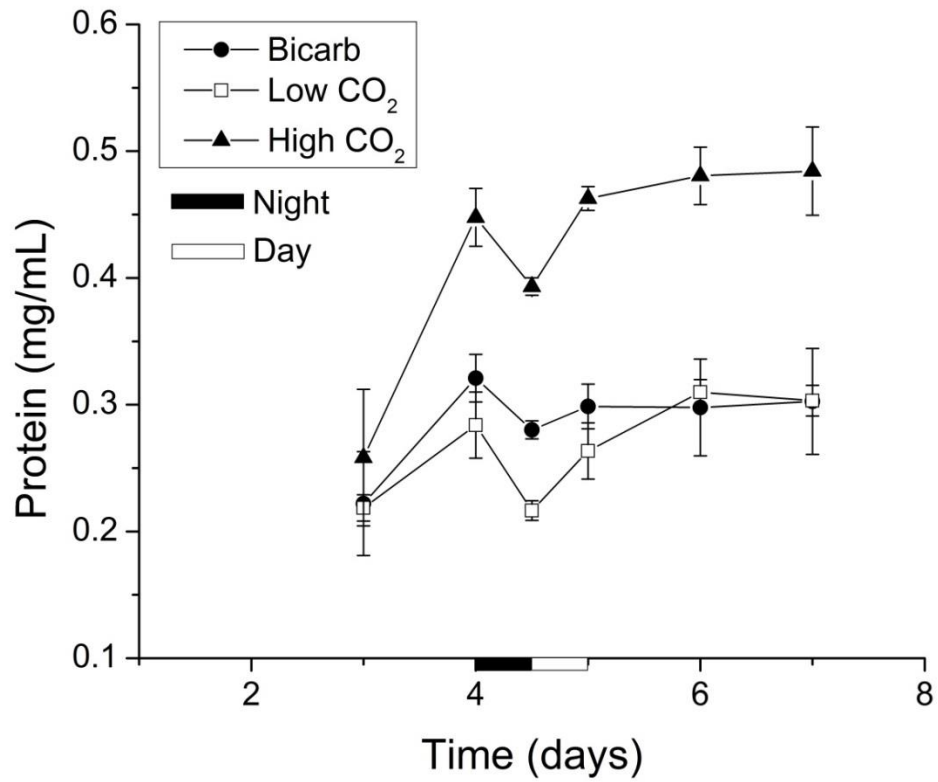
Supplemental Material

Figure 3.S1: Mean and standard deviation of protein concentration for cultures of *C. reinhardtii* when cultured with low CO₂ (ambient air), high CO₂ (5% v/v), and low CO₂ with 50 mM HCO₃⁻ (n=3).

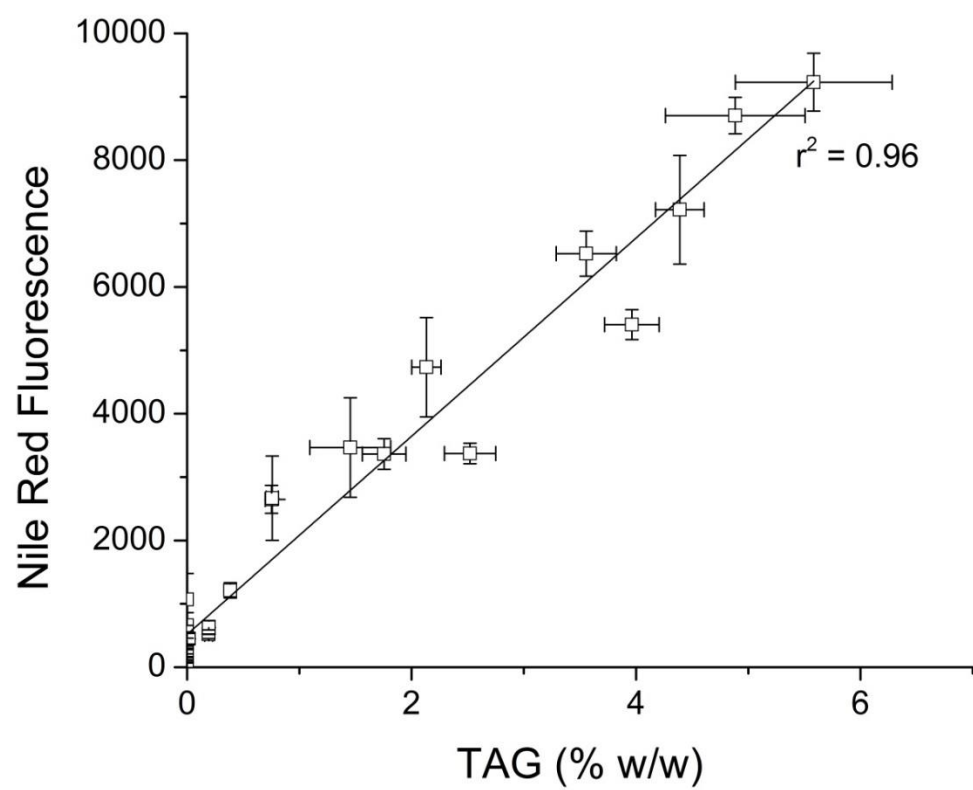


Figure 3.S2: Relationship between Nile Red fluorescence intensity and TAG content (% w/w) for *Chlamydomonas reinhardtii*. Each data point represents the average of three replicates.

CHAPTER 4

AN OPTIMIZED INORGANIC CARBON REGIME FOR ENHANCED GROWTH
AND LIPID ACCUMULATION IN *CHLORELLA VULGARIS*

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Manuscript in Chapter 4

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Contributions: Conceived the study, collected and analyzed data, assisted with study design, discussed the results, and edited the manuscript.

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Contributions: Assisted with study design, analyzed data, and edited the manuscript.

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Contributions: Assisted with study design, analyzed data, and edited the manuscript.

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Title: Optimized Inorganic Carbon Regime for Enhanced Growth and Lipid Accumulation in *Chlorella vulgaris*

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Abstract

Background

Industrial scale-up of algal biofuel production thus far has been cost prohibitive due in part to costs associated with providing the necessary nutritional requirements for enhanced growth of the algal culture. Specifically, carbon dioxide is a substrate that should be supplemented above atmospheric concentrations to achieve maximum biomass productivity. Life cycle analyses have concluded that energy costs associated with pumping and mixing CO₂ into algal cultures are significant contributors to the overall energy consumption (Campbell et al., 2011; Jorquera et al., 2010; Liu et al., 2013; Yang et al., 2011). Developing strategies which minimize these costs will inherently increase the economic feasibility of commercial scale algal biofuel production. Here we present an optimal growth and lipid accumulation scenario that enhances the growth rate and triacylglyceride (TAG) accumulation rate in *Chlorella vulgaris* sp. strain UTEX 395. By growing the organism in the presence of low concentrations of sodium bicarbonate and controlling the pH of the system with a periodic gas sparge of 5% CO₂ (v/v) in air, specific growth rates were significantly increased above the control group. Once cultures had reached desired cell densities, which can be “fine-tuned” based on initial nutrient concentrations, cultures were switched to a lipid accumulation metabolism by triggering TAG accumulation through the addition of 50 mM NaHCO₃. Additionally, various bicarbonate salts were evaluated for their capacity to induce lipid accumulation in the industrially relevant green Chlorophyte *C. vulgaris*.

Results

The two phase, optimized growth and lipid accumulation scenario increased the specific growth rate of *C. vulgaris* by 69% compared to the 5% CO₂ (v/v) continuous control system, while biomass productivity (g L⁻¹ day⁻¹) was increased by 27%. Total biodiesel potential (measured as total FAME) was increased from 53.3% (FAME biomass⁻¹), for the control cultures, to 61% (FAME biomass⁻¹) under the optimized conditions, and biodiesel productivity (g L⁻¹ day⁻¹) was increased by 7.6%. Screening various bicarbonate salts revealed that ACS (American Chemical Society) grade and industrial grade NaHCO₃ induces the highest TAG yield per biomass weight (% w/w), whereas Na₂CO₃ did not induce a significant amount of TAG accumulation, and NH₄HCO₃ had an inhibitory effect on cell growth. The raw, unrefined form of trona, NaHCO₃·Na₂CO₃ (sodium sesquicarbonate) induced TAG accumulation, albeit at a slightly lower yield than more refined grades of sodium bicarbonate.

Conclusions

The strategic addition of sodium bicarbonate was found to enhance growth rates and lipid accumulation rates in cultures of *C. vulgaris*, as compared to traditional growth regimes which only supply elevated concentrations of CO_{2(g)} as the sole inorganic carbon source. Further, various forms of bicarbonate were evaluated for their capacity to enhance lipid accumulation and it was determined that sodium sesquicarbonate, being the natural, and hence least expensive form of bicarbonate adequately induces TAG accumulation in cultures of *C. vulgaris*. This work presents an industrially relevant, two-phase, optimized photoautotrophic growth and lipid accumulation scenario which may

result in a significant reduction in commercial scale operating costs and an overall increase in algal biofuel productivity.

Background

The extraction and refinement of petroleum as a natural resource for fuel and specialty chemicals has enabled human innovation and led to unprecedented technological growth in industrialized nations; however, these technological advancements are not without cost. The current rate of producing enough crude petroleum to satisfy global demand has led to political, economic and environmental controversy (Dismukes et al., 2008). The US alone currently consumes over 18 million barrels of crude oil each day, with a significant percentage being imported from foreign sources (EIA, 2012). Additionally, atmospheric concentrations of carbon dioxide have risen drastically due to the increasing combustion rate of fossil fuels (NRC, 2010a). Carbon dioxide is one of the most prevalent greenhouse gases responsible for global climate change and ocean acidification. Apart from the natural carbon cycle, industrial processes currently contribute 29 billion tonnes of carbon dioxide to the atmosphere each year (NRC, 2010a). This is more than 400x the current rate of global carbon fixation achieved by primary biota (Dukes, 2003).

Renewable sources of energy rich, biologically produced feed stocks are needed to offset this trend. Corn, soybean and sugar cane are the current primary sources for bioethanol and biodiesel, however these crops compete for arable land needed to produce food (Ferrell and Sarisky-Reed, 2010). Algal derived biofuels, especially biodiesel, have

received increased attention in recent decades as a potential renewable energy resource (Dismukes et al., 2008; Hu et al., 2008; Sheehan, 1998; Yang et al., 2011). Based on life cycle analyses and conservative estimates of biodiesel yields, it has been projected that less than 5% of currently available arable land would be required to displace 50% of fossil fuel consumption in the US (Chisti, 2008). This estimate is even more appealing when considering the fact that microalgae do not require arable land as they are not a conventional crop; rather, algal ponds simply need flat terrain, water, appropriate temperature, abundant sunlight and nutrients.

One of these nutrients is inorganic carbon, which is necessary for photoautotrophic cell growth as well as lipid synthesis. Biodiesel, defined as fatty acid methyl ester (FAME) has chemical properties similar to traditional diesel, and can be produced from energy rich storage compounds such as triacylglycerol (TAG), which can be synthesized by microalgae in relatively high abundance (Chisti, 2008).

TAG is comprised of long chain hydrocarbons (fatty acids), and a fundamental requirement for their synthesis is inorganic carbon availability to the organism. To realize commercial scale development of algal derived biofuels, optimized processes for the delivery and biological uptake of inorganic carbon is paramount to reducing cost and increasing productivity. This study focuses on 1) determining the necessary concentration and speciation of dissolved inorganic carbon (DIC) for optimal photosynthetic activity in cultures of *Chlorella vulgaris* sp. strain UTEX 395 and 2) developing an optimized two-phase growth and lipid accumulation strategy to increase the specific growth rate and induce TAG accumulation in *C. vulgaris*. The results

presented within this study provide an industrially relevant synopsis for optimizing productivity and reducing resource costs.

Results and Discussion

Experimental Design and Rationale

Although microalgae will grow under atmospheric concentrations of inorganic CO₂ (~400 ppm), improved biomass yields can be achieved by supplementing with additional inorganic carbon (Chi et al., 2013, 2011). It is often cited that this additional carbon source could come from industrial waste such as power plant flue gas (Aresta et al., 2005; Collet et al., 2011). Unfortunately, studies have suggested that this process may be economically cost prohibitive (Cooney et al., 2011). Moreover, recent life cycle analyses have shown that due to the inefficiency of aerating open algal ponds with CO₂ gas, there may not be enough CO₂ produced from waste streams to reach the biomass-carbon ratio required for large scale algae biofuel production (Pate et al., 2011). This is due, in part, to the fact that the mass transfer of CO₂ in an aqueous solution is 10,000 times slower than the mass transfer of CO₂ in air, and hence the majority of CO₂ gas which is sparged into an open pond escapes back into the atmosphere (Moroney and Ynalvez, 2007). Additionally, the enzyme responsible for fixing CO₂ into biomass, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), has a relatively low affinity for CO₂ and can only function at about 25% of its catalytic capacity at atmospheric concentrations (Giordano et al., 2005; Moroney and Ynalvez, 2007). Because of this, many microalgae species have developed CO₂ concentrating mechanisms (CCMs) which allow them to utilize both carbon dioxide and bicarbonate as carbon species dissolved in

the culture medium (Chi et al., 2013; Giordano et al., 2005; Reinfelder, 2011; Thielmann et al., 1990). Depending on the pH of a system in equilibrium, inorganic carbon can be in one of three forms; dissolved carbon dioxide ($\text{CO}_{2(\text{aq})}$), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}). Research has suggested that the preferential species of DIC for microalgae is $\text{CO}_{2(\text{aq})}$ (Raven, 2010), however at the optimal pH range for growth (pH 7 - 9), the predominant species is HCO_3^- . Many species of microalgae are capable of actively transporting HCO_3^- and converting it into $\text{CO}_{2(\text{aq})}$ for utilization by Rubisco (Bozzo et al., 2000; Coleman and Colman, 1981; Hopkinson et al., 2011; Matsuda and Colman, 1991; Moroney and Somanchi, 1999; Radmer and Ollinger, 1980).

Additionally, previous research has revealed that the timely addition of bicarbonate to microalgae cultures induces a significant increase in TAG accumulation (Gardner et al., 2012). This “bicarbonate trigger” has been tested and optimized on various species; including Chlorophytes and diatoms (Gardner et al., 2013a, 2012; White et al., 2012).

In this study, we compare how various bicarbonate salts effectively induce TAG accumulation, and present a two-phase, enhanced growth and lipid accumulation scenario for cultures of *C. vulgaris*. By supplementing cultures with low doses of bicarbonate to first enhance the specific growth rate, and secondly by adding elevated concentrations of bicarbonate in concert with medium nitrogen depletion, both growth and lipid accumulation rates are significantly increased. The discussion is broken into two parts: the first of which details the results of using various bicarbonate salts to induce lipid accumulation and the second details an optimized growth and lipid accumulation scenario.

Part 1: Bicarbonate Salt Study

To the best of our knowledge, only ACS grade NaHCO_3 has been used to induce TAG accumulation in microalgae (Gardner et al., 2013a, 2012; White et al., 2012). From an industrial cost perspective, a less refined and hence cheaper source of inorganic bicarbonate will be preferable. To further elucidate the mechanisms of the bicarbonate triggering phenomenon, and evaluate the potential use of other bicarbonate salts, 6 compounds (Table 4.1) were evaluated using cultures of *C. vulgaris*. Briefly, batch triplicate cultures of *C. vulgaris* were grown in Bold's basal medium until just prior to nitrogen depletion, at which time the cultures were harvested and transferred into medium deplete of any nitrogen source. Seven experimental conditions were designed to compare extractable lipid accumulation as monitored by gas chromatography-flame ionization detection (GC-FID) and total biodiesel potential via gas chromatography-mass spectroscopy (GC-MS). Extractable lipid is defined here as intracellular lipids which have either been incorporated into the energy storage compound triacylglyceride (TAG), or are precursor compounds such as free fatty acids (FFA), monoacylglyceride (MAG) or diacylglyceride (DAG), which can be further processed into TAG or membrane-bound lipid compounds. Extractable lipids can be liberated from a lysed cell, using non-polar solvents. Previous work (Lohman et al., 2013) has shown that GC-FID analysis can be effectively used for classification and carbon chain length identification of extractable lipid from microalgae cultures. Additionally, total biodiesel is the sum of all fatty acids which have been transesterified into FAME, which can be quantified based on carbon-chain length and saturation via GC-MS (Lohman et al., 2013).

Table 4.1: Culture characteristics after medium nitrogen depletion and supplementation with various bicarbonate salts: 0 mM HCO_3^- Control, 50 mM ACS grade NaHCO_3 , 50 mM industrial grade NaHCO_3 , 50 mM KHCO_3 , 50 mM Na_2CO_3 , 50 mM NH_4HCO_3 and 25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (25 mM of sesquicarbonate was used to be equimolar Carbon). Values are reported for the completion of the experiment (5.75 d) (n=3).

