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Comparison of CO_2 and Bicarbonate as Inorganic Carbon Sources for Triacylglycerol and Starch Accumulation in *Chlamydomonas reinhardtii*

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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 diox-ide and bicarbonate, and the effects these have on inducing lipid and starch accumulation during nitrogen depletion. High concentrations of CO_2 (5%; v/v) produced the highest amount of biofuel precursors, transesterified to fatty acid methyl esters, but exhibited rapid accumulation and degra-dation characteristics. Low CO_2 (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.

KEYWORDS: algal biofuel; triacylglycerol (TAG); fatty acid methyl ester (FAME); Nile Red fluorescence

Introduction

Algal-based biofuels have received increasing attention as an alternative to traditional fossil-based fuel sources, which are becoming environmentally and politically more controver-sial. Research is still necessary, both from the private and public sectors, before algal biofuels can truly compete economically with fossil-based products (Chisti, 2007; Hu et al., 2008). However, rapid progress is being made on all levels, from high-yielding strain identification to improved harvesting and extraction techniques (Greenwell et al., 2010). It has also been pointed out that near-term research on maximizing lipid content, compared to maximizing growth yield, has a more substantial cost reduction potential for industrial use of microalgae for biofuel production (Davis et al., 2011). The focus of this report is characteriza-tion of the model green alga Chlamydomonas reinhardtii CC124 and its response to various concentrations and species of inorganic carbon. C. reinhardtii is one of the most widely studied phototrophic eukaryotes. With a fully mapped genome and an extensive library of peer-reviewed literature, it is an ideal organism to continue our investigation into the means by which various species of inorganic carbon affect growth, lipid and starch synthesis as well as other changes in metabolic activity.

It is widely accepted that commercial scale growth reactors, either open pond systems or enclosed photo-bioreactors, will require supplemented inorganic carbon sources. Traditionally, this has been achieved by aeration using increased concentrations of CO_2 in air. Recently, we showed that inorganic carbon, in the form of bicarbonate (HCO^-), is an effective lipid accumulation trigger. This trigger effect [3] has been demonstrated on two other distinct species of algae: the green alga Scenedesmus sp. strain WC-1 and the marine diatom Phaeodactylum tricornutum (Gardner et al., 2012). Bicarbonate arrests cellular replica-tion in both green algae (C. reinhardtii and WC-1), but not in the diatom, and immediately induces carbon storage

metabolic activity. Furthermore, it was recently shown that the addition of sodium bicarbonate is a viable strategy to increase lipid accumulation in marine Chlorophytes (White et al., 2012). Here, we report the physiological differences in *C. reinhardtii* when different concentrations and forms of inorganic carbon were used during triacylglycerol (TAG) and starch accumulation. This provides further insight into potential industrial application of microalgae for biofuel production.

Materials and Methods

Strain, Culturing Conditions, and Biomass Analysis

C. reinhardtii CC124, obtained from the Chlamydomonas 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 (Harris, 1989). 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. Batch experiments were conducted in triplicate using 70 mm × 500 mm glass tubes containing 1.25 L Sager's minimal medium with environmental parameters controlled as described previously (Gardner et al., 2012) and in the Supplementary Materials. Scientific grade sodium bicarbonate was used in all experiments with bicarbonate addition (Sigma–Aldrich, St. Louis, MO).

An optical hemocytometer and a transmitted/epifluorescence light microscope (Nikon Eclipse E800) with an Infinity 2 color camera were used to determine cell concentrations and collect micrographs or fluorescence images, respectively, according to previous protocols (Gardner et al., 2012). Dry weight yields were determined at the end of the experiments by filtering 10 mL of culture using 1-μm pore size glass fiber filters (Fisher Scientific, Pittsburgh, PA) to collect the biomass. The biomass was washed with 10 mL diH_2O, 18 MΩ, to remove media salts and excess bicarbonate. Algal cells were dried for 18 h on the filter in a 70°C oven until the filter weight was constant. Dry cell weight (DCW) yields were calculated by subtracting the dry weight of the clean filter from the oven dried weight of the filter with biomass.

Analysis of Media Components

Medium pH was measured using a standard bench top pH meter. Ammonium and nitrate concentrations were measured by Nessler reagent (HACH, Loveland, CO) and ion chromatography (Dionex, Sunnyvale, CA), respectively, using previous protocols with modifications (Gardner et al., 2012, see Supplementary Material for modifications). Media dissolved inorganic carbon (DIC, sum of dissolved carbon dioxide, bicarbonate, and carbonate), was measured on 8 mL, 0.2-μm pore size filtered, supernatant with a Skalar Formacs TOC/TN Analyzer using a Skalar LAS-160 autosampler. In this protocol, 100 μL of sample is dispensed in 2% (v/v) phosphoric acid to liberate the inorganic carbon as CO_2. The CO_2 was detected on an IR detector and concentration determined from peak area fit to a standard curve derived from bicarbonate and carbonate mix standards (Sigma–Aldrich).

