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

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Abstract

Inorganic carbon is a fundamental component for microalgal lipid biosynthesis. Understanding how the concentration and speciation of dissolved inorganic carbon (DIC) influences lipid metabolism in microalgae may help researchers optimize the production of these high value metabolites. 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. Specifically, cultures sparged with atmospheric air were compared to cultures which were sparged with 5% CO₂ (v/v) and cultures supplemented with 50 mM NaHCO₃ just prior to medium nitrogen depletion. All three conditions exhibited similar lipid profiles prior to nitrogen depletion in the medium, with FFA and MAG being the predominant lipid metabolites. However, these precursors 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. The methodologies and findings presented here may be adapted to other organisms with high industrial relevance.

Key words

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

1. 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 (US Department of Energy, 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, conversion, and refinement. However, overcoming cultivation challenges involving contamination of growth reactors, microalgae strain selection, and techniques to promote faster growth rates and higher lipid yields may potentially increase production in the future (National Research Council, 2012). In fact, maximizing culture lipid content has been identified as one of the greatest potential targets for cost reduction and should be a near-term research focus (Davis et al., 2011). Recent developments in biomass conversion to biofuels using thermochemical processes have yielded extremely promising results, and may offset the need for high lipid containing cultures (Elliott et al., 2013; Zhu et al., 2013). However, from an energy sustainability perspective, utilizing the free energy of sunlight to biologically reduce carbon into energy storage compounds such as triacylglyceride (TAG) should remain a key focus. Furthermore, if higher value products such as polyunsaturated fatty acids, pigments or pharmaceuticals are to be produced, thermochemical post-processing methods can most likely not be used.

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*. Combined with our previously published work (Gardner et al., 2013a), an improved understanding of carbon flux in *C. reinhardtii* is gained which may be used to improve the productivity of *C. reinhardtii* and possibly other, more industrially relevant, green algae.

2. Materials and Methods

2.1. Strain and Culturing Conditions

Chlamydomonas reinhardtii CC124, obtained from the Chlamydomonas Resource Center (University of Minnesota, 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.

2.1.1. Low CO₂

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

2.1.2. 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.

2.1.3. 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).

2.2. 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.

2.3. 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).

2.4. Total Protein and Chlorophyll Measurements

Total protein content of algal cultures was 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).

2.5. 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 (Section 2.6.) and direct *in situ* transesterification for total FAME analysis (Section 2.7.), respectively.

2.6. 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 (Section 2.8.2).

2.7. 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) (Section 2.8.3). 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 trifluoride 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.

2.8. Lipid analysis

2.8.1. 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) (Supplemental Data Fig. S3). Briefly, 1 mL aliquots were removed from cultures and assayed directly with Nile Red (4 μL from 250 $\mu\text{g mL}^{-1}$ in acetone) or by diluting 1:5 with dH_2O before staining with Nile Red (20 μL from 250 $\mu\text{g mL}^{-1}$ in acetone). To maintain linearity of the Nile Red assay, dilution was required when population counts exceeded 1×10^7 cells mL^{-1} . 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 μL of sample. Unstained samples were used for background medium and cellular autofluorescence correction.

2.8.2. 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).

2.8.3. 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 described in Section 2.8.4. 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.

2.8.4. Lipid Quantification and Analysis

Lipid quantification was performed using a custom program developed specifically for this purpose (Lohman et al., 2013). 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.

3. Results and Discussion

Lipid content of microalgal cultures are typically analyzed at time-of-harvest by extraction followed 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 (Bigelow et al., 2011; Griffiths et al., 2010; Laurens et al., 2012a, 2012b; Lohman et al., 2013). 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.

3.1 Background

Chlamydomonas reinhardtii CC124 has been extensively studied because the organism was one of the first microalgae to be genetically sequenced. Thus, much is known about the organism's metabolism and its capacity for fixing inorganic carbon into energy storage compounds such as triacylglycerides or starch (Lee and Fiehn, 2008; Moroney and Ynalvez, 2007; Work et al., 2010). For instance, Moroney and Ynalvez proposed a novel carbon-concentrating mechanism for *C. reinhardtii* by which the organism can utilize inorganic carbon as either dissolved CO₂ or HCO₃⁻ and, through enzymatic conversion, concentrate the substrate as carbon dioxide around the Rubisco enzyme (Moroney and Ynalvez, 2007). Work et al. determined that starchless mutant strains of *C. reinhardtii* accumulate significantly higher concentrations of lipids on a cellular basis during nitrogen deprivation as compared to the wild-type CC124 strain (Work et al., 2010). Our previous work has revealed that the timely addition of high concentrations of sodium bicarbonate can result in significant increases in intracellular lipid accumulation in many microalgal species, both greens and diatoms (Gardner et al., 2013a, 2013b, 2012). Regarding *C. reinhardtii* specifically, our previous research suggests that not only the concentration of dissolved inorganic carbon (DIC) influences starch and lipid metabolism, but the speciation of the substrate can play an important role as well. For example, it was determined that cultures of *C. reinhardtii* CC124 rapidly accumulated both starch and lipid directly after medium nitrogen depletion when supplemented with additional DIC (either as gaseous CO₂ or HCO₃⁻). However, cultures which were supplemented with CO₂ quickly catabolized their starch reserves within 24 h of maximum accumulation; whereas, cultures supplemented with HCO₃⁻ continued to accumulate starch well into the stationary phase (Supplemental Data, Fig. S1, data previously reported) (Gardner et al., 2013a).