Treatment	Cell concentration ($\times 10^7$ cells mL^{-1})	Cell Doublings	Dry weight (g L^{-1} ; DCW) ^a	Endpoint pH	Total Chlorophyll (mg L^{-1})
0 mM HCO_3^- Control	3.48 ± 0.17	4.61 ± 0.92	0.37 ± 0.03	8.08 ± 0.07	1.82 ± 0.08
50 mM ACS Grade NaHCO_3	2.15 ± 0.17	1.57 ± 0.35	0.58 ± 0.06	9.82 ± 0.03	2.00 ± 0.34
50 mM Industrial Grade NaHCO_3	1.78 ± 0.13	0.60 ± 0.40	0.60 ± 0.02	9.82 ± 0.06	1.72 ± 0.18
50 mM KHCO_3	1.56 ± 0.13	0.86 ± 0.33	0.54 ± 0.03	9.77 ± 0.03	2.01 ± 0.21
50 mM NH_4HCO_3	0.62 ± 0.04	-0.13 ± 0.28	0.15 ± 0.04	9.31 ± 0.05	0.31 ± 0.17
50 mM Na_2CO_3	1.21 ± 0.22	0.78 ± 0.19	0.35 ± 0.01	9.98 ± 0.02	1.37 ± 0.15
25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$	1.21 ± 0.16	0.34 ± 0.39	0.49 ± 0.04	9.83 ± 0.03	1.77 ± 0.22

^a Dry cell weight (DCW) determined gravimetrically with lyophilized biomass

The control group received no additional inorganic carbon aside from what is naturally available in equilibrium with the atmosphere. The other six groups received an additional source of dissolved inorganic carbon (DIC, final concentration) as follows: 50 mM C from 1) ACS grade NaHCO_3 , 2) Industrial grade NaHCO_3 , 3) KHCO_3 , 4) Na_2CO_3 , 5) NH_4HCO_3 , and 6) 25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (25 mM of sesquicarbonate was used to be equimolar in carbon as compared to the other treatments). Table 4.1 presents physiological characteristics for each treatment at the conclusion of each experiment. As expected, the control group continued to increase in cell concentration from 6.3×10^6 cells mL^{-1} until reaching stationary growth at 3.5×10^7 cells mL^{-1} (Table 4.1), whereas cellular replication in cultures supplemented with additional DIC was either significantly retarded or completely arrested. This phenomenon has been well documented (Gardner et al.,

2013a, 2012; White et al., 2012) and is believed to be the result of a fundamental metabolic shift from growth metabolism to lipid accumulation metabolism. Figure 4.1 presents lipid profiles (FFA, MAG, DAG, TAG, and *in situ* transesterified FAME as biodiesel potential) for each culture after maximum TAG accumulation as quantified via GC-FID and GC-MS (Lohman et al., 2013).

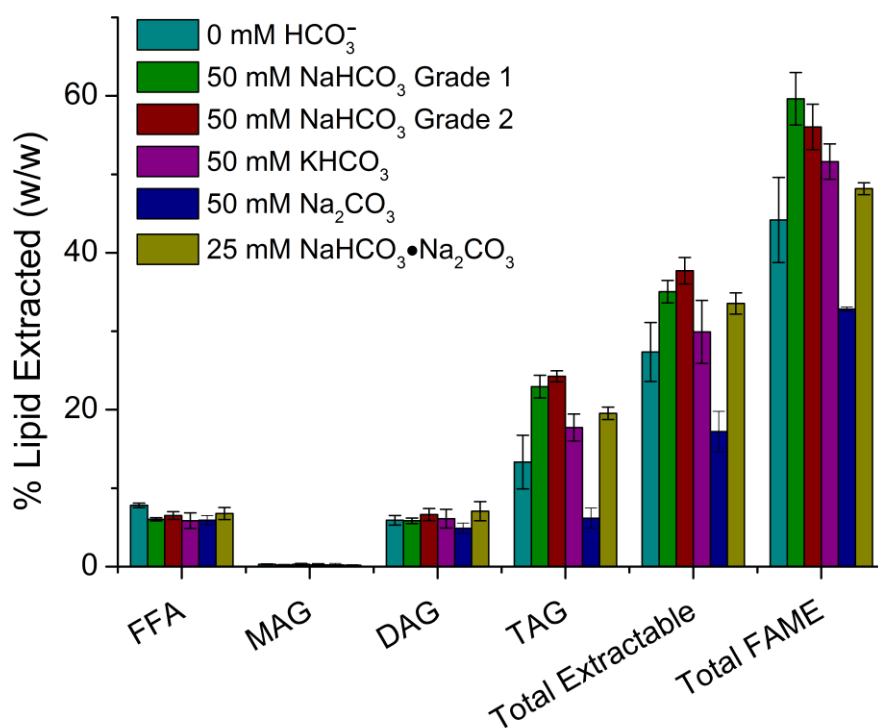


Figure 4.1: Extractable lipid class and FAME profiles for cultures of *C. vulgaris* when re-suspended into medium deplete of nitrogen and supplemented with various bicarbonate salts: 0 mM HCO_3^- Control, 50 mM ACS grade 1 NaHCO_3 , 50 mM industrial grade 2 NaHCO_3 , 50 mM KHCO_3 , 50 mM Na_2CO_3 and 25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (25 mM of sesquicarbonate was used to be equimolar Carbon). Values are reported for the completion of the experiment (n=3). All values expressed as weight percent (% weight extractable lipid or weight FAME/weight biomass).

As shown in Figure 4.1, cultures supplemented with 50 mM ACS or industrial grades of bicarbonate accumulated the most TAG after 5 d of N-deplete culturing (22.9 ± 1.5 and 24.3 ± 0.7 % w/w respectively), as compared to the 0mM HCO_3^- control (13.3 ± 3.4 % w/w) which did not receive any additional DIC. Cultures supplemented with 50 mM KHCO_3 or 25 mM $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3$ also accumulated a significant amount of TAG (17.7 ± 1.7 and 19.5 ± 0.797 % w/w respectively), albeit at lower final percentages than cultures provided with ACS grade NaHCO_3 (Figure 4.1). It is interesting to note here that sodium sesquicarbonate is essentially the raw material (*i.e.*, trona) used for the production of the more refined grades of carbonates/bicarbonates. Trona typically contains high concentrations of silicates and trace amounts of other metals and salts, hence it is the cheapest form of bicarbonate available. Cultures treated with 50 mM NH_4HCO_3 not only did not accumulate TAG (data not shown), but began to decline in cell concentration after 2 days of incubation (Table 4.1). The pH of that system was above 9.3 (Table 4.1) which is the pKa for the $\text{NH}_4^+/\text{NH}_3$ equilibrium, indicating the predominant nitrogen species was NH_3 . The decline in cell concentration was presumably due to the effects of ammonia, which at concentrations above 1.2 mM has been shown to inhibit photosynthesis in microalgal cultures (Azov and Goldman, 1982). Finally, cultures supplemented with 50 mM Na_2CO_3 show a significantly lower percentage of TAG (6.2 ± 1.3 % w/w) as compared to the control (Figure 4.1). There are no known metabolic pathways in which *C. vulgaris* can assimilate CO_3^{2-} into biomass, which suggests that the small amount of TAG which accumulated in that system was due to HCO_3^- availability. The pH of that system was 10 (Table 4.1), and the pKa for the

$\text{HCO}_3^-/\text{CO}_3^{2-}$ equilibrium is pH 10.3, indicating the predominant species of inorganic carbon available in that system was HCO_3^- , albeit at a lower concentration than for the other treatments. Free fatty acid, MAG and DAG percentages were statistically the same in all treatments (6.5 ± 0.3 , 0.24 ± 0.06 , 6.1 ± 0.34 % w/w, respectively), thus the total extractable lipid percentage (sum of FFA, MAG, DAG and TAG) for each culture follows the same trend as TAG percentages. Total biodiesel potential, defined as total FAME (% w/w) (Eustance et al., 2013) also followed the same trend as the TAG percentage, with cultures treated with 50 mM ACS and industrial grade NaHCO_3 accumulating the most FAME at 59.6 ± 3.3 and 56.0 ± 2.9 % w/w, respectively (Table 4.2). FAME profiles are further expanded in Table 4.2, which presents each fatty acid by carbon chain length and saturation. Overall, *C. vulgaris* primarily synthesizes C_{18} fatty acids, specifically mono-unsaturated C_{18} (Laurens et al., 2012b; Lohman et al., 2013). Interestingly, the control condition which did not receive additional inorganic carbon produced equivalent concentrations of fully saturated $\text{C}_{16:0}$ fatty acid and poly-unsaturated $\text{C}_{18:3}$ fatty acid, compared to treatments which received additional bicarbonate (Table 4.2). However, regarding $\text{C}_{18:1}$ fatty acid, the control produced approximately half (16.8 ± 3 % w/w) as much as the treatment which received 50 mM ACS grade NaHCO_3 (31.8 ± 1.7 % w/w), indicating this fatty acid was synthesized in abundance due to elevated concentrations of DIC (Table 4.2).

Table 4.2: FAME profiles after medium nitrogen depletion and supplementation with various bicarbonate salts: 0 mM HCO_3^- Control, 50 mM ACS grade NaHCO_3 , 50 mM industrial grade NaHCO_3 , 50 mM KHCO_3 , 50 mM Na_2CO_3 and 25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (25 mM of sesquicarbonate was used to be equimolar Carbon). Values are reported for the completion of the experiment (5.75 d) (n=3). All values expressed as weight percent (% weight FAME/weight biomass).

Treatment	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Other ^a	Total biodiesel potential (%) ^b
0 mM HCO_3^- Control	7.51 ± 0.69	1.59 ± 0.16	4.72 ± 0.5	16.82 ± 2.97	3.92 ± 0.43	9.3 ± 0.75	0.4 ± 0.1	44.19 ± 5.42
50 mM ACS Grade NaHCO_3	7.78 ± 0.46	2.33 ± 0.18	2.46 ± 0.14	31.82 ± 1.73	5.61 ± 0.35	9.06 ± 0.43	0.58 ± 0.06	59.63 ± 3.34
50 mM Industrial Grade NaHCO_3	7.38 ± 0.37	2.11 ± 0.05	2.28 ± 0.18	30.06 ± 1.39	5.16 ± 0.13	8.43 ± 0.74	0.61 ± 0.05	56.04 ± 2.9
50 mM KHCO_3	6.8 ± 0.22	1.79 ± 0.05	2.64 ± 1.11	27.01 ± 2.08	5.15 ± 0.07	7.74 ± 0.3	0.54 ± 0.13	51.62 ± 2.25
50 mM Na_2CO_3	5.12 ± 0.11	1.26 ± 0.03	2.56 ± 0.04	14.47 ± 0.12	3.4 ± 0.02	5.84 ± 0.05	0.2 ± 0.05	32.82 ± 0.28
25 mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$	6.69 ± 0.08	1.84 ± 0.03	3.82 ± 0.09	23.02 ± 0.62	4.67 ± 0.09	7.72 ± 0.12	0.47 ± 0.08	53.33 ± 1.35

^a Sum of other compounds detected

^b Total FAMES

The results of this study provide further evidence that the timely addition of bicarbonate, when coupled with nitrogen depletion, can trigger significant lipid accumulation in cultures of *C. vulgaris*. Further, these data show that not only is the species of inorganic carbon critical to the process, but also the concentration of available bicarbonate.

Part 2: Optimized Growth and Lipid Accumulation

Based on previous observations regarding the use of bicarbonate as a suitable TAG inducing mechanism (Gardner et al., 2013a, 2012; White et al., 2012), it was envisioned that specific growth rates might also be increased if cultures were provided

with an additional source of soluble inorganic carbon during the exponential growth phase. Therefore, experiments were designed to investigate optimal growth parameters. Traditional methods of supplying inorganic carbon to algae cultures typically involves a gas sparge which provides elevated levels of CO₂ (1-5% v/v) in an air-lift photo-bioreactor or open pond systems (Concas et al., 2012; de Moraes and Costa, 2007; Laurens et al., 2012b). Industrial systems often use CO₂ to regulate pH, by automating a process to turn on CO₂ gas sparge when pH reaches undesirably high levels, and then turn off after the CO₂ has acidified the system, typically fluctuating pH between 8.0-8.3. It is interesting to note here that at pH 8.3 virtually 100% of the DIC in a system is in the form of the bicarbonate ion. In an effort to optimize this process, a combination of 5mM NaHCO₃ (final concentration at inoculation) was added to the medium, and a pH controller was incorporated to maintain pH between 8.4-8.7 by automatically sparging the system with 5% CO₂ (v/v) when the pH reached 8.7 and turning off when the pH reached 8.4. This higher pH range was specifically chosen to maintain an inorganic carbon speciation of predominantly bicarbonate relative to CO_{2(aq)}. This system was then evaluated against the following 4 conditions: 1) Cultures aerated with atmospheric air only; 2) Cultures aerated with atmospheric air and the medium supplemented with 5 mM NaHCO₃ prior to inoculation; 3) Cultures aerated with atmospheric air and pH regulated with 5% CO₂ (v/v) to maintain a pH range of 8.4-8.7; 4) Cultures aerated with 5% CO₂ (v/v) continuously during the daylight hours. In this way, each inorganic carbon regime (*e.g.* 5 mM NaHCO₃, 5% CO₂, pH regulated and atmospheric air only) was evaluated

individually, and the combined system utilizing bicarbonate plus pH regulated CO₂ could be compared to those individual systems.

Figure 4.2 shows cell density increasing over time for all five systems as measured by absorbance (440nm).

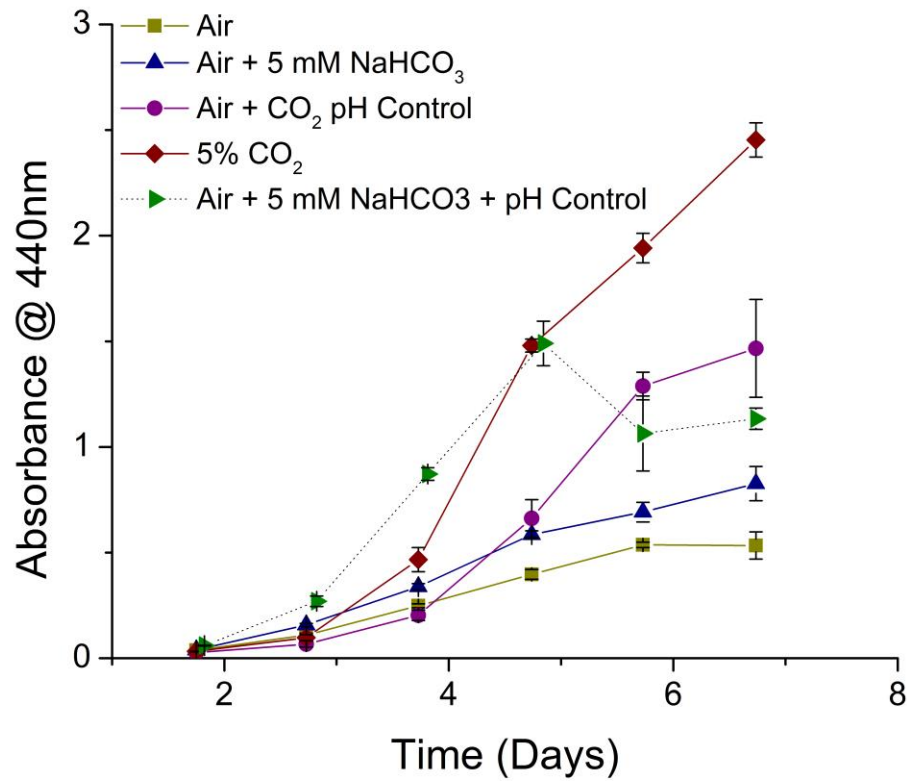


Figure 4.2: Absorbance measurements (440nm) from cultures of *C. vulgaris* when cultured under various inorganic carbon regimes: (■) Continuous sparge of atmospheric air, (▲) Continuous sparge of atmospheric air and supplemented with 5mM NaHCO₃ at inoculation, (●) Continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7, (◆) Continuous sparge of atmospheric air supplemented with 5% CO₂ (v/v) during daytime hours, (►) Continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO₃ at inoculation (n=3).

Initially, the cultures which received 5mM NaHCO_3 and were pH controlled with CO_2 (dashed line) were significantly denser than the other four conditions (3.8 d). However, after the medium became deplete of nitrogen (day 5), cultures began to secrete a surfactant which lifted cells into the headspace of the bioreactor. This phenomenon was exacerbated in the denser cultures and caused absorbance measurements to decrease (Figure 4.2). Surfactant formation has been observed in green microalgae before (Concas et al., 2012; Gardner et al., 2012), and is believed to be the result of protein secretion from cultures deplete of nutrients. Interestingly, cultures which received 5mM NaHCO_3 without pH control did not form an observable surfactant. It was surmised this was a result of the high pH manifested by these cultures, which due to photosynthesis, reached levels as high as 11. It has been previously reported that the timely addition of 50mM NaHCO_3 directly before nitrogen depletion not only stopped cellular replication and induced TAG accumulation, but also inhibited surfactant formation (Gardner et al., 2012). Therefore, it was expected that by combining the enhanced growth regime detailed above with a TAG inducing bicarbonate addition may result in a two-phase, optimized growth and lipid accumulation scenario.