Starch and Chlorophyll Measurements

Cellular starch was determined by EnzyChrom starch assay kit (BioAssay Systems, Hayward, CA) using the manufacturer's protocol with modifications (see Supplementary Material for modifications).

Chlorophyll *a*, *b*, and total were determined using 95% ethanol extraction and absorption correlation. One milliliter of culture was centrifuged at 6,000*g* for 5 min, after which the supernatant was discarded. One milliliter 95% ethanol was added to the centrifuged pellet, which was vortexed and lightly sonicated (bath) to dispense the pellet. The cellular debris was seperated by centrifugation at 14,000*g* for 3 min and absorption was read at 649 and 665 nm on the supernatant. Calculations of chlorophyll ($\mu g\,mL^{-1}$) were conducted as described previously (Harris, 1989).

Lipid Analysis

Cellular TAG accumulation was monitored throughout the experiments using the Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one) (Sigma–Aldrich) 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 (Chen et al., 2009; Cooksey et al., 1987; da Silva et al., 2009; Elsey et al., 2007; Lee et al., 1998; Liu et al., 2008; Yu et al., 2009). Furthermore, Nile Red fluorescence correlated to % TAG from gas chromatography (GC) analysis on extracted lipid from *C. reinhardtii* ($r^2 = 0.998$), correlation not shown. TAG accumulation over time was monitored using Nile Red and was measured using a previously described protocol (Gardner et al., 2012); however, a 7–15 min Nile Red staining time was found to be optimal for *C. reinhardtii*. Imaging with Bodipy 505/515 followed previous protocols (Bigelow et al., 2011).

At the conclusion of the experiments, biomass was harvested by centrifugation at 5,000*g* for 10 min. The supernatant fraction was discarded and the pellets were washed to remove media salts and bicarbonate from the biomass. Biomass pellets were frozen and lyophilized (Labconco lyophilizer, Kansas City, MO) for GC lipid analysis. *C. reinhardtii* free fatty acids (FFAs), monoglycerides (MAGs), di-glycerides (DAGs), and tri-glycerides (TAGs) were extracted using a 1:1:1 chloroform/hexane/tetrahydrofuran solvent mixture combined with bead beating (Gardner et al., 2011, see Supplementary Materials for

bead beading and extraction protocol), and analyzed by gas chromatography—flame ionization detection (GC–FID; Agilent 6890N, Santa Clara, CA). GC–FID analysis was accomplished using 1 μL injections onto a 15 m (fused silica) RTX biodiesel column (Restek, Bellefonte, PA) where the column temperature ramped from 100 to 370°C at a rate of 10°C/min (1 min hold temperature at 100°C) with a 320°C injection temperature. Helium was used as the carrier gas and column flow was ramped at 0.2 mL min^{-2} from 1.3 (0–22 min) to 1.5 (22–24 min) to 1.7 mL min^{-1} (24–36 min). Calibration curves were constructed by combining C16:0, C19:0, and C23:0 fatty acid methyl esters (FAMEs); C12:0, C14:0, C16:0, C18:0 MAGs; C12:0, C14:0, C16:0, C18:0 DAGs; and C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, C20:0 TAGs (all from Sigma–Aldrich) for quantification ($r^2 > 0.99$). This GC method allows for quantification of FFA, FAMEs, MAGs, DAGs, and TAGs in a single analysis.

Biofuel potential, defined as total FAMEs produced directly from the biomass, and fatty acid compositions of these FAMEs were determined from direct in situ transesterification of dried biomass using previous protocols with modifications (Griffiths et al., 2010, see Supplementary Material for modifications), and analyzed with gas chromatography—mass spectroscopy detection (GC–MS; Agilent 6890N and 5973 Network MS). GC–MS analysis was done according to a previously published protocol (Bigelow et al., 2011).