3.2. 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 this 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 (Fig. 1a) and experimental results are tabulated for two time points in Table 1; directly before nitrogen depletion in the bulk medium (2.8 d) and at the end of the experiments (7 d). 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) (Fig. 1a). However, significant differences were observed after extended culturing in nitrogen-deplete media. 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 1). The cessation or delayed cell cycle in cultures supplemented with NaHCO₃ is a phenomenon that has been observed and documented for other species of green microalgae (Gardner et al., 2013a, 2013b, 2012; White et al., 2012). Interestingly, the final DCW of cultures supplemented with NaHCO₃ exceeded those of cultures grown under ambient air (0.99 g L⁻¹ vs. 0.84 g L⁻¹) due to higher TAG content. However, cultures grown on 5% CO₂ achieved the overall highest DCW of 1.56 g L⁻¹ (Table 1). Dry cell weights decreased during the dark cycle for all cultures (Fig. 1a) due to a decrease in protein concentration (Supplemental Data, Fig. S2, d 4.5). Protein

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 CO₂ “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 1). The concentration of protein (g protein per liter of culture) at the end of the light cycle remained roughly the same in the cultures after nitrogen depletion (Supplemental Data, Fig. S2) 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 NaHCO₃ reduced their total chlorophyll content by 3%; whereas, cultures grown on ambient air exhibited an 8% increase in chlorophyll content. Cultures grown on 5% CO₂ remained relatively constant in chlorophyll content (Table 1). Nitrogen deprivation has been shown to down-regulate protein and chlorophyll biosynthesis (Miller et al., 2010) and can result in the catabolism of proteins 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, Fig. S2, d 4.5).

3.3. 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 (Bertozzini et al., 2011; Chen et al., 2011, 2009; Cooksey et al., 1987; da Silva et al., 2009). 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 Fig. 1b has been correlated to extracted TAG, as quantified by GC-FID analysis (Section 3.4) for all cultures in this experiment ($r^2 = 0.96$, Supplemental Data, Fig. S3). As expected, cellular TAG was below detection before nitrogen depletion but increased in cultures supplemented with additional inorganic carbon after nitrogen depletion (Fig. 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 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 1.79 x higher specific fluorescence than cultures grown on 5% CO₂, indicating a higher TAG content per cell (Table 1), which was further verified by GC-FID analysis and is discussed below (Section 3.4).

3.4. 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 Fig. 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 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 (Fig. 2, Table 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 (Fig. 2, Table 2). TAGs increased directly after nitrogen depletion in cultures grown on 5% CO₂ (Fig. 2c) and cultures amended with 50 mM NaHCO₃ (Fig. 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 (Fig. 2b). These data suggest a reallocation of free fatty acid into TAG biosynthesis once cultures undergo nutrient stress, and they 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).

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 Fig. 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 Supplemental Data Table S1. 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 undetectable. 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 (Fig. 3d, e, and f). C₁₆ DAGs only began to accumulate after nitrogen depletion and to a much lower concentration (~0.5% w/w, Supplemental Data Table S1) than C₁₈ DAGs (~1.5% w/w, Supplemental Data Table S1) (Fig. 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 (Fig. 3, Supplemental Data Table S1). 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).

3.5. 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). Fig. 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. 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 in Section 3.4, C₁₆ TAG contributes the majority to overall TAG in all three conditions. This preferential shift towards fully saturated C₁₆ fatty acids after nitrogen depletion could indicate that unsaturated C₁₆ fatty acids were being

hydrogenated for energy storage (*i.e.* C₁₆ TAG). 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.

Most interesting is the change in total FAME over time. Fig. 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 (Fig. 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 (2 sample T test $p < 0.05$) higher for all treatments due to a decrease in DCW (Fig. 1a) caused by a decrease in protein concentration (Supplemental Data, Fig. S2 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 NaHCO₃. 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.

4. Conclusions

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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Table 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 (x10 ⁶ cells mL ⁻¹)	Nile Red fluorescence (x10 ³ 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 (x10 ⁶ cells mL ⁻¹)	Nile Red fluorescence (x10 ³ 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)

Table 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

Table 3. 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

Figure Captions

Fig. 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.

Fig. 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.

Fig. 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). Line indicates time of nitrogen depletion of the culture medium.

Fig. 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.

Fig. 5. Comparison of total FAME values for *C. reinhardtii* when reporting data as (a) a concentration (mg FAME/mL culture), (b) a weight percent (mg FAME/mg biomass), and (c) FAME per cell (pg FAME/1000 cells) (n=3).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5