Figure 4.3 presents cell concentrations over time for cultures grown under the same conditions as specified above, however, cultures which were amended with 5mM NaHCO_3 at inoculation and pH controlled with 5% CO_2 (v/v) were also supplemented with 50mM NaHCO_3 at day 4, just prior to nitrogen depletion (black arrow, Figure 4.3). The addition of 50mM bicarbonate resulted in a cessation of cellular replication (Figure 4.3, Table 4.3), as expected, and also inhibited surfactant formation. However, prior to

stopping the cell cycle (Figure 4.3, black arrow), these cultures exhibited a 69% increase in the specific growth rate as compared to the 5% CO₂ (v/v) continuous control system (Table 4.3).

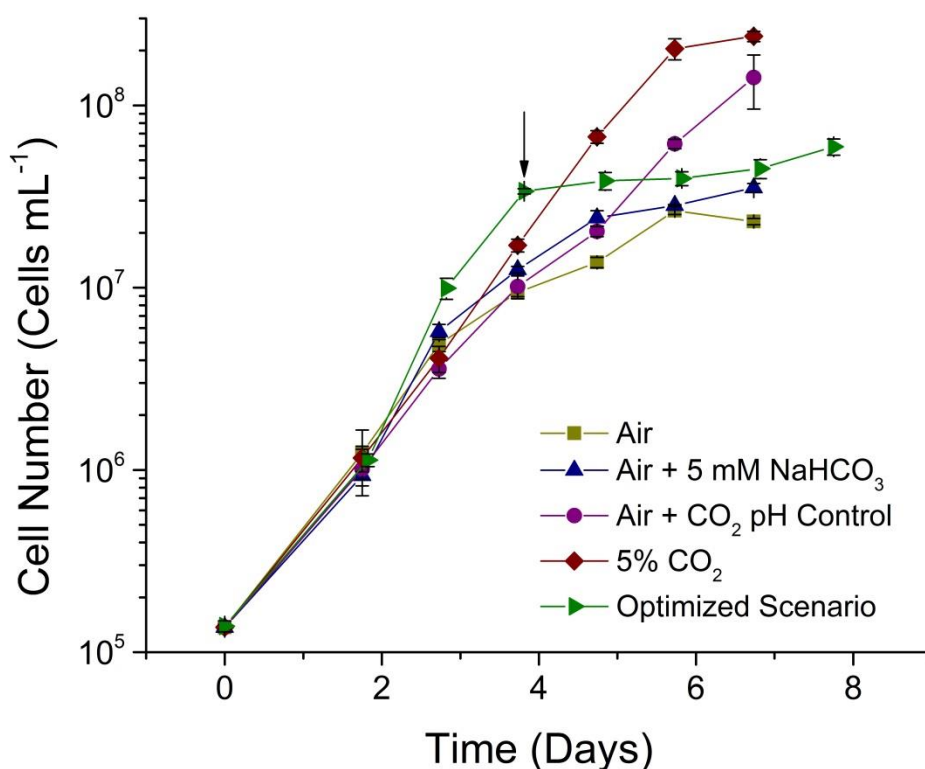


Figure 4.3: Growth (cells mL⁻¹) of cultures of *C. vulgaris* when cultured under various inorganic carbon regimes: (■) Continuous sparge of atmospheric air, (▲) Continuous sparge of atmospheric air and supplemented with 5mM NaHCO₃ at inoculation, (●) Continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7, (◆) Continuous sparge of atmospheric air supplemented with 5% CO₂ (v/v) during daytime hours, (►) The optimized scenario of a continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO₃ at inoculation plus an additional 50mM NaHCO₃ just prior to nitrogen depletion to stimulate TAG accumulation (n=3). Arrow indicates time of 50mM NaHCO₃ addition just prior to nitrogen depletion of the culture medium.

Furthermore, biomass productivity ($\text{g L}^{-1} \text{ day}^{-1}$) was increased by 27% above the 5% CO_2 control (Table 4.3). The photosynthetic capacity of each culture was measured in proxy by monitoring the total chlorophyll content over time. Most interestingly, the optimized scenario produced significantly higher concentrations of chlorophyll (Figure 4.4), indicating an increased capacity for electron transport and inorganic carbon fixation.

Table 4.3: Characteristics of cultures of *C. vulgaris* when grown under various inorganic carbon regimes: 1) continuous sparge of atmospheric air, 2) continuous sparge of atmospheric air and supplemented with 5mM NaHCO_3 at inoculation, 3) continuous sparge of atmospheric air supplemented periodically with 5% CO_2 (v/v) to maintain pH between 8.4-8.7, 4) continuous sparge of atmospheric air supplemented with 5% CO_2 (v/v) during daytime hours and 5) the optimized scenario of a continuous sparge of atmospheric air supplemented periodically with 5% CO_2 (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO_3 at inoculation plus an additional 50mM NaHCO_3 just prior to nitrogen depletion to stimulate TAG accumulation (n=3). All growth yields are calculated for the exponential growth phase (*i.e.* from inoculation until depletion of nitrogen).

Treatment	Cell concentration ($\times 10^7$ cells mL^{-1})	Specific Growth Rate (μ_{max} Day^{-1})	Biomass Productivity ($\text{g L}^{-1} \text{ Day}^{-1}$; DCW) ^a	Maximum Chlorophyll (mg L^{-1})
Air only Control	2.31 ± 0.09	0.61 ± 0.09	0.04 ± 0.00	3.52 ± 1.0
Air + 5 mM NaHCO_3	3.53 ± 0.2	0.82 ± 0.02	0.06 ± 0.01	5.72 ± 1.0
Air + 5% (v/v) CO_2 pH Control	14.21 ± 4.67	0.76 ± 0.05	0.11 ± 0.02	8.01 ± 1.23
5% (v/v) CO_2 Continuous	23.92 ± 1.55	1.02 ± 0.02	0.11 ± 0.01	8.13 ± 1.46
Optimized Scenario	5.93 ± 0.61^b	1.72 ± 0.06	0.14 ± 0.00	10.7 ± 0.84

^a Dry cell weight (DCW) determined gravimetrically with lyophilized biomass

^b 50mM NaHCO_3 addition results in cessation of cellular division

Lipid profiles were compared for cultures grown on 5% CO_2 continuously during the daylight hours against cultures grown under the optimized conditions outlined above. Additionally, to assess how cultures grown under optimized conditions would perform

when using a lower grade of bicarbonate, 50mM $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (sesquicarbonate) was supplemented as a third treatment.

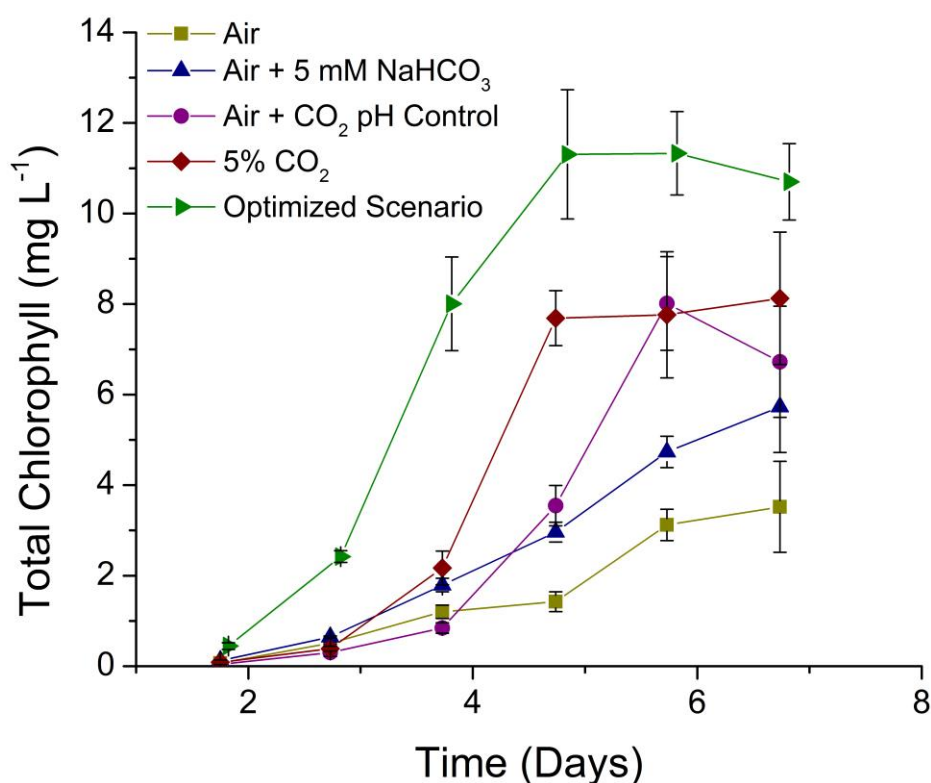


Figure 4.4: Total chlorophyll concentration (mg L^{-1}) for cultures of *C. vulgaris* when cultured under various inorganic carbon regimes: (■) Continuous sparge of atmospheric air, (▲) Continuous sparge of atmospheric air and supplemented with 5mM NaHCO_3 at inoculation, (●) Continuous sparge of atmospheric air supplemented periodically with 5% CO_2 (v/v) to maintain pH between 8.4-8.7, (◆) Continuous sparge of atmospheric air supplemented with 5% CO_2 (v/v) during daytime hours, (►) The optimized scenario of a continuous sparge of atmospheric air supplemented periodically with 5% CO_2 (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO_3 at inoculation plus an additional 50mM NaHCO_3 just prior to nitrogen depletion to stimulate TAG accumulation (n=3).

Figure 4.5 presents extractable lipid profiles for the three conditions as well as total extractable lipid (sum of FFA, MAG, DAG and TAG) and total biodiesel potential (FAME). No statistical difference was observed in TAG content between cultures grown on 5% CO₂ (23.2 ± 0.77 % w/w) and cultures supplemented with 50mM NaHCO₃·Na₂CO₃ (23.5 ± 1.5 % w/w).

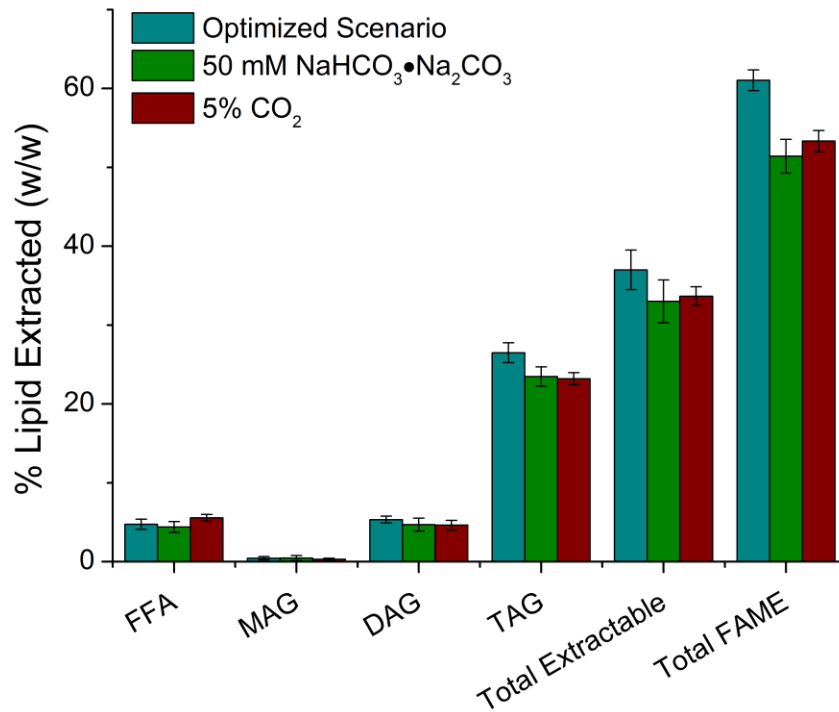


Figure 4.5: Extractable lipid class and FAME profiles for cultures of *C. vulgaris* when cultured under various inorganic carbon regimes: 1) The optimized scenario of a continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO₃ at inoculation plus an additional 50mM of ACS grade NaHCO₃ just prior to nitrogen depletion to stimulate TAG accumulation. 2) The same culture conditions as scenario 1 listed above, except TAG accumulation was induced by adding 50 mM NaHCO₃·Na₂CO₃ (sesquicarbonate). 3) Continuous sparge of atmospheric air supplemented with 5% CO₂ (v/v) during daytime hours. Values are reported for the completion of the experiment (n=3). All values expressed as weight percent (% weight extractable lipid or weight FAME/weight biomass).

However, cultures supplemented with 50mM ACS grade NaHCO_3 (Figure 4.5, optimized scenario) increased in TAG content by 3.3% (w/w) over the 5% CO_2 control. Free fatty acid, MAG and DAG content was statistically equivalent between all three cultures; hence total extractable lipid followed the same trend as TAG percentage. Total biodiesel potential (% FAME biomass⁻¹) was increased from 53.3 ± 1.34 (% w/w) (5% CO_2 control) to 61 ± 1.3 (% w/w) under the optimized condition (Figure 4.5), and biodiesel productivity was increased by 7.7% (Table 4.4).

This increase in total biodiesel potential is partially attributed to increased concentrations of TAG in the optimized condition; however a significant portion may also have come from residual membrane-bound lipids. Table 4.4 presents FAME profiles for each of the three cultures, separated by carbon chain length and saturation. Interestingly, cultures supplemented with ACS grade sodium bicarbonate made significantly more of each fatty acid except mono-unsaturated C_{18} , which reached the highest concentration in cultures grown on 5% CO_2 .

Table 4.4: Lipid characteristics for cultures grown under various inorganic carbon regimes: 1) The optimized scenario of a continuous sparge of atmospheric air supplemented periodically with 5% CO₂ (v/v) to maintain pH between 8.4-8.7 and an initial addition of 5mM NaHCO₃ at inoculation plus an additional 50mM of ACS grade NaHCO₃ just prior to nitrogen depletion to stimulate TAG accumulation. 2) The same culture conditions as scenario 1 listed above, except TAG accumulation was induced by adding 50 mM NaHCO₃·Na₂CO₃ (sesquicarbonate). 3) Continuous sparge of atmospheric air supplemented with 5% CO₂ (v/v) during daytime hours. (n=3). All biodiesel yields are calculated for the stationary growth phase (*e.g.* from depletion of nitrogen until termination of experiment).

Treatment	C16:0 ^a	C16:1 ^a	C18:0 ^a	C18:1 ^a	C18:2 ^a	C18:3 ^a	Other ^b	Total biodiesel potential (%) ^c	Total biodiesel productivity (g L ⁻¹ Day ⁻¹) ^c
50 mM ACS Grade NaHCO ₃	10.66 ± 0.3	2.76 ± 0.03	5.17 ± 0.09	24.42 ± 0.44	7.17 ± 0.14	10.21 ± 0.33	0.64 ± 0.06	61.04 ± 1.31	0.098 ± 0.002
50 mM NaHCO ₃ ·Na ₂ CO ₃	9.27 ± 0.34	2.64 ± 0.1	3.97 ± 0.11	19.85 ± 1.2	6.56 ± 0.18	8.45 ± 0.15	0.68 ± 0.09	51.41 ± 2.14	0.07 ± 0.005
5% (v/v) CO ₂ Continuous	9.45 ± 0.7	1.85 ± 0.14	2.36 ± 0.2	26.59 ± 2.1	4.58 ± 0.4	7.88 ± 0.76	0.63 ± 0.12	53.33 ± 1.35	0.091 ± 0.005

^a All values expressed as weight percent (% weight FAME/weight biomass)

^b Sum of other compounds detected

^c Total FAMES

Conclusions

We describe here a comprehensive study comparing various bicarbonate salts and their effect on lipid accumulation in the model Chlorophyte *Chlorella vulgaris* sp. strain UTEX 395. Further, we present an optimized, two-phase enhanced growth and lipid accumulation scenario with industrial relevance. The strategic addition of sodium bicarbonate was found to enhance growth rates and lipid accumulation rates in cultures of *C. vulgaris*, as compared to traditional growth regimes which only supply elevated concentrations of CO_{2(g)} as the sole inorganic carbon substrate. From an industrial perspective, the cost of transport, storage and delivery of gaseous CO₂ has been suggested to be uneconomical for large scale algal production (Campbell et al., 2011; Jorquera et al., 2010; Liu et al., 2013; Yang et al., 2011). However, improved growth rates in microalgae cultures are often achieved when elevated concentrations of dissolved inorganic carbon are present in the medium. By supplementing cultures with low doses of bicarbonate to first enhance the specific growth rate, and secondly by adding elevated concentrations of bicarbonate in concert with medium nitrogen depletion, both growth and lipid accumulation rates are significantly increased above the control. Additionally, under these optimized growth and lipid accumulation strategies, it was found that chlorophyll content was significantly increased, suggesting higher photosynthetic activity and hence greater electron transport and carbon fixation rates. Finally, the raw, unprocessed form of bicarbonate (sesquicarbonate) was found to be an adequate source of inorganic carbon for both enhanced growth and lipid accumulation in cultures of *C. vulgaris*. These findings present an industrially relevant approach to optimizing

dissolved inorganic carbon usage in algal biofuel production. However, the logistical feasibility of this technology will depend on a techno-economic analysis comparing costs associated with bicarbonate versus CO₂, strain selection, requisite DIC concentrations and culturing times. This work is in progress.