Results and Discussion

Cellular Growth and Carbon Utilization

To advance our knowledge of inorganic carbon utilization during TAG accumulation in *C. reinhardtii*, batch cultures were grown under 5% CO_2 sparge until near ammonium depletion. At which time, experiments were initiated that analyzed *C. reinhardtii* while being sparged with atmospheric air (0.04% CO_2), with and without 50 mM bicarbonate added, and cultures that were maintained at 5% CO_2. This allowed for comparison of cells while utilizing CO_2, at high and low concentrations, or low CO_2 with supplementary bicarbonate as an inorganic carbon source. Figure 1 shows cell growth (A), medium ammonium concentrations (B), and medium nitrate concentrations (B) for *C. reinhardtii* grown under 14:10 h light/dark cycle in Sager's minimal medium. Sager's minimal medium was chosen, in contrast to Tris–acetate–phosphate medium (commonly known as TAP), to minimize the heterotrophic activity and thus maximize the autotrophic properties of *C. reinhardtii*. Prior to ammonium depletion, the cultures maintained exponential growth and exhibited a 1.6 day^{-1} maximum specific growth rate (10.4 h doubling time). Medium ammonium became depleted near 2.8 days and further cell cycling was arrested in the cultures to which bicarbonate was added. However, cultures sparged with 5% CO_2 or atmospheric air, without added bicarbonate,

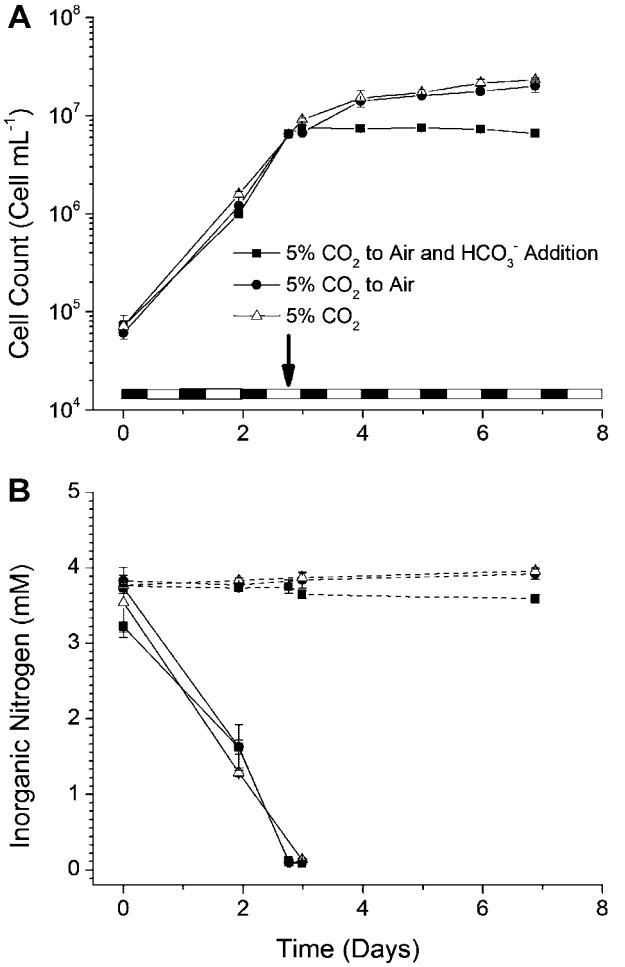

Figure 1. *C. reinhardtii* CC124 batch growth average and standard deviation of cellular density (**A**) and medium inorganic nitrogen concentration (**B**), solid line for NH_4^+ and dashed line for NO_3^-. Arrow indicates time of medium NH_4^+ depletion and the bar represents the light and dark times of the light cycle. Growth was maintained in Sager's minimal medium illuminated with a 14:10 h L:D cycle ($n = 3$).

continued to divide an average of 1.7 more times. Previous studies on the Chlorophyte *Scenedesmus* sp. WC-1 showed a similar cessation of cell cycling upon a 50 mM bicarbonate addition and is comparable with the cell cycle arrest observed in *C. reinhardtii* (Gardner et al., 2012). Medium nitrate was not utilized by any of the cultures and the slight concentration decrease in the bicarbonate added cultures, visible in Figure 1B, is attributed to culture media dilution from the sodium bicarbonate addition.