Methods

Strain and Culturing Conditions

Chlorella vulgaris sp. strain UTEX 395 (University of Texas at Austin) was cultured on Bold's basal medium (Nichols and Bold, 1965) with pH adjusted to 8.7 prior to autoclaving. Cultures were either grown in batch airlift tube reactors or baffled flasks, depending on the experimental conditions, as outlined below.

Part 1: Bicarbonate Salt Study Cultures of *C. vulgaris* were grown in 100 mL of medium in a 250 mL baffled shaker flask (Fisher Scientific, Palatine, IL) until just prior to medium nitrogen depletion. The biomass was then concentrated via centrifugation at 4800x *g* and 4°C for 10 minutes (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). Biomass pellets were re-suspended in 100 mL of Bold's basal medium deplete of nitrogen and amended with one of the following bicarbonate salts (final concentrations): 0 mM HCO₃⁻ (control), 50 mM ACS grade NaHCO₃, 50 mM ACS grade Na₂CO₃ (both from Sigma-Aldrich, St. Louis MO), 50 mM industrial grade NaHCO₃, 50 mM NH₄HCO₃, 50 mM KHCO₃, 25 mM natural grade NaHCO₃·Na₂CO₃ (sesquicarbonate) (all from Church & Dwight Co., Inc.; Princeton, NJ). All studies were conducted in triplicate for statistical reliability.

Part 2: Inorganic Carbon Growth
and Lipid Accumulation Study

Experiments were conducted in triplicate batch cultures using 70×500 mm glass tubes containing 1.2 L medium submersed in a water bath to control temperature. Rubber stoppers, containing ports for aeration and sampling, were used to seal the tubes. Temperature was maintained at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Light ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was maintained on a 14:10 L/D cycle using a light bank containing T5 tubes. Aeration (400 mL min^{-1}) was supplied by humidified compressed air (supplemented with 5% CO_2 (v/v) for high CO_2 or pH controlled conditions) and controlled using individual rotameters for each bioreactor (Cole-Parmer, USA). ACS grade sodium bicarbonate was used in all experiments involving bicarbonate addition (Sigma-Aldrich, St. Louis MO) and natural grade $\text{NaHCO}_3 \cdot \text{Na}_2\text{CO}_3$ (Church & Dwight, Princeton, NJ) was used in all experiments involving sesquicarbonate.

Culture Analysis

Cultures were checked for bacterial contamination by inoculation into Bold's Basal medium supplemented with 0.05% yeast extract and 0.05% glucose and incubated in the dark. Cell concentrations were determined using an optical hemacytometer with a minimum of 400 cells counted per sample for statistical reliability. Dry cell weights (DCWs) were determined by harvesting 50 mL of culture into a tared 50 mL centrifuge tubes (Fisher Scientific, Palatine, IL) followed by centrifugation at $4800 \times g$ at 4°C for 10 minutes (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H_2O (diH_2O), $18 \text{ M}\Omega$, to remove media salts and excess bicarbonate, before centrifuging again. Remaining algal pellets were frozen and

lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours. DCWs were calculated by subtracting the weight of the biomass-free centrifuge tube from the weight of the centrifuge tube with lyophilized biomass.

Analysis of Media Components

Medium pH was measured using a standard bench top pH meter. Ammonium and nitrate concentrations were measured using Nessler reagent (HACH, Loveland, CO) and ion chromatography (Dionex, Sunnyvale, CA), respectively, using previously described protocols (Gardner et al., 2013a).

Dissolved Inorganic Carbon Analysis

Medium dissolved inorganic carbon (DIC, sum of dissolved carbon dioxide, bicarbonate, and carbonate), was measured using previously described protocols (Gardner et al., 2013a). Briefly, 8 mL of culture was filtered, 0.2 μm pore size, and the supernatant was analysed with a Skalar Formacs TOC/TN analyser using a Skalar LAS-160 autosampler. DIC quantification was conducted by correlating peak area to a calibrated standard curve from bicarbonate and carbonate mix standards (Sigma-Aldrich).

Chlorophyll Measurements

Chlorophyll a, b, and carotenoids were determined using the methanol extraction and absorption correlation described in (Ördög et al., 2011). One mL of culture was centrifuged at 16,000x *g* for 5 min, after which the supernatant was discarded. One mL

methanol was added to the centrifuged pellet, which was then vortexed and lightly sonicated to disperse the pellet. The slurry was heated at 70°C for 5 min in a water bath followed by centrifugation at 16,000x *g* for 3 min. Absorption was read at 666 nm, 653 nm and 470 nm. Calculations for chlorophyll and carotenoids ($\mu\text{g mL}^{-1}$) were conducted as previously described in (Ördög et al., 2011).

Harvesting

Cultures were harvested just prior to medium nitrogen depletion and at the conclusion of each experiment. Each time, two aliquots of 50 mL were dispensed into 50-mL centrifuge tubes (Fisher Scientific, Palatine, IL) and centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 4800x *g* at 4°C for 10 minutes. The concentrated biomass was rinsed with diH₂O to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were then frozen and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours and stored at -20°C for subsequent lipid analysis.

Bead Beating Extraction of Lipids from Dry Biomass

Extraction and analysis of extractable lipid was conducted as previously reported (Lohman et al., 2013). Roughly ~30 mg of dried biomass was combined with 1 mL of chloroform in a 1.5 mL stainless steel bead beating microvial with silicone cap (BioSpec Products, Bartlesville, OK). Three types of beads (0.6 g of 0.1 mm zirconium/silica beads, 0.4 g of 1.0 mm glass beads, two 2.7 mm glass beads) were added to each vial before capping. A FastPrep bead beater (Bio101/Thermo Savant, Carlsbad, CA) was used

to agitate the vials for six 20 s pulses at power level 6.5 followed by a 1 min cool down period between pulses. Total bead beating exposure time was 2 min. The mixture of solvent, residual biomass and beads was then transferred to a Pyrex test tube with a Teflon lined screw cap (Kimble-Chase, Vineland, NJ), and the steel microvial was rinsed twice with 1 mL of chloroform, which was also added to the test tube, bringing the total solvent volume to 3 mL. One mL of 15% NaCl (v/v) was added to enhance phase separation. Samples were then centrifuged at 1,200x *g* for 2 min to pellet the residual biomass. One mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Transesterification for FAME Analysis

Transesterification of fatty acid was conducted using a previously described protocol (Griffiths et al., 2010) with modifications. FAME composition was analyzed using gas chromatography – mass spectroscopy detection. Briefly, approximately 20 mg of dried algae biomass was transferred to a Pyrex test tube with a Teflon lined screw cap (Kimble-Chase, Vineland, NJ). One mL of toluene and 2 mL sodium methoxide (Fisher Scientific, Pittsburgh PA) were added to each test tube along with 10 μ L of a 10mg/mL standard mix (C11:0 and C17:0 TAG) to monitor transesterification efficiency of the TAG into FAME. Samples were heated in an oven for 30 min at 90°C and vortexed every 10 min. Samples were allowed to cool to room temperature before 2 mL of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) were added and samples were heated again for an additional 30 min. Samples were again allowed to cool before 10 μ L of a 10 mg/mL of C23:0 FAME was added to assess the completeness of

partitioning FAME into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated salt water solution (NaCl in diH₂O) were added. Samples were heated for 10 min at 90°C to facilitate FAME partitioning into the organic phase, vortexed for 10 s and centrifuged at 1,200x *g* for 2 min to enhance phase separation. One mL of the organic phase was removed from the top layer using a glass syringe and transferred to a 2 mL GC vial for GC-MS analysis.

Lipid Analysis

GC-FID

GC-FID analysis was performed according to our previously published protocol (Lohman et al., 2013). Briefly, a 1 µL splitless injection was performed via an autosampler into a GC-FID (Agilent 6890N, Santa Clara CA) equipped with a 15 m (fused silica) RTX biodiesel column (Restek, Bellefonte PA). The initial column temperature was held at 100°C for 1 min, before being increased to 370°C at a rate of 10°C min⁻¹. The injector temperature was held constant at 320°C. Helium was used as the carrier gas and column flow was held at 1.3 mL min⁻¹ for 22 min, increased to 1.5 mL min⁻¹, held for 2 min, increased to 1.7 mL min⁻¹ and held for 12 min. All flow rate increases were set to 0.2 mL min⁻². Calibration curves were constructed for each of the following standards: C12:0, C14:0, C16:0, C18:0, and C20:0 FFAs; C12:0, C14:0, C16:0, and C18:0 MAGs; C12:0, C14:0, C16:0, and C18:0 DAGs; along with C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, and C20:0 TAGs (Sigma-Aldrich, St. Louis MO) for quantification ($r^2 > 0.99$). This GC method allows for an estimate of the amounts of FFA, MAG, DAG and TAGs in a single analysis as detailed in (Lohman et al., 2013).

GC-MS

GC-MS analysis was performed as follows. Briefly, 1 μL split (2:1) injections were performed via an autosampler into a GC-MS (Agilent 6890N GC and Agilent 5973 Networked MS) equipped with a 60 m x 0.25 mm x 0.15 mm Agilent DB-23 column (0.25 μm phase thickness). The injector temperature was 280°C and the detector temperature was 150°C. The initial column temperature was 50°C (held for 1 min) and was increased to 175°C at a rate of 25.0°C min⁻¹, immediately followed by a ramp at 4.0°C min⁻¹ to a final temperature of 230°C which was held for 10 min before run termination. Helium was used as the carrier gas and column flow was held at 0.5 mL min⁻¹. Quantities of FAMES were determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275), with additional analyses performed using a custom program described below. A 28-component fatty acid methyl ester standard prepared in methylene chloride (NLEA FAME mix; Restek, Bellefonte, PA) was used for GC-MS retention time identification and response curve generation ($r^2 > 0.99$).

Lipid Quantification and Analysis

Lipid quantification was performed using a custom program developed specifically for this purpose (Appendix B). Chromatogram data from the GC-FID and GC-MS were exported from the instrument software (Chemstation Ver. B.02.01-SR1) as a Microsoft Excel spreadsheet. The spreadsheet was then imported into a web-based software application written in Microsoft's ASP.NET framework using C# and MVC as the coding language and paradigm, respectively. Data were stored with unique identifiers

in a Microsoft SQL database. Six point calibration curves were constructed using the LINEST function which is built into the ASP.NET framework library. Chromatogram peaks were quantified based on the closest standard, as determined by retention time of the sample peak and standard peak, respectively. All chromatograms were manually integrated and inspected prior to export to ensure accuracy and reliability. Manual calculations were performed periodically using Excel to verify software results.

Calculations

Specific growth rates were calculated using the following equation:

$$\mu = K' = \ln \left(\frac{mt_2}{mt_1} \right) / (t_2 - t_1) \quad t_2 > t_1$$

Where m_t are biomasses at the different timepoints (t_1 and t_2) respectively.

Abbreviations

FFA: Free Fatty Acid; MAG: Monoacylglyceride; DAG: Diacylglyceride; TAG: Triacylglyceride; FAME: Fatty Acid Methyl Ester; DIC: Dissolved Inorganic Carbon; GC-FID: Gas chromatography – Flame ionization detection; GC-MS: Gas chromatography – Mass spectroscopy; Chl a: Chlorophyll a; Chl b: Chlorophyll b; CCM: Carbon concentrating mechanism; Rubisco: Ribulose-1, 5-Bisphosphate carboxylase oxygenase;

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

EL carried out growth and biomass harvest assays, conducted lipid extraction and analysis and drafted the manuscript. TP and RDG carried out DIC, pH, chlorophyll and nutrient assays and participated in study design, study coordination, data analysis/interpretation and drafting the manuscript. KEC, BP and RG participated in study design, study coordination, data analysis/interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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CHAPTER 5

PROJECT CONCLUSION AND FUTURE WORK

Project Rationale

During the 1970's, the US Department of Energy's Aquatic Species Program initiated a quest to develop an alternative medium for transportation fuel by harnessing the biologically synthesized energy stored in microalgae. Four decades later, research and development has advanced to the point of commercial scale applications. Although many technological hurdles have been addressed, the economic feasibility of algae derived biofuel is still uncertain. Optimized system processes which consider geobiological resource allocation, infrastructure, and energy tradeoffs are needed to fully realize a truly marketable and sustainable production process. At the fundamental level, more research and development is also needed to address downstream processes such as harvesting, dewatering, extraction, refinement, resource reallocation and recycling.

This dissertation represents the summary of work completed to develop robust methods for quantifying and analyzing the lipid content of microalgal cultures, and demonstrates how these methods can be used to elucidate lipid metabolism in both Chlorophytes and diatoms. Additionally, an optimized process was developed to enhance growth and lipid accumulation rates in the model Chlorophyte *Chlorella vulgaris* by supplementing cultures with additional, low cost, dissolved inorganic carbon in the form of sodium bicarbonate, which may result in an industrially relevant approach to reduce costs and increase production. The methods developed for quantifying and analyzing

lipids allow for the chromatographic separation of compounds based on carbon chain length and saturation and can be used to define the free fatty acid, monoacylglyceride, diacylglyceride and triacylglyceride content of live cultures. Additionally, through a modified direct *in situ* transesterification technique, total cellular fatty acids which have been chemically altered into fatty acid methyl esters can be separated by mass, saturation and bond orientation. Combined, these techniques can be used to develop comprehensive lipid profiles throughout the course of a growth cycle, and provide insight into how lipid metabolism changes due to nutrient deprivation and changes in inorganic carbon availability.

Attainment of Project Aims

The project goals were to:

- (i) Develop robust methods for quantifying and analyzing lipid precursor compounds such as FFA, MAG, DAG and TAG, as well as total cellular fatty acids transesterified into FAME.
- (ii) Verify method extraction efficacy by comparing against traditional extraction and analysis techniques using two Chlorophyta and one diatom.
- (iii) Elucidate inorganic carbon partitioning into lipid fractions in cultures of *Chlamydomonas reinhardtii* when grown under three unique inorganic carbon regimes.
- (iv) Determine an optimized inorganic carbon regime for enhanced growth and lipid accumulation in cultures of *Chlorella vulgaris*.

(i) Method Development

A rapid method for the efficient extraction of lipids from live cultures of microalgae for the purpose of quantifying the lipid content and characterizing the lipid carbon chain length and saturation was developed. Additionally, methods were developed to determine the fraction of total biodiesel potential (as FAME) derived either from extractable lipid or the residual fraction. Furthermore, the methods frequently determine from which lipid class (FFA, MAG, DAG or TAG) a particular FAME was derived. Since properties of biodiesel depend heavily on the length and degree of saturation of the FAME, the methods developed can assist in ‘fine-tuning’ the final product, be it biodiesel or a higher value product, through the choice of certain algae or growth conditions (Lohman et al., 2013).

(ii) Method Validation

The efficacy and usefulness of the methods described above were demonstrated by comparing lipid profiles from three frequently studied microalgae. It was demonstrated that the marine diatom *P. tricornutum* preferentially produced unsaturated C₁₆ lipid compounds, whereas the two freshwater green microalgae, *C. vulgaris* and *C. reinhardtii*, predominantly synthesized unsaturated C₁₈ fatty acid compounds. From an industrial standpoint, it will be beneficial to choose the appropriate algae and culture conditions to produce the largest possible amount of biodiesel with the appropriate FAME profile or to screen for algal products with higher value than biodiesel. For instance *P. tricornutum* was shown to produce an omega-3 fatty acid, specifically eicosapentaenoic acid (C_{20:5}), which has been suggested to benefit human health. It was

determined that these compounds were primarily derived from TAG (Lohman et al., 2013).

(iii) Inorganic Carbon Partitioning into Lipid

Lipid content of microalgal cultures are traditionally analyzed at time-of-harvest by extraction and subsequent gravimetric analysis or by transesterification and subsequent quantification of the resultant fatty acid methyl ester (FAME) content. Modern analyses include direct or *in situ* transesterification of dry biomass and subsequent FAME analysis. These approaches however are unable to identify how inorganic carbon is being partitioned into fatty acid or where a specific compound was derived from. Further, these approaches do not provide a comprehensive evaluation of lipid profiles over time. By using the relatively straight-forward methods for quantifying the extractable lipid classes outlined above, including free fatty acids, mono-, di-, and triacylglycerides via GC-FID, and total FAME via GC-MS, lipid profiles over time were established for *C. reinhardtii* when grown under three unique inorganic carbon regimes.

Extractable lipids were characterized to include lipid class (FFA, MAG, DAG, TAG) and carbon chain length for comparison against FAMEs. Data points were collected each day throughout the growth cycles to provide comprehensive lipid profile analyses for *C. reinhardtii* when grown with atmospheric concentrations of CO₂ (0.04% v/v), elevated CO₂ (5% v/v), and for cultures aerated with ambient air and supplemented with 50 mM sodium bicarbonate. FFAs and to a lower degree MAGs were the predominant metabolites prior to nitrogen depletion, as compared to DAG and TAGs, but were reduced over time as the organism began synthesizing DAGs and TAGs; suggesting

a reallocation of FFA and MAG into DAG and TAG. All three conditions produced equal concentrations of saturated and unsaturated C₁₆ fatty acids until nitrogen in the growth medium was depleted, after which a shift towards increasing concentrations of saturated C₁₆ fatty acids was observed. This trend was more significant in cultures with increased availability of inorganic carbon. Unsaturated C₁₈ fatty acids were the predominant FAMES in all three conditions even though C₁₆ TAGs were twofold more abundant than C₁₈ TAGs, indicating that C₁₈ FAMES were derived from compounds other than C₁₈ TAGs, such as membrane lipids. In all three conditions the total FAME content on a weight per weight basis (mass of FAME per biomass - as dry cell weight) did not significantly change over time, however lipid production per culture volume increased over time in all three treatments, reaching the highest concentration in cultures grown on 5% CO₂ (v/v). Conversely, lipid production per cell was highest in cultures supplemented with sodium bicarbonate. These results demonstrate the importance of reporting lipid and FAME productivity in at least the various ways highlighted here since each of these values provides insight into differences between culture treatments and might be uniquely valuable to researchers in industry and academia, respectively. Additionally, these results provide insight into which lipid compounds *C. reinhardtii* synthesizes and how the organism's metabolism changes due to nitrogen depletion and inorganic carbon source availability. These results help elucidate how inorganic carbon is being partitioned between lipid classes (*i.e.* FFAs, MAGs, DAGs and TAGs). This approach can be utilized for determining the optimal time-point of harvest during biodiesel production as well as during screening for high value product accumulation

(such as Ω -3 fatty acids). This work will be submitted to the Journal of Applied Phycology.

(iv) Optimized Process for Enhanced Growth and Lipid Accumulation

A comprehensive study comparing various bicarbonate salts and their effect on lipid accumulation in the model Chlorophyte *Chlorella vulgaris* strain UTEX 395 was conducted. Based on these results, an optimized, two-phase enhanced growth and lipid accumulation scenario with industrial relevance was developed. The strategic addition of sodium bicarbonate was found to enhance growth rates and lipid accumulation rates in cultures of *C. vulgaris*, as compared to traditional growth regimes which only supply elevated concentrations of $\text{CO}_{2(g)}$ as the sole inorganic carbon substrate. From an industrial perspective, the cost of transporting, storage and delivery of gaseous CO_2 has been suggested to be uneconomical for large scale algal production (Campbell et al., 2011; Jorquera et al., 2010; Liu et al., 2013; Yang et al., 2011). However, improved growth rates in microalgae cultures may only be achieved when elevated concentrations of carbon are present in the medium. By supplementing cultures with low doses of bicarbonate to first enhance the specific growth rate, and secondly by adding elevated concentrations of bicarbonate in concert with medium nitrogen depletion, both growth and lipid accumulation rates were significantly increased above the control. Additionally, under the optimized growth and lipid accumulation conditions, it was found that chlorophyll content was significantly increased, suggesting higher photosynthetic activity and hence greater electron transport and carbon fixation rates. Finally, the raw, unprocessed form of bicarbonate (sesquicarbonate) was found to be an adequate source of

inorganic carbon for both enhanced growth and lipid accumulation in cultures of *C. vulgaris*.

Future Work

To summarize the rationale for the work presented in this dissertation; the current demand for traditional petroleum based transportation fuel is increasing whereas the supply chain is stressed by diminishing reserves and increased costs associated with exploiting these natural resources. Due to the inherent advantages of their growth characteristics and energy storage potential, microalgae may provide a potent alternative for the mass production of transportation fuel. However, fundamental research is still necessary to fully develop an economically sustainable and scalable commercial application.

Herein, I present an optimized growth and lipid accumulation scenario which may help to alleviate costs associated with system processes and resources. However, this work will need to be expanded and tested on a variety of microalgae species, and further validated with robust life cycle analysis to prove its feasibility. Additional research and development is needed to incorporate nutrient recycling and resource reallocation strategies in order to achieve a sustainable and scalable process. Further, these studies will need to be demonstrated at scale with “real world” parameters taken into account such as outdoor cultivation and variations in climate and nutrient availability. At the fundamental level, further investigation into how these organisms fix inorganic carbon

and how variations in DIC speciation and concentration affect photosynthetic activity, growth and lipid accumulation is still needed.

Expanding the Optimized Scenario to Additional Organisms

Growth and lipid accumulation rates were significantly enhanced in cultures of *Chlorella vulgaris* when supplemented with 5mM NaHCO₃ and pH regulated with a periodic sparge of 5% CO₂ (v/v) until near nitrogen depletion, which was coupled with a high dose (50mM) of sodium bicarbonate. This study may present an industrially relevant scenario for reducing system process costs associated with pumping and mixing CO₂ gas. However, further research is necessary to determine how effective this strategy is with other relevant organisms and mixed cultures. For example, the marine green microalga *Nannochloropsis oceanica* and the marine diatom *Phaeodactylum tricornutum* have both been used in scale-up applications due to their inherently fast growth rates and ability to accumulate significant concentrations of TAG or other high value co-products such as Ω -3 fatty acids (Chiu et al., 2009; Fernández Sevilla et al., 2004). Determining if the optimized growth and lipid accumulation scenario is applicable to these industrially relevant organisms will further validate its commercial feasibility.

Life Cycle Analysis

Intuitively it makes sense that if a commercial scale algal biofuel facility reduces its CO₂ usage by supplementing cultures with bicarbonate for enhanced growth and lipid accumulation, there will also be a subsequent reduction in costs associated with transporting, storage, pumping and mixing of CO₂. However, a robust life cycle analysis

is required to validate these claims. A number of assumptions are made regarding the costs associated with either form of inorganic carbon. An appropriate LCA model would address costs associated with building the infrastructure needed to transport CO₂ from waste stream processes such as power plant flue gas, as well as the costs associated with storing and delivering CO₂ to outdoor algal ponds. Likewise, costs associated with mining, processing, refining and delivering bicarbonate will need to be evaluated. Alternatively, it may be more economical to produce bicarbonate from CO₂ absorbed into NaOH. Either way, a comprehensive evaluation of these processes needs to be undertaken before implementation at commercial scale. Additionally, an assessment regarding a two-phase, growth and lipid accumulation reactor design should be developed which takes advantage of reduced areal land requirements for the lipid accumulation stage through concentrating algal slurries.

Nutrient Recycling

Although it has been shown that energy requirements associated with pumping and mixing CO₂ into algal bioreactors contributes significantly to the overall system process costs (*e.g.* Jorquera et al., 2010), reducing this overhead will not be enough to compete economically with the current price of traditional fossil based fuels. Therefore, additional cost reductions are necessary and may be found by intelligent designs incorporating nutrients derived from industrial and municipal waste streams. Research has shown that many species of microalgae are capable of growth in municipal wastewater and formation water produced from coal bed methane operations (Feng et al., 2011; Pittman et al., 2011). This work should be expanded to determine if these water

sources can be continually reused after the dewatering process, and if nutrients can be replenished from sources such as anaerobic digester sludge. Furthermore, inorganic carbon may be efficiently replenished in recycled water which is high in alkalinity, due to the addition of elevated concentrations of sodium bicarbonate for enhanced TAG accumulation. Cultures which have been “triggered” to induce lipid accumulation via bicarbonate addition utilize the HCO_3^- ion for fatty acid synthesis (Gardner et al., 2012); however the Na^+ counter ion remains in solution. This may present an opportunity to replenish the DIC by sparging exhausted medium with CO_2 . Additionally, many species of microalgae are capable of utilizing organic carbon as an energy source (Neilson, 1974). Research has shown increased growth yields and lipid production when certain organisms are grown mixotrophically, *i.e.* with both inorganic and organic carbon substrates. During a recent screening study of mine, significant increases in growth rate and TAG biosynthesis were observed in three out of four diatoms when grown mixotrophically on 1% glycerol (v/v). At the scale necessary to displace 50% of traditional fossil-based transportation fuels with biodiesel from microalgae, a significant amount of glycerol will also be produced (Fan et al., 2010), making glycerol a waste product. However, if glycerol can be recycled by feeding it back to the microalgae as an organic carbon source for growth and lipid accumulation, it would help reduce cost and significantly improve the economics of algal derived-biofuels.

Scalability

All of the aforementioned studies focusing on optimization strategies, nutrient recycling and life cycle analysis should be validated for commercial feasibility by scaling

experiments to more “real world” outdoor pond environments. It has been shown that mesoscale pond reactors (~200L) adequately mimic large scale systems with regards to parameters such as growth rates and biomass and biofuel productivities (Putt et al., 2011). Further, outdoor cultivation provides insight into how changes in climate, such as temperature and light availability affect culture growth and sustainability.

Elucidation of Photosynthetic Activity and Inorganic Carbon Fixation

Fundamental research is still required to fully elucidate the biochemistry involved in microalgal inorganic carbon utilization. It has been observed that the total chlorophyll content of cultures grown under the optimized growth scenario is significantly higher than that of control cultures, suggesting that the photosystem II electron transport rate (ETR) is also increased. Pulse-Amplitude Modulated (PAM) fluorometry can be used to determine the effective ETR in algal cultures (Napoléon and Claquin, 2012). Additionally, O₂ evolution can be measured in proxy through dissolved oxygen and correlated to inorganic carbon utilization (Gardner et al., 2013c). Combining these techniques has provided insight into HCO₃⁻ consumption, however further research is necessary. Determining where HCO₃⁻ derived carbon is incorporated into lipid is being investigated through collaboration with Washington State University (WSU) and members of the Algal Biofuel Research Group at Montana State University (MSU). By using ¹H high resolution magic angle spinning (HR-MAS) NMR to monitor coupled and decoupled measurements on ¹³C labeled metabolites, in concert with ¹³C NMR to monitor carbon uptake from H¹³CO₃⁻, we have begun to determine the onset, rate and pathways associated with ¹³C incorporation. Furthermore, by effectively doing the reverse

experimental design (*e.g.*, labeling biomass during optimized exponential growth and then inducing TAG accumulation using unlabeled bicarbonate) information on carbon recycling from the biomass can be acquired. Future work should continue these fundamental investigations into how bicarbonate enhances photosynthetic activity and lipid accumulation.

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APPENDICES

APPENDIX A

CO-AUTHOR PUBLICATION ABSTRACTS

Title

Comparison of CO₂ and Bicarbonate as Inorganic Carbon Sources for Triacylglycerol and Starch Accumulation in *Chlamydomonas reinhardtii*

Authors

Robert D. Gardner, Egan J. Lohman, Robin Gerlach, Keith E. Cooksey, Brent M. Peyton

Journal

Biotechnology and Bioengineering, 2012

Abstract

Microalgae are capable of accumulating high levels of lipids and starch as carbon storage compounds. Investigation into the metabolic activities involved in the synthesis of these compounds has escalated since these compounds can be used as precursors for food and fuel. Here, we detail the results of a comprehensive analysis of *Chlamydomonas reinhardtii* using high or low inorganic carbon concentrations and speciation between carbon dioxide and bicarbonate, and the effects these have on inducing lipid and starch accumulation during nitrogen depletion. High concentrations of CO₂ (5%; v/v) produced the highest amount of biofuel precursors, transesterified to fatty acid methyl esters, but exhibited rapid accumulation and degradation characteristics. Low CO₂ (0.04%; v/v) caused carbon limitation and minimized triacylglycerol (TAG) and starch accumulation. High bicarbonate caused a cessation of cell cycling and accumulation of both TAG and starch that was more stable than the other experimental conditions. Starch accumulated prior to TAG and then degraded as maximum TAG was reached. This suggests carbon reallocation from starch-based to TAG-based carbon storage.

Title

Cellular Cycling, Carbon Utilization, and Photosynthetic Oxygen Production during Bicarbonate-Induced Triacylglycerol Accumulation in *Scenedesmus* sp.

Authors

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Journal

Energies, 2013

Abstract

Microalgae are capable of synthesizing high levels of triacylglycerol (TAG) which can be used as precursor compounds for fuels and specialty chemicals. Algal TAG accumulation typically occurs when cellular cycling is delayed or arrested due to nutrient limitation, environmental challenge (*e.g.*, pH, light, temperature stress), or by chemical addition. This work is a continuation of previous studies detailing sodium bicarbonate-induced TAG accumulation in the alkaline Chlorophyte *Scenedesmus* sp. WC-1. It was found that a culture amendment of approximately 15 mM sodium bicarbonate was sufficient to arrest the cellular cycle (process of a parent cell splitting into daughter cells) and switch the algal metabolism from high growth to a TAG accumulating state. However, the cultures were limited in dissolved inorganic carbon (DIC) one day after the amendment, suggesting additional carbon supplementation was necessary. Therefore, additional abiotic and biotic experimentation was performed to evaluate in- and out-gassing of CO₂. Cultures to which 40-50 mM of sodium bicarbonate was added to arrest the cell cycling while maintaining DIC in the culture medium consumed DIC faster than CO₂ could ingas and photosynthetic oxygen production was elevated in these bicarbonate-enhanced cultures.

APPENDIX B

GC-ONLINE DOCUMENTATION

Overview

GCOOnline is a proprietary web-based software application developed by Egan Jackson Lohman for the specific purpose of quantifying and analyzing lipid compounds, either as free fatty acid (FFA), monoacylglyceride (MAG), diacylglyceride (DAG), triacylglyceride (TAG) or fatty acid methyl ester (FAME), after separation and detection via GC-FID or GC-MS. The following provides general usage, functionality and documentation for the application.

Software Beta

Technology

GCOOnline was written using the following coding languages and paradigms:

1. ASP.NET 4.0 Framework
2. C# Object Oriented Coding language
3. Model, View, Controller (MVC) 4.0 coding paradigm
4. Entity Framework 5.0 Model First coding paradigm
5. SQL Server 2010
6. HTML 4
7. jQuery

Source Code

The source code for GCOOnline has been made public and is hosted under Google Code Project. GCOOnline is licensed under the MIT License. Code can be downloaded via Subversion Repository source control software. We recommend using TortoiseSVN Client.

1. Code URL: <http://code.google.com/p/gc-online/>
2. SVN: <https://gc-online.googlecode.com/svn/trunk/>
3. The MIT License (MIT)

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Workflow

Once you have downloaded and compiled the source code using Microsoft's Visual Studio 2010 Express or later, you will need to configure IIS to run the application as a website. Configuring IIS is outside the scope of this documentation, please refer to online materials or consult your IT administrator.

GCOOnline requires data collected from a GC to be imported into the application as a Microsoft Excel workbook file (.xlsx). The organization of these workbooks and subsequent worksheets is critical for the application to function properly.

Each workbook should contain only worksheets containing GC data. These data can be either sample data or calibration data, but no other worksheets should exist in the workbook. Each spreadsheet should correspond to one and only one Run of a Sequence. A workbook can contain as many Run worksheets as desired and they do not have to correspond to the actual runs per sequence from the GC. In other words, you can mix and match runs from any given sequence; however for organizational purposes this is not recommended. There is an example workbook included in the root directory of the source code repository.

Each worksheet, which corresponds to one Run, must have the following columns with the headers spelled and capitalized precisely.

1. SequenceName
2. RunName
3. ExperimentDate
4. Dilution
5. CDW
6. SampleVolume
7. CDWVolume
8. BMCmmol
9. CellCount
10. Time
11. Area

- 12. Height
- 13. Width
- 14. Area%
- 15. Symmetry

Columns 1-9 correspond to your specific sample information, all of these fields are required, but you can set them to 1 if you don't require a specific calculation: The header column must have the exact text string of the column name (ex. SequenceName), the cell directly below the header row holds the actual value.