Cellular properties such as cell concentration, degree of aggregation, and cell size can be monitored over the course of a batch growth experiment using an optical hemocytometer. It was observed that air aeration, without bicarbonate addition, resulted in cells that were smaller than the cells maintained at 5% CO_2. This can be observed in the micrographs and fluorescent images of Figure 2 (comparison of A and B). However, as time progressed these smaller cells gradually grew into larger cells and this difference

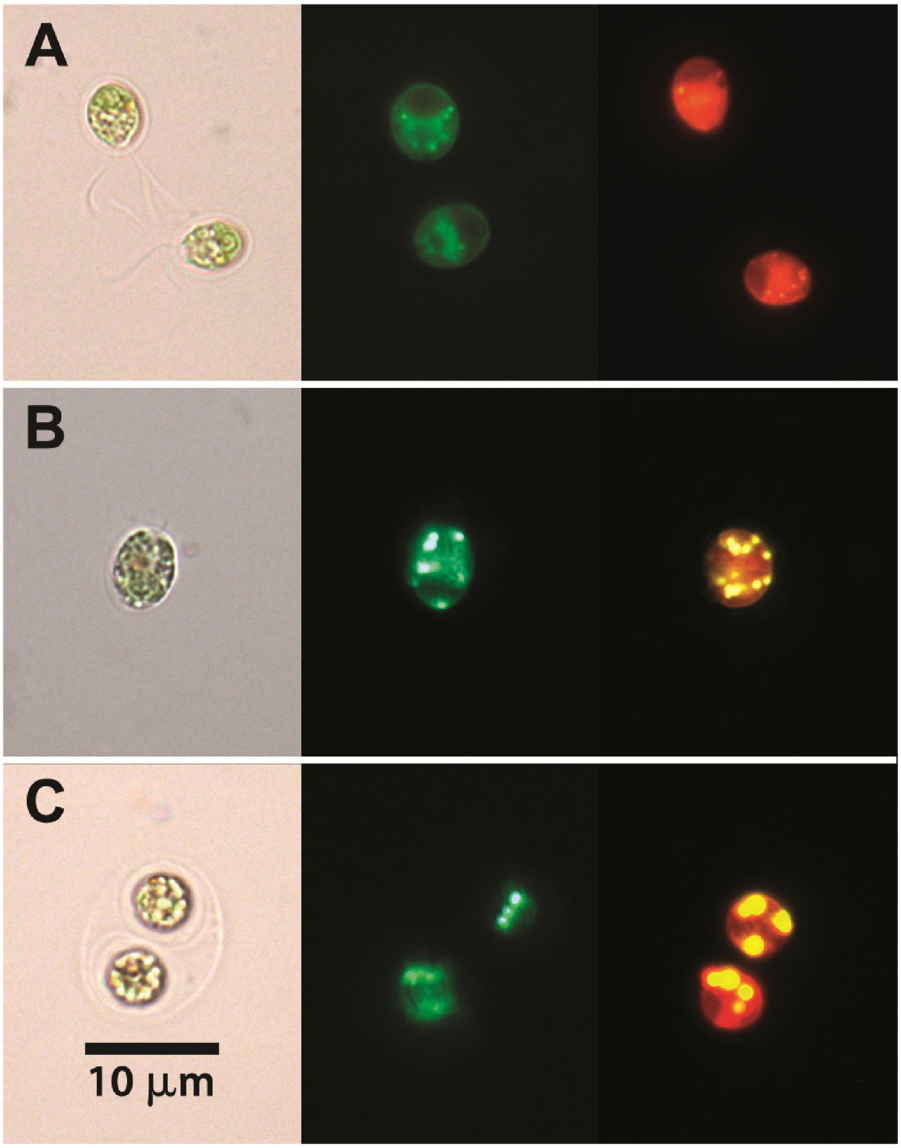

Figure 2. Transmitted micrographs, Bodipy 505/515 and Nile Red epifluorescent images (left to right) of *C. reinhardtii* CC124, lipid vacuoles stain blue and yellow with Bodipy 505/515 and Nile Red, respectively. Images were taken prior to culture harvest for cultures grown on 5% CO_2 switched to air (**A**), maintained on 5% CO_2 (**B**), and grown on 5% CO_2 which was switched to air and the addition of 50 mM sodium bicarbonate (**C**). Cells imaged are representative cells for each respective culture and all micrographs are at the same magnification.

became less evident (note—the images taken in Fig. 2 were captured within 1 day of culture harvest). Additionally, both cultures maintained in air or on 5% CO_2, without added bicarbonate, retained their flagella and motility. However, cultures to which bicarbonate was added shed their flagella and formed membrane bound, incompletely divided cells (Fig. 2C). This caused a cessation of cellular motility and increased cell size. Furthermore, the cells maintained an incomplete division state until the end of the experiment. This would not be evident if only cell number were reported.