1. SequenceName – ex. 20130906_Calibration, This is the name of the sequence, you can name this whatever you want, but it needs to be the same on each worksheet (type = string)
2. RunName – ex. Run_1A (Name of run) (type = string)
3. ExperimentDate – ex. 20130906 (type = DateTime)
4. Dilution – ex. 3 (unit = mL, type = integer, double or float) This is the dilution, if any you used
5. CDW – ex. 1.23 (unit = mg/mL, type = integer, double or float) This is the overall cell dry weight of the CULTURE. This is not the mass you weighed out from which you extracted. This is multiplied by the SampleVolume to derive the actual sample weight.
6. SampleVolume – ex. 45 (unit = mL, type = integer, double or float) This is the sample volume you actually extracted from. This is multiplied by CDW to determine the concentration (mg/mL) of extracted biomass.
7. CDWVolume – ex. 45 (unit = mL, type = integer, double or float) This is the overall culture volume you measured to determine your CDW.
8. BMCmmol ex 0.1 (unit = C mmol, type = integer, double or float) If you don't require a Carbon ratio you do not need to specify this. Just enter 1.
9. CellCount – 1e7 (unit = cells/mL, type = integer, double or float) This is used to calculate lipid per 1000 cells. You can set this to 1 if not required.

Columns 10-15 correspond to data obtained from the GC. All of these fields are required:

1. Time – Retention time for individual peak
2. Area – Response area for individual peak
3. Height – Height of individual peak
4. Width – Width of individual peak
5. Area% – Percent area of individual peak
6. Symmetry – General symmetry rating

Again, each worksheet should contain columns for the six fields generated from the GC software (*e.g.* Agilent Chemstation). A header row should contain the name of the field,

spelled and capitalized precisely as written above. Each row below the header row should contain the corresponding data for each peak in the chromatogram. There is no limit on how many peaks per run can be analyzed.

Sequence Management

The home page of GCOOnline is the sequence management section. Here you can upload new workbooks, delete existing sequences, or view existing sequences.

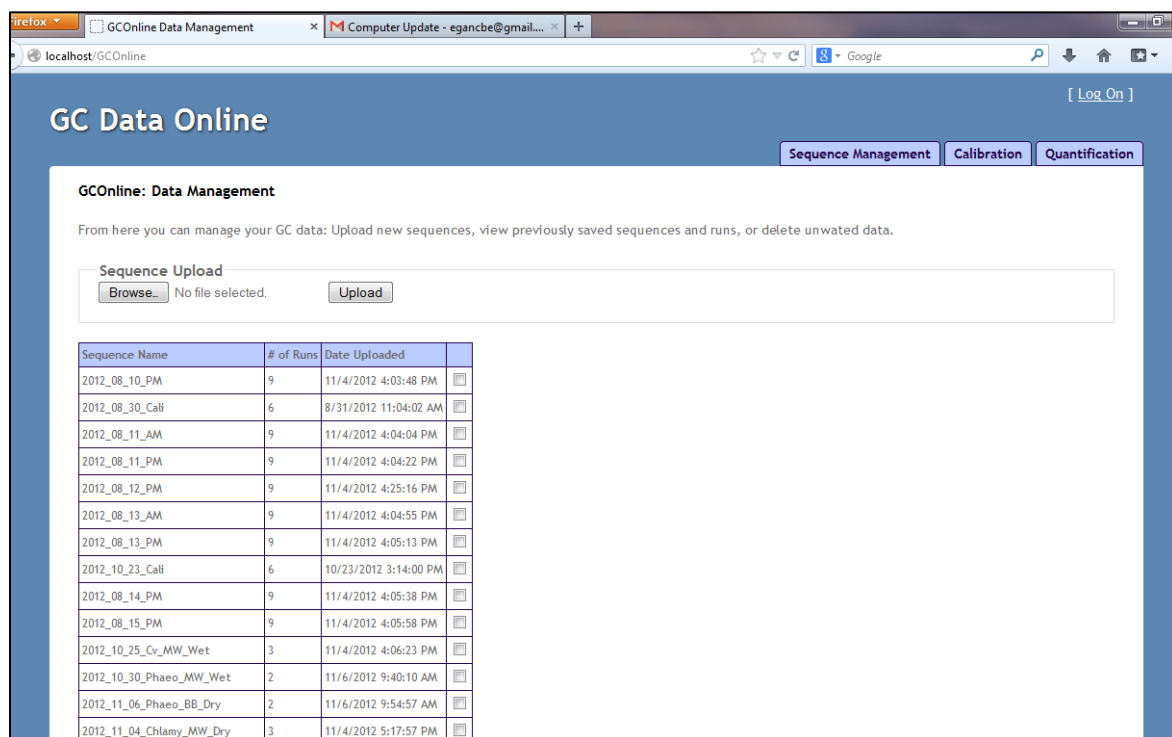


Figure B.1: GCOOnline Sequence Management Screenshot.

Your first order of business should be to upload a workbook containing a sequence and subsequent runs for a calibration. Use the browse button to locate the workbook on your local or networked hard drive, and upload it to the database. Once the sequence has uploaded, it will appear in the list below the upload button. You can then check the box next to the sequence and use the navigation at the bottom to View Runs, Get Details on the Sequence, or Delete the Sequence.

Check the box next to your calibration sequence, and then click View Runs.

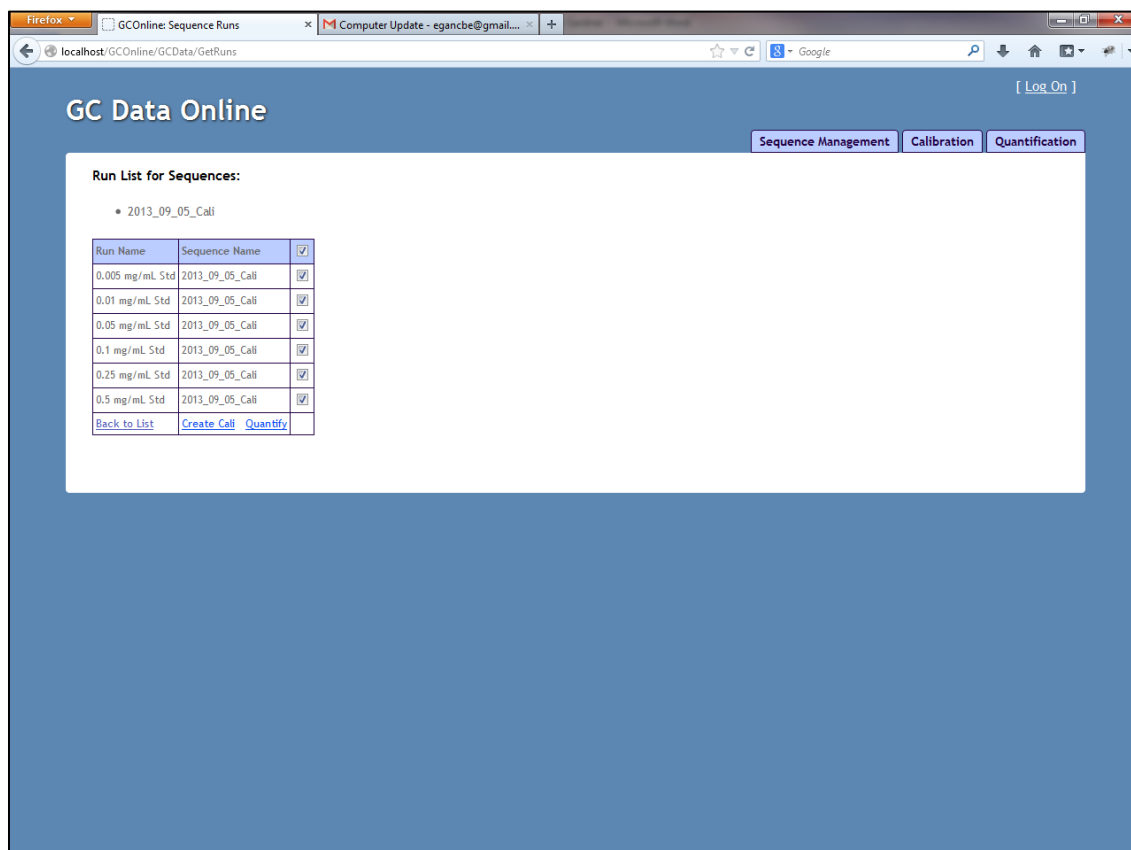


Figure B.2: GCOOnline View Runs Screenshot.

From the View Runs screen, you can choose the runs you wish to analyze by checking the box corresponding to each run. The checkbox at the top selects or deselects all runs. You can then either click Create Cali, or Quantify. Create Cali will allow you to define which standard each peak in each run corresponds to, and then generates a calibration curve using Microsoft's LINEST function. This is the same code used in Microsoft Excel's LINEST function for linear regression fit.

Select all runs in the sequence and then click Create Cali. A new table will appear assigning each run to a standard concentration. The algorithm will try to identify these concentrations from the name of the Run, so it is best practice to include the concentration in the Run Name (see Figure B.2). If the algorithm makes a mistake, simply change the concentration value for each run in the Concentration (mg/mL) column.

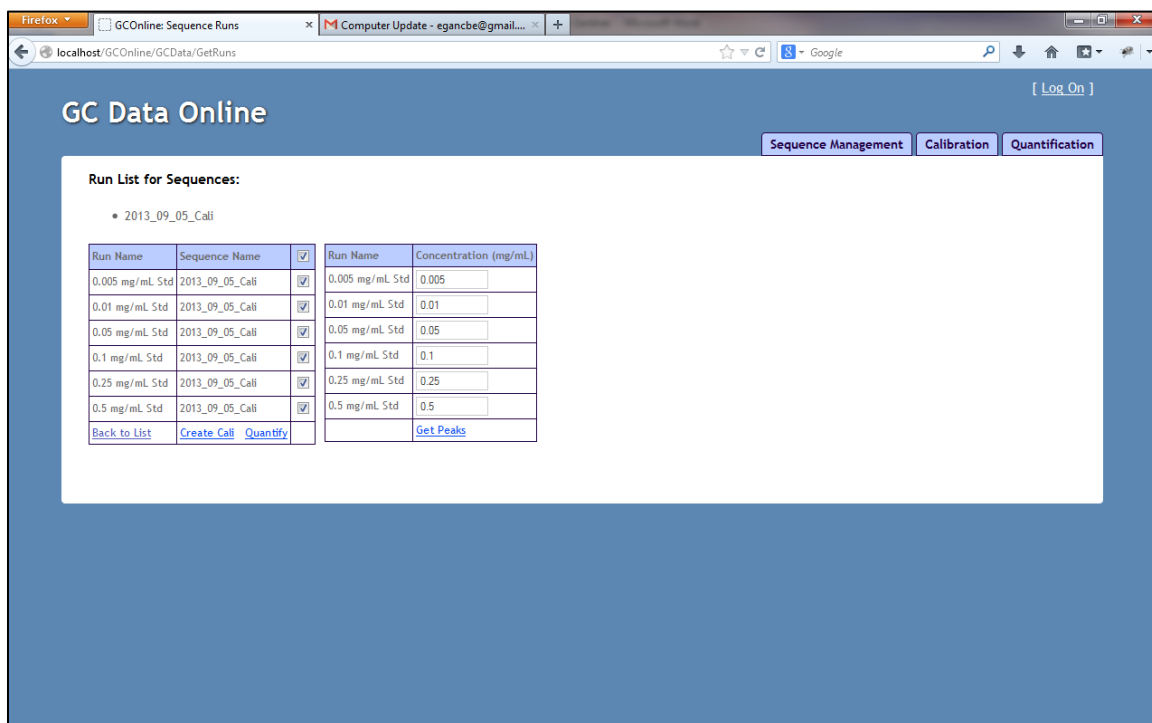


Figure B.3: GCOnline Create Calibration Screenshot.

Clicking Get Peaks, will generate individual charts for each calibration run. From here you can specify which peak correlates to each standard in your calibration set. By default the standards are hardcoded into the software and currently use a Biodiesel standard mix containing C10:0, C12:0, C14:0, C16:0, C18:0, and C20:0 FFAs; C12:0, C14:0, C16:0, and C18:0 MAGs; C12:0, C14:0, C16:0, and C18:0 DAGs; along with C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, C20:0 and C22:0 TAGs (Sigma-Aldrich, St. Louis MO). If you need to use different standards you will have to modify the code (See Files, Classes, Methods and Functions below).



Figure B.4 GCOOnline Chromatogram Chart Screenshot

Methodically assign each peak to its corresponding standard for each calibration chromatogram by clicking the peak in the graph and assigning a standard from the popup window. Once you have assigned all peaks/run, you will have a complete table at the top of the screen assigning each standard a value per concentration range you used for your calibration (e.g. 0.005 -0.5 mg/mL).

Click the Generate Cali Curve link under the calibration table. GCOOnline will use the assignments and the LINEST function to generate a linear regression best fit to your data. This will appear as a table. NOTE: This may take a few minutes and it will appear that nothing is happening. Be patient, your regression fit will appear shortly.

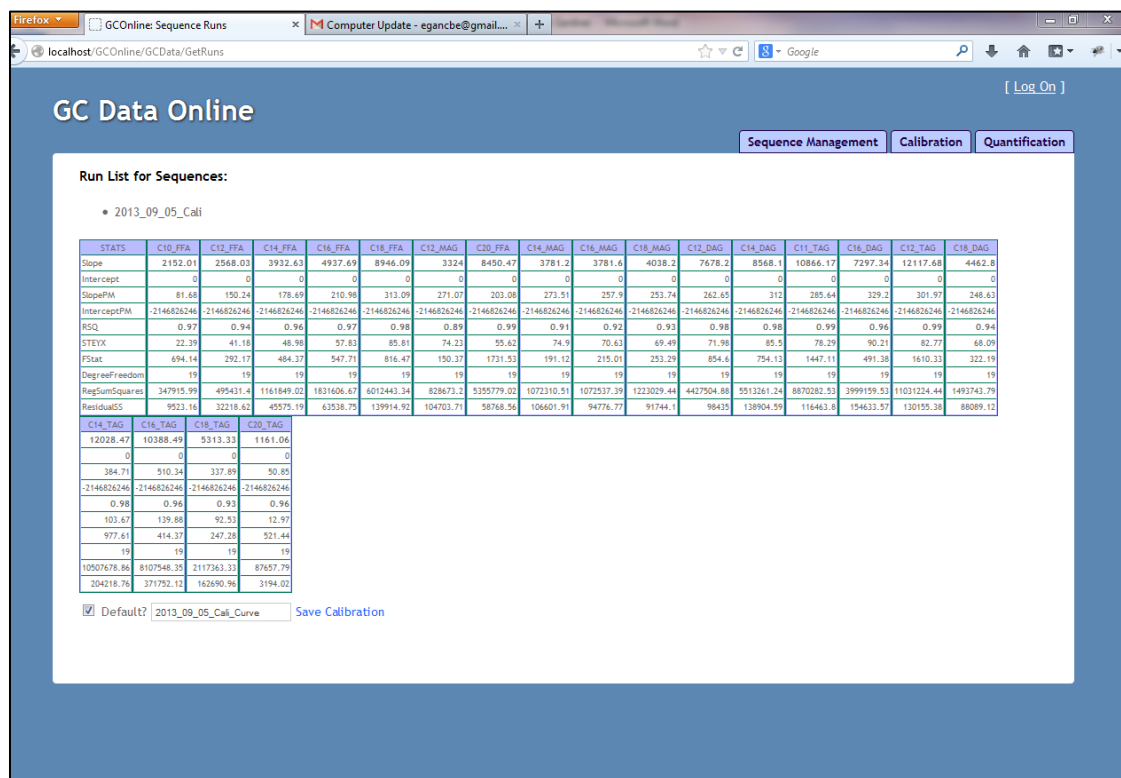


Figure B.5: GCOOnline Generate Calibration Curve Screenshot

This table will contain the following for each standard:

1. Slope
2. Intercept
3. SlopePM
4. InterceptPM
5. RSQ
6. STEYX
7. FStat
8. DegreeFreedom
9. RegSumSquares
10. ResidualSS

These are the typical statistical values generated when running a linear regression model. If you are satisfied with the results, Enter a name for your calibration and click save. NOTE, you can also check the Default checkbox (selected by default) which assigns this calibration curve as the default calibration to use by the software. Click Save Calibration.

Analyzing and Quantifying Samples

Once you have saved a calibration curve to the database, you can analyze a sample sequence. Follow the steps outlined above to upload a sample sequence workbook and then from the Sequence Manager click View Runs. As outlined above, you can then select the runs you wish to quantify by clicking the Quantify link under the run table.

GC Data Online [Log On]

GCOOnline: Quantification Management

From here you can create new quantification ranges, or quantify runs.

Create a new Quantification Range: [Show Table](#)

Select a Calibration:

Calibration List
☐ 2012_08_30_Cali_Chlamy
☐ 2012_10_23_Cali
☐ 2012_11_12_Cali
☐ 2012_12_02_Cali
☐ 2013_01_04_Cali_Curve
☐ 2013_07_19_Calibration_Curve
☐ 2013_09_04_Calibration_Curve
☐ 2013_09_05_Calibration_Curve

Select a Quantification Range:

Quantification Range List
☐ 2012_10_23_Range
☐ 2013_01_04_Cali_Range
☐ 7_25_2013_Cali_Range
☐ 2012_08_10_Range_Chlamy
☐ 2013_09_05_Cali_Range

Select runs to quantify:

Run Name	Sequence Name	Dilution (nL)	CDW (mg)	Sample Volume (mL)	Biomass (C-mmol)	Cell Count (Cells/mL)	Group
☒ 3A	09_05_2013_5%	3	1.5980000000000001	12.891113892	1	1	1
☒ 3B	09_05_2013_5%	3	1.7579999999999999	10.580204778	1	1	1
☒ 3C	09_05_2013_5%	3	1.6740000000000001	11.469534050	1	1	1

[Quantify](#)

Figure B.6: GCOOnline Quantification Management

You will now see a screen with two or three tables. The first table is a list of calibration curves, the second is a list of quantification ranges, and the third is a list of sample runs to be quantified. If this is your first time using the application, then you will only have the calibration list table, and the run list table. You will need to generate a quantification range before quantifying your samples. You can do this by clicking Show Table at the top of this screen (see Figure B.6). This will generate an empty table with each standard as a row. You will need to assign a retention time range which GCOOnline will use to determine which standard calibration slope to use for quantifying each peak in your sample data. These time ranges should not overlap. To avoid overlap, but also avoid gaps, simply add 001 to the start of the next range. For example: If you wish to quantify all peaks for C10 FFA as any peak that falls between 2.45 – 3.65 minutes, then the range

for C12 FFA should start at 3.65001. Assign a Calibration Range Name and click Create. Your new range will automatically show up in the Quantification Range table and be selected by default. You will get an error if you try to analyze a sequence without selecting both a Calibration and Quantification Range.

The Run List table has many columns, most of which have been filled out from the data you provided in the original worksheet. The final column Group is used to assign replicate samples for averaging and standard deviation. Assign the same group to samples you wish to be averaged. Click Quantify. GCOOnline will attempt to analyze and quantify your data, and create an Excel workbook containing the results. The location the application uses to save this workbook to is hardcoded in the code. You will want to change this location to something suitable for your method of organizing files on your local hard drive. Please see the following sections for modifying the code.

Files, Classes, Methods and Functions

This section details the most pertinent files, Classes, methods and functions used by GCOOnline. To begin, GCOOnline is written using the Model, View, Controller programming paradigm. An in-depth discussion of MVC is outside the scope of this documentation; please refer to online material for more information. Essentially however, the MVC paradigm separates tasks into three categories. All user interface functionality, (*i.e.* HTML, form fields, client side scripts, etc..) are encompassed in the View. All business logic is encompassed in the Model, and the Controller is responsible for aligning a View with its respective Model.

Views

1. Calibration – Includes all views used for creating, modifying and deleting calibrations.
2. GCData – Includes all views used for uploading, saving, modifying and deleting GC data.
3. Quantification – Includes all views used for quantification

Scripts

1. GCOOnline relies heavily on the javascript library jQuery. <http://www.jquery.com>
2. Common.js – This javascript file contains all the frontend scripting code used to manipulate the document object model (DOM), send and receive data via asynchronous javascript (AJAX), and store relevant object models as javascript object notation (JSON).

Models

1. There is only one model used for GCOOnline. DataModel.cs contains all the properties, Classes and relevant Entity Framework database context needed for the application.

Controllers

1. CalibrationController.cs – responsible for connecting Calibration views with the DataModel.
2. GCDDataController.cs – responsible for connecting GCDData views with the DataModel.
3. QuantificationController.cs – responsible for connecting Quantification views with the DataModel.

All classes, methods and functions are documented in the code itself. Additional information is provided by Microsoft Visual Studio Intellisense for usage information.

Customizing the Application

It may be desirable to modify GCOOnline for various purposes. Two such instances immediately come to mind and are outlined below. 1) You may wish to use different standards for your calibration and quantification. 2) You may wish to save quantification data to a different location in your local hard drive. Other modifications are outside the scope of this documentation.

Modifying the Default Standards

You will need to modify the code in three places:

1. Scripts/Common.js – line 13. Here you will find an empty JSON object (this.standardsArray) containing a name, value pair for each standard. Modify this at will.
2. Quantification/Index.cshtml – line 6. Here you will find a string array (standardsArray) containing a list of standards. Modify this at will. NOTE: Your naming convention must match with the standardsArray in common.js.
3. Models/ModelData.cs – line 168. Here you will find a Dictionary called lookup which contains a name value pair for each standard. Modify this at will. NOTE: Your naming convention must match with standardsArray in common.js.

Modifying the Local Hard Drive Location

1. Models/DataModel.cs – line 20. Here you will find a instance of ExcelQueryFactory which accepts 1 parameter, which is a string specifying the location to save the output Excel workbook. Modify this at will.

APPENDIX C

TABULATED DATA

Chapter 2-4

Table C.1: Data for Figure 2.2

<i>P. tricornutum</i>					
Extraction Technique	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)	Total Biofuel Potential (% w/w)
Wet MW	1.4 ± 0.2	2.04 ± 0.02	4.12 ± 0.22	31.51 ± 0.32	
Beads	2.62 ± 0.09	4.52 ± 0.09	7.37 ± 0.1	32.06 ± 0.05	
Dry MW	1.89 ± 0.01	2.58 ± 0.01	5.18 ± 0.01	31.47 ± 0.57	
Oven	0.88 ± 0.07	1.51 ± 0.01	2.47 ± 0.12	24.54 ± 0.94	
Sonication	1.32 ± 0.03	1.7 ± 0.05	3.35 ± 0.17	25.09 ± 2.05	
Bligh & Dyer	1.87 ± 0.07	3.62 ± 0.11	6.82 ± 0.16	27.65 ± 1.05	
Direct trans.					52.76 ± 4.32
<i>C. vulgaris</i>					
Extraction Technique	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)	Total Biofuel Potential (% w/w)
Wet MW	1.88 ± 0.54	1.94 ± 0.01	4.2 ± 0.36	21.56 ± 0.95	
Beads	1.73 ± 0.37	2.24 ± 0.11	6.45 ± 0.47	21.31 ± 2.46	
Dry MW	2.46 ± 0	2.66 ± 0	4.71 ± 0.1	21.32 ± 0.06	
Oven	0.5 ± 0.04	0.51 ± 0	1.14 ± 0	4.05 ± 0.13	
Sonication	0.5 ± 0.15	0.51 ± 0.01	1.14 ± 0.31	4.69 ± 1.53	
Bligh & Dyer	1.24 ± 0.17	1.58 ± 0.05	5.91 ± 0.32	12.63 ± 0.74	
Direct trans.					32.96 ± 0.93
<i>C. reinhardtii</i>					
Extraction Technique	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)	Total Biofuel Potential (% w/w)
Wet MW	2.56 ± 0.15	2.63 ± 0.01	5.07 ± 0.03	8.53 ± 0.28	
Beads	2.49 ± 0.17	3.08 ± 0.11	6.34 ± 0.06	8.52 ± 0.19	
Dry MW	2.31 ± 0.13	2.39 ± 0	4.66 ± 0	7.89 ± 0.36	
Oven	2.37 ± 0.05	2.43 ± 0.01	4.61 ± 0.14	7.58 ± 0	
Sonication	1.95 ± 0.06	2.36 ± 0.02	5.41 ± 0.31	8.09 ± 0.07	
Bligh & Dyer	2.03 ± 0.21	2.4 ± 0.01	5.4 ± 0.09	6.96 ± 0.03	
Direct trans.					14.45 ± 0.95

Table C.2: Data for Figure 2.3

Compound	Total FAME (% w/w)	Extractable FAME (% w/w)	Residual FAME (% w/w)
C12:0	0 ± 0	0 ± 0	0 ± 0
C14:1	0 ± 0	0 ± 0	0 ± 0
C14:0	1.99 ± 0.02	1.16 ± 0.04	0.83 ± 0.06
C16:1	24.81 ± 0.4	13.52 ± 0.22	11.29 ± 0.62
C16:0	14.66 ± 0.59	7.59 ± 0.12	7.07 ± 0.7
C18:1-3	3.62 ± 0.32	3.89 ± 0.52	-0.27 ± 0.2
C18:0	0.39 ± 0.01	0.25 ± 0.02	0.14 ± 0.03
C20:5	4.52 ± 0.07	2.45 ± 0.12	2.07 ± 0.19
C20:1	0.37 ± 0.01	0.23 ± 0.01	0.14 ± 0.01
C20:0	0.12 ± 0	0 ± 0	0.12 ± 0
C22:6	0.34 ± 0.01	0.16 ± 0.02	0.18 ± 0.02
C22:1	0 ± 0	0 ± 0	0 ± 0
C22:0	0.07 ± 0.01	0 ± 0	0.07 ± 0.01
C24:1	0.06 ± 0.01	0 ± 0	0.06 ± 0.01
C24:0	0.25 ± 0	0 ± 0	0.25 ± 0

Table C.3: Data for Figure 2.4

Compound	Total FAME (% w/w)	Extractable FAME (% w/w)	Residual FAME (% w/w)
C12:0	0 ± 0	0 ± 0	0 ± 0
C14:1	0.03 ± 0.02	0 ± 0	0.03 ± 0
C14:0	0.04 ± 0	0 ± 0	0.04 ± 0
C16:1	3.07 ± 0.07	2.57 ± 0.16	0.49 ± 0.05
C16:0	6.88 ± 0.17	4.04 ± 1.08	2.84 ± 0.42
C18:1-3	22.18 ± 0.72	14.72 ± 0.19	7.46 ± 1.1
C18:0	0.67 ± 0.02	0.65 ± 0.05	0.02 ± 0.02
C20:5	0 ± 0	0 ± 0	0 ± 0
C20:1	0.05 ± 0.03	0 ± 0	0.05 ± 0.03
C20:0	0.04 ± 0	0 ± 0	0.04 ± 0
C22:6	0 ± 0	0 ± 0	0 ± 0
C22:1	0 ± 0	0 ± 0	0 ± 0
C22:0	0 ± 0	0 ± 0	0 ± 0
C24:1	0 ± 0	0 ± 0	0 ± 0
C24:0	0.05 ± 0	0 ± 0	0.05 ± 0

Table C.4: Data for Figure 2.5

Compound	Total FAME (% w/w)	Extractable FAME (% w/w)	Residual FAME (% w/w)
C12:0	0 ± 0	0 ± 0	0 ± 0
C14:1	0.02 ± 0	0 ± 0	0.02 ± 0
C14:0	0.04 ± 0	0.03 ± 0	0.02 ± 0
C16:1	2.01 ± 0.1	1.99 ± 0.31	0.02 ± 0.21
C16:0	3.35 ± 0.22	1.7 ± 0.14	1.65 ± 0.23
C18:1-3	8.64 ± 0.53	4.28 ± 0.27	4.36 ± 0.21
C18:0	0.33 ± 0.03	0.03 ± 0	0.3 ± 0.03
C20:5	0 ± 0	0 ± 0	0 ± 0
C20:1	0.07 ± 0.04	0.04 ± 0.02	0.04 ± 0.06
C20:0	0.05 ± 0.03	0.03 ± 0.02	0.01 ± 0.01
C22:6	0 ± 0	0 ± 0	0 ± 0
C22:1	0 ± 0	0 ± 0	0 ± 0
C22:0	0 ± 0	0 ± 0	0 ± 0
C24:1	0 ± 0	0 ± 0	0 ± 0
C24:0	0 ± 0	0 ± 0	0 ± 0

Table C.5: Data for Figure 3.1a

Cell Counts (cells/mL)			
Time (days)	Bicarb (% w/w)	Low CO ₂ (% w/w)	High CO ₂ (% w/w)
2	1340000 ± 134536.24	1173330 ± 61101.01	1096670 ± 66583.28
2.5	6830000 ± 453100.43	7146670 ± 185831.46	6160000 ± 52915.03
3	7233330 ± 635085.3	8116670 ± 642910.05	8250000 ± 492442.89
4	7416670 ± 579511.29	9566670 ± 275378.53	14066700 ± 908753.73
4.5	7083330 ± 332916.41	9516670 ± 419324.85	17033300 ± 2020730
5	7550000 ± 304138.13	9166670 ± 378593.89	16866700 ± 1594780
6	7266670 ± 404145.19	11566700 ± 503322.3	18800000 ± 1609350
7	7483330 ± 388372.67	12466700 ± 763762.62	17100000 ± 360555.13
Dry Cell Weights (mg/mL)			
Time (days)	Bicarb (% w/w)	Low CO ₂ (% w/w)	High CO ₂ (% w/w)
2	0.21 ± 0.02	0.21 ± 0.02	0.17 ± 0.02
2.5	0.45 ± 0.04	0.46 ± 0.03	0.39 ± 0.03
3	0.61 ± 0.05	0.56 ± 0.03	0.79 ± 0.03
4	0.92 ± 0.04	0.65 ± 0.02	1.36 ± 0.09
4.5	0.82 ± 0.02	0.51 ± 0.05	1.2 ± 0.12
5	0.88 ± 0.05	0.65 ± 0.04	1.47 ± 0.08

6	0.95 ± 0.01	0.77 ± 0.02	1.6 ± 0.11
7	0.99 ± 0.02	0.85 ± 0.02	1.56 ± 0.09

Table C.6: Data for Figure 3.1b

Cell Counts (cells/mL)			
Time (days)	Bicarb (% w/w)	Low CO ₂ (% w/w)	High CO ₂ (% w/w)
2	109.67 ± 44.28	97 ± 14.11	96 ± 20.81
2.5	326.67 ± 25.66	228.33 ± 60.48	243.33 ± 33.29
3	658.33 ± 201.89	308.33 ± 27.54	1068.33 ± 409.4
4	2648.33 ± 220.36	448.33 ± 85.49	3465 ± 784.52
4.5	3363.33 ± 240.9	521.67 ± 68.25	4733.33 ± 783.73
5	3371.67 ± 161.74	626.67 ± 108.67	6525 ± 355.11
6	5405 ± 235.64	1211.67 ± 121.07	8703.33 ± 287.33
7	7218.33 ± 857.24	2666.67 ± 665.1	9230 ± 456.7

Table C.7: Data for Figure 3.2a

Time (days)	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)
2	2.27 ± 0.07	0.82 ± 0.08	0.4 ± 0.11	0 ± 0
2.5	2.22 ± 0.2	0.52 ± 0.03	0.86 ± 0.17	0 ± 0
3	2.2 ± 0.22	0.39 ± 0.05	1.06 ± 0.28	0 ± 0
4	1.54 ± 0.11	0.17 ± 0.07	2.05 ± 0.11	0.75 ± 0.06
4.5	2.05 ± 0.2	0.09 ± 0.06	1.93 ± 0.12	1.76 ± 0.19
5	1.86 ± 0.08	0.05 ± 0.01	2.07 ± 0.05	2.52 ± 0.23
6	1.32 ± 0.09	0.08 ± 0.01	2.64 ± 0.12	3.96 ± 0.24
7	1.48 ± 0.19	0.13 ± 0.02	2.53 ± 0.37	4.39 ± 0.22

Table C.8: Data for Figure 3.2b

Time (days)	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)
2	2.27 ± 0.29	0.74 ± 0.11	0.5 ± 0.1	0 ± 0
2.5	2.16 ± 0.28	0.52 ± 0.05	0.93 ± 0.12	0 ± 0
3	2.46 ± 0.45	0.44 ± 0.1	0.89 ± 0.35	0 ± 0
4	2.06 ± 0.1	0.05 ± 0.01	1.87 ± 0.08	0.02 ± 0.01
4.5	1.88 ± 0.19	0.01 ± 0	2.41 ± 0.29	0.19 ± 0.04
5	1.95 ± 0.25	0.02 ± 0.01	2.14 ± 0.08	0.19 ± 0.02
6	1.08 ± 0.05	0.06 ± 0.01	2.48 ± 0.05	0.38 ± 0.04
7	1.21 ± 0.14	0.05 ± 0.01	2.49 ± 0.21	0.76 ± 0.05

Table C.9: Data for Figure 3.2c

Time (days)	FFA (% w/w)	MAG (% w/w)	DAG (% w/w)	TAG (% w/w)
2	2.46 ± 0.22	0.98 ± 0.12	0.36 ± 0.05	0 ± 0
2.5	2.51 ± 0.18	0.57 ± 0.04	0.92 ± 0.08	0 ± 0
3	2.32 ± 0.12	0.36 ± 0.05	1.2 ± 0.08	0 ± 0
4	1.81 ± 0.14	0.21 ± 0.03	1.76 ± 0.55	1.45 ± 0.36
4.5	1.62 ± 0.17	0.09 ± 0.04	1.76 ± 0.19	2.13 ± 0.13
5	1.42 ± 0.16	0.04 ± 0.02	1.77 ± 0.06	3.56 ± 0.27
6	0.96 ± 0.06	0.09 ± 0.04	2.97 ± 0.19	4.88 ± 0.62
7	1.24 ± 0.22	0.09 ± 0.01	3.09 ± 0.14	5.58 ± 0.7

Table C.10: Data for Figure 3.3a

Time (days)	C ₁₆ FFA (% w/w)	C ₁₆ MAG (% w/w)	C ₁₆ DAG (% w/w)	C ₁₆ TAG (% w/w)
2	1.46 ± 0.02	0.69 ± 0.08	0 ± 0	0 ± 0
2.5	1.39 ± 0.1	0.34 ± 0	0.02 ± 0.01	0 ± 0
3	1.38 ± 0.13	0.22 ± 0.02	0.02 ± 0	0 ± 0
4	0.65 ± 0.04	0.12 ± 0.06	0.37 ± 0.04	0.53 ± 0.04
4.5	0.92 ± 0.08	0.05 ± 0.05	0.33 ± 0.03	1.02 ± 0.12
5	0.82 ± 0.03	0.01 ± 0.01	0.33 ± 0.01	1.49 ± 0.09
6	0.89 ± 0.04	0.01 ± 0	0.52 ± 0.04	2.56 ± 0.11
7	0.96 ± 0.13	0.03 ± 0.01	0.54 ± 0.07	2.85 ± 0.25

Table C.11: Data for Figure 3.3b

Time (days)	C ₁₆ FFA (% w/w)	C ₁₆ MAG (% w/w)	C ₁₆ DAG (% w/w)	C ₁₆ TAG (% w/w)
2	1.41 ± 0.16	0.61 ± 0.1	0 ± 0	0 ± 0
2.5	1.33 ± 0.13	0.33 ± 0.03	0.02 ± 0.01	0 ± 0
3	1.47 ± 0.24	0.28 ± 0.05	0.03 ± 0.02	0 ± 0
4	0.97 ± 0.01	0.04 ± 0.01	0.2 ± 0.06	0.02 ± 0.01
4.5	1.01 ± 0.1	0.02 ± 0.01	0.21 ± 0.06	0.03 ± 0
5	0.81 ± 0.1	0.02 ± 0.01	0.19 ± 0.05	0.06 ± 0.02
6	0.71 ± 0.04	0.02 ± 0	0.32 ± 0	0.3 ± 0.03
7	0.77 ± 0.11	0.02 ± 0	0.3 ± 0.02	0.62 ± 0.06

Table C.12: Data for Figure 3.3c

Time (days)	C ₁₆ FFA (% w/w)	C ₁₆ MAG (% w/w)	C ₁₆ DAG (% w/w)	C ₁₆ TAG (% w/w)
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2	1.64 ± 0.14	0.78 ± 0.1	0 ± 0.01	0 ± 0
2.5	1.66 ± 0.08	0.39 ± 0.03	0.02 ± 0.06	0 ± 0
3	1.45 ± 0.08	0.19 ± 0.03	0.06 ± 0.05	0 ± 0
4	0.74 ± 0.08	0.16 ± 0.02	0.35 ± 0.37	0.94 ± 0.18
4.5	0.68 ± 0.09	0.05 ± 0.02	0.43 ± 0.1	1.3 ± 0.07
5	0.53 ± 0.04	0.02 ± 0.01	0.31 ± 0.04	2.07 ± 0.08
6	0.67 ± 0.04	0.03 ± 0.02	0.56 ± 0.12	3.21 ± 0.23
7	0.79 ± 0.15	0.02 ± 0	0.63 ± 0.19	3.71 ± 0.15

Table C.12: Data for Figure 3.3d

Time (days)	C ₁₈ FFA (% w/w)	C ₁₈ MAG (% w/w)	C ₁₈ DAG (% w/w)	C ₁₈ TAG (% w/w)
2	0.82 ± 0.06	0.13 ± 0.01	0.1 ± 0.06	0 ± 0
2.5	0.83 ± 0.09	0.18 ± 0.03	0.35 ± 0.13	0 ± 0
3	0.82 ± 0.1	0.17 ± 0.03	0.49 ± 0.22	0 ± 0
4	0.88 ± 0.1	0.04 ± 0.02	1.29 ± 0.06	0.22 ± 0.02
4.5	1.13 ± 0.13	0.06 ± 0.02	1.16 ± 0.07	0.73 ± 0.08
5	1.04 ± 0.06	0.05 ± 0	1.29 ± 0.05	1.02 ± 0.13
6	0.43 ± 0.05	0.07 ± 0.01	1.48 ± 0.06	1.4 ± 0.13
7	0.52 ± 0.06	0.1 ± 0.01	1.3 ± 0.23	1.53 ± 0.07

Table C.12: Data for Figure 3.3e

Time (days)	C ₁₈ FFA (% w/w)	C ₁₈ MAG (% w/w)	C ₁₈ DAG (% w/w)	C ₁₈ TAG (% w/w)
2	0.86 ± 0.13	0.12 ± 0.01	0.18 ± 0.05	0 ± 0
2.5	0.82 ± 0.16	0.18 ± 0.02	0.43 ± 0.1	0 ± 0
3	0.99 ± 0.21	0.17 ± 0.06	0.34 ± 0.22	0 ± 0
4	1.09 ± 0.1	0.01 ± 0	1.27 ± 0.13	0 ± 0
4.5	0.87 ± 0.1	0.01 ± 0.01	1.74 ± 0.21	0.17 ± 0.04
5	1.14 ± 0.15	0.01 ± 0	1.57 ± 0.06	0.13 ± 0.03
6	0.37 ± 0.01	0.04 ± 0	1.66 ± 0.03	0.08 ± 0.02
7	0.43 ± 0.03	0.03 ± 0.01	1.61 ± 0.17	0.14 ± 0.01

Table C.13: Data for Figure 3.3f

Time (days)	C ₁₈ FFA (% w/w)	C ₁₈ MAG (% w/w)	C ₁₈ DAG (% w/w)	C ₁₈ TAG (% w/w)
2	0.83 ± 0.08	0.21 ± 0.03	0.07 ± 0.01	0 ± 0
2.5	0.86 ± 0.1	0.18 ± 0.01	0.33 ± 0.06	0 ± 0
3	0.87 ± 0.06	0.17 ± 0.02	0.63 ± 0.05	0 ± 0

4	1.07 ± 0.2	0.05 ± 0.01	0.98 ± 0.17	0.52 ± 0.18
4.5	0.94 ± 0.09	0.04 ± 0.02	0.89 ± 0.1	0.83 ± 0.07
5	0.89 ± 0.12	0.02 ± 0.01	1 ± 0.04	1.49 ± 0.19
6	0.28 ± 0.03	0.07 ± 0.02	1.71 ± 0.12	1.59 ± 0.21
7	0.46 ± 0.08	0.07 ± 0.01	1.67 ± 0.19	1.79 ± 0.18

Table C.14: Data for Figure 3.4a

Time (days)	C16:1	C16:0	C18:1-3	C18:0
2	0.005 ± 0	0.004 ± 0	0.016 ± 0	0.001 ± 0
2.5	0.009 ± 0	0.008 ± 0	0.022 ± 0.001	0.001 ± 0
3	0.013 ± 0.001	0.015 ± 0.001	0.034 ± 0.001	0.001 ± 0
4	0.013 ± 0	0.019 ± 0.001	0.05 ± 0.001	0.002 ± 0
4.5	0.013 ± 0.001	0.019 ± 0.001	0.05 ± 0.002	0.002 ± 0
5	0.012 ± 0.001	0.02 ± 0.001	0.048 ± 0.003	0.002 ± 0
6	0.015 ± 0.001	0.026 ± 0.002	0.058 ± 0.004	0.003 ± 0
7	0.016 ± 0.001	0.03 ± 0.002	0.065 ± 0.003	0.003 ± 0

Table C.15: Data for Figure 3.4b

Time (days)	C16:1	C16:0	C18:1-3	C18:0
2	0.005 ± 0	0.004 ± 0	0.014 ± 0	0 ± 0
2.5	0.009 ± 0	0.008 ± 0.001	0.023 ± 0.001	0.001 ± 0
3	0.011 ± 0	0.013 ± 0	0.03 ± 0	0.001 ± 0
4	0.011 ± 0	0.013 ± 0.001	0.04 ± 0.002	0.001 ± 0
4.5	0.011 ± 0	0.013 ± 0	0.041 ± 0.001	0.001 ± 0
5	0.01 ± 0	0.013 ± 0	0.04 ± 0.001	0.001 ± 0
6	0.012 ± 0.001	0.016 ± 0.001	0.047 ± 0.001	0.002 ± 0
7	0.013 ± 0.001	0.018 ± 0.002	0.049 ± 0.002	0.002 ± 0

Table C.16: Data for Figure 3.4c

Time (days)	C16:1	C16:0	C18:1-3	C18:0
2	0.003 ± 0	0.003 ± 0	0.012 ± 0	0 ± 0
2.5	0.007 ± 0	0.006 ± 0	0.017 ± 0	0.001 ± 0
3	0.013 ± 0	0.016 ± 0	0.037 ± 0.001	0.001 ± 0
4	0.015 ± 0	0.028 ± 0	0.058 ± 0.001	0.002 ± 0
4.5	0.015 ± 0	0.033 ± 0	0.063 ± 0	0.002 ± 0
5	0.015 ± 0.001	0.037 ± 0.002	0.068 ± 0.003	0.003 ± 0

6	0.018 ± 0.001	0.049 ± 0.002	0.083 ± 0.003	0.003 ± 0
7	0.02 ± 0.001	0.055 ± 0.002	0.091 ± 0.002	0.004 ± 0

Table C.17: Data for Figure 3.5a

Time (days)	Bicarb	Low CO ₂	High CO ₂
2	0.026 ± 0	0.023 ± 0.001	0.019 ± 0.001
2.5	0.04 ± 0.001	0.041 ± 0.002	0.031 ± 0
3	0.064 ± 0.003	0.056 ± 0	0.068 ± 0.001
4	0.085 ± 0.002	0.065 ± 0.003	0.104 ± 0.001
4.5	0.084 ± 0.004	0.067 ± 0.001	0.115 ± 0.001
5	0.083 ± 0.006	0.065 ± 0.001	0.125 ± 0.006
6	0.102 ± 0.008	0.076 ± 0.003	0.154 ± 0.005
7	0.114 ± 0.006	0.083 ± 0.005	0.171 ± 0.005

Table C.18: Data for Figure 3.5b

Time (days)	Bicarb	Low CO ₂	High CO ₂
2	11.225 ± 1.001	10.915 ± 0.989	12.34 ± 1.637
2.5	9.134 ± 0.639	9.198 ± 0.259	8.219 ± 0.135
3	10.115 ± 0.984	9.819 ± 0.538	8.657 ± 0.554
4	8.942 ± 0.248	10.184 ± 0.298	7.877 ± 0.073
4.5	10.262 ± 0.466	12.883 ± 1.433	9.486 ± 0.35
5	9.186 ± 0.785	10.165 ± 0.285	8.339 ± 0.279
6	10.772 ± 0.608	9.958 ± 0.262	9.872 ± 0.182
7	11.519 ± 0.726	9.807 ± 0.531	11.09 ± 0.611

Table C.19: Data for Figure 3.5c

Time (days)	Bicarb	Low CO ₂	High CO ₂
2			
2.5	5.936 ± 0.488	5.764 ± 0.416	5.055 ± 0.071
3	8.88 ± 1.075	6.937 ± 0.542	8.26 ± 0.371
4	11.506 ± 1.046	6.842 ± 0.371	7.448 ± 0.588
4.5	11.885 ± 0.421	7.046 ± 0.439	6.825 ± 0.864
5	10.972 ± 0.589	7.071 ± 0.221	7.43 ± 0.747
6	14.152 ± 1.893	6.613 ± 0.074	8.255 ± 1.028
7	15.301 ± 0.919	6.683 ± 0.727	10.025 ± 0.29

Table C.20: Data for Figure 4.1

Compound	0 mM HCO ₃ Control	50 mM Scientific Grade NaHCO ₃	50 mM Industrial Grade NaHCO ₃	50 mM KHCO ₃	50 mM Na ₂ CO ₃	25 mM NaHCO ₃ Na ₂ CO ₃
FFA	7.815 ± 0.288	6.027 ± 0.2	6.509 ± 0.495	5.857 ± 0.991	5.926 ± 0.581	6.768 ± 0.77
MAG	0.304 ± 0.039	0.221 ± 0.029	0.305 ± 0.096	0.221 ± 0.145	0.215 ± 0.182	0.175 ± 0.035
DAG	5.913 ± 0.611	5.845 ± 0.351	6.637 ± 0.77	6.102 ± 1.198	4.895 ± 0.664	7.066 ± 1.212
TAG	13.324 ± 3.412	22.942 ± 1.449	24.262 ± 0.712	17.734 ± 1.715	6.179 ± 1.294	19.531 ± 0.79
Total Extractable	27.356 ± 3.765	35.036 ± 1.433	37.712 ± 1.688	29.914 ± 4.012	17.214 ± 2.591	33.54 ± 1.359
Total FAME	44.189 ± 5.425	59.63 ± 3.343	56.045 ± 2.902	51.619 ± 2.252	32.823 ± 0.279	48.166 ± 0.767

Table C.21: Data for Figure 4.2

Time (days)	Air	Air + 5 mM NaHCO ₃	Air + CO ₂ pH Control	5% CO ₂	Air + 5 mM NaHCO ₃ + pH Control
1.75	0.037 ± 0.002	0.039 ± 0.004	0.026 ± 0.007	0.032 ± 0.001	0.06 ± 0.002
2.73	0.109 ± 0.01	0.158 ± 0.007	0.066 ± 0.014	0.097 ± 0.014	0.269 ± 0.025
3.73	0.248 ± 0.009	0.339 ± 0.015	0.203 ± 0.025	0.467 ± 0.057	0.872 ± 0.03
4.74	0.397 ± 0.023	0.585 ± 0.018	0.662 ± 0.089	1.48 ± 0.03	1.49 ± 0.105
5.73	0.537 ± 0.013	0.692 ± 0.046	1.288 ± 0.065	1.942 ± 0.07	1.063 ± 0.177
6.74	0.533 ± 0.064	0.827 ± 0.081	1.467 ± 0.232	2.453 ± 0.081	1.133 ± 0.05

Table C.22: Data for Figure 4.3

Time (days)	Air	Air + 5 mM NaHCO ₃	Air + CO ₂ pH Control	5% CO ₂	Optimized Scenario
1.75	1236670 ± 418847.625	930000 ± 43588.989	1010000 ± 289309.523	1163330 ± 185831.465	1133330 ± 90737.717
2.73	4900000 ± 438634.244	5733330 ± 546015.873	3580000 ± 400000	4093330 ± 670919.767	9933330 ± 1312300
3.73	9423330 ± 503620.227	12566700 ± 503322.296	10133300 ± 1457170	17066700 ± 1365040	33800000 ± 1113550
4.74	13766700 ± 896288.644	24133300 ± 2328810	20333300 ± 1285820	67300000 ± 5408330	38600000 ± 4275510
5.73	26466700 ± 1242310	28233300 ± 251661.148	61466700 ± 3629510	204667000 ± 27061700	39733300 ± 3477550
6.74	23066700 ± 923760.431	35266700 ± 2003330	142133000 ± 46677300	239200000 ± 15450600	45000000 ± 5370290
7.75					59333300 ± 6057500

Table C.23: Data for Figure 4.4

Time (days)	Air	Air + 5 mM NaHCO ₃	Air + CO ₂ pH Control	5% CO ₂	Optimized Scenario
1.75	0.067 ± 0.014	0.122 ± 0.014	0.039 ± 0.042	0.086 ± 0.052	0.444 ± 0.073
2.73	0.511 ± 0.024	0.644 ± 0.024	0.301 ± 0.084	0.387 ± 0.053	2.422 ± 0.13
3.73	1.202 ± 0.148	1.793 ± 0.148	0.845 ± 0.115	2.171 ± 0.374	8.007 ± 1.035

4.74	1.425 +- 0.218	2.962 +- 0.218	3.547 +- 0.446	7.688 +- 0.608	11.307 +- 1.428
5.73	3.119 +- 0.347	4.733 +- 0.347	8.012 +- 1.035	7.761 +- 1.393	11.33 +- 0.921
6.74	3.52 +- 1.004	5.727 +- 1.004	6.724 +- 1.228	8.125 +- 1.463	10.701 +- 0.843

Table C.24: Data for Figure 4.5

Treatment	Optimized NaHCO ₃	Optimized Sequicarbonate	5% CO ₂
FFA	4.738 +- 0.653	4.383 +- 0.69	5.552 +- 0.425
MAG	0.44 +- 0.202	0.454 +- 0.324	0.297 +- 0.125
DAG	5.328 +- 0.449	4.692 +- 0.815	4.619 +- 0.612
TAG	26.494 +- 1.258	23.472 +- 1.222	23.193 +- 0.765
Total Extractable	37 +- 2.509	33 +- 2.714	33.661 +- 1.2
Total FAME	61.035 +- 1.314	51.407 +- 2.136	53.333 +- 1.345