Final cell concentration and DCW are given in Table I. Cultures maintained on 5% CO_2 and those sparged with air without added bicarbonate had the highest number of cells. Cultures to which bicarbonate was added had less than half as many cells. In contrast, cultures to which bicarbonate was added had twice the biomass yield compared to "no-bicarbonate" added cultures. This argues that the added bicarbonate caused a change in metabolism to shift the cells from a growth state to a product formation state as evident by the cessation of cellular division and higher biomass yield. Additional evidence of increased TAG and starch storage supporting this observation is further discussed below.

Since carbonate speciation and carbon species concentrations are a function of pH and total DIC, these parameters were monitored throughout the experiments. Figure 3

Table I. Comparison of final average and standard deviation of culture cell number, biomass yield, fluorescence TAG accumulation, and starch properties of *C. reinhardtii* cultured in Sager's minimal medium during 14:10 h light–dark cycling ($n = 3$).

Gas-sparge during NH_4^+ depletion	Time of HCO_3 addition (day)	Cell concentration ($\times 10^7$ cells mL^{-1})	Dry weight ($g\,L^{-1}$; DCW)[a]	Total Nile Red fluorescence ($\times 10^3$ units)	Nile Red specific fluorescence (units $cell^{-1}$)[b]	Total starch ($g\,L^{-1}$)	Specific starch ($\mu g\,cell^{-1}$)[c]
5% CO_2	N/A	2.31 ± 0.09	0.48 ± 0.05	4.5 ± 1.4	2.0 ± 0.6	0.06 ± 0.007	0.3 ± 0.04
Air	N/A	2.01 ± 0.30	0.55 ± 0.06	1.8 ± 0.4	0.9 ± 0.3	0.04 ± 0.005	0.2 ± 0.01
Air	2.8	0.66 ± 0.04	1.14 ± 0.11	8.8 ± 1.1	13.5 ± 2.2	0.79 ± 0.21	11.7 ± 3.3

N/A, not applicable.
[a]Dry cell weight (DCW) determined gravimetrically with filtered samples dried at 70°C.
[b]Calculated by fluorescence signal/cell density × 10,000 (scaling factor).
[c]Calculated by total starch/cell density × 100,000 (scaling factor).

shows total DIC (A) and medium pH (B) for the *C. reinhardtii* cultures. Again, all cultures were initially grown on 5% CO_2 and began with 1.4 mM C and a pH of 6.8. Through 2.8 days, there was a decrease in pH to 5.0 along with a DIC decrease to 0.2 mM C. At the time of medium ammonium depletion (2.8 days), the cultures to which bicarbonate was added increased in DIC to 52.1 mM C (50 mM C targeted) and showed an initial increase in pH to 7.9. By the end of the light cycle (3.0 days), the pH had risen to pH 9.3 and DIC had decreased to 50.2 mM C. Over the next 14:10 h light/dark cycle, beginning with 10 h dark, the DIC decreased at a rate of 0.87 mM C h^{-1} to 29.9 mM C and the pH increased to 10.0. This decrease in DIC concentration during the dark cycle is presumably due to CO_2 off gassing as the medium was not in carbon equilibrium due to the high bicarbonate addition and algal photosynthesis was not active. In contrast, the DIC decrease in the light could be a combination of CO_2 off gassing and algal photosynthesis consuming the DIC. At 4 days, the DIC increased due to in-gassing during the dark and decreased due to photosynthetic utilization during the light hours. The remainder of DIC data points were taken at the end of the light cycle, thus increased DIC from dark cycle in-gassing is not shown. By the end of the experiment, there were 26.2 mM C remaining with a final medium pH of 10.5. Abiotic DIC equilibrium was calculated for Sager's minimal medium with a 50 mM sodium bicarbonate addition, using the chemical equilibrium model Visual Minteq (ver 3.0, KTH Department of Land and Water Research Engineering) and is shown by the dashed line in Figure 3A. Comparison of the abiotic carbon equilibrium model with the DIC data, from 4.4 d through the remainder of the experiment, suggests active bicarbonate utilization by *C. reinhardtii* due to the carbon concentration being below equilibrium and the pH remaining high, indicating bicarbonate was the predominate DIC species.

The medium pH in the cultures to which bicarbonate was not added and the cultures sparged with 5% CO_2 remained low, but there was an increase in pH from 5.0 to 5.7 after ammonium in the medium became depleted. The DIC concentration in the 5% CO_2 sparged cultures increased after ammonium depletion and remained at 0.55 mM C throughout the remainder of the experiment. The DIC concentration in the cultures without added bicarbonate was below the detection limit of 0.01 mM C for the remainder of the experiment after the gas-sparging was switched from 5% CO_2 to air. This suggests that the air sparged cultures, without added bicarbonate, were carbon limited after the aeration shift and could be the reason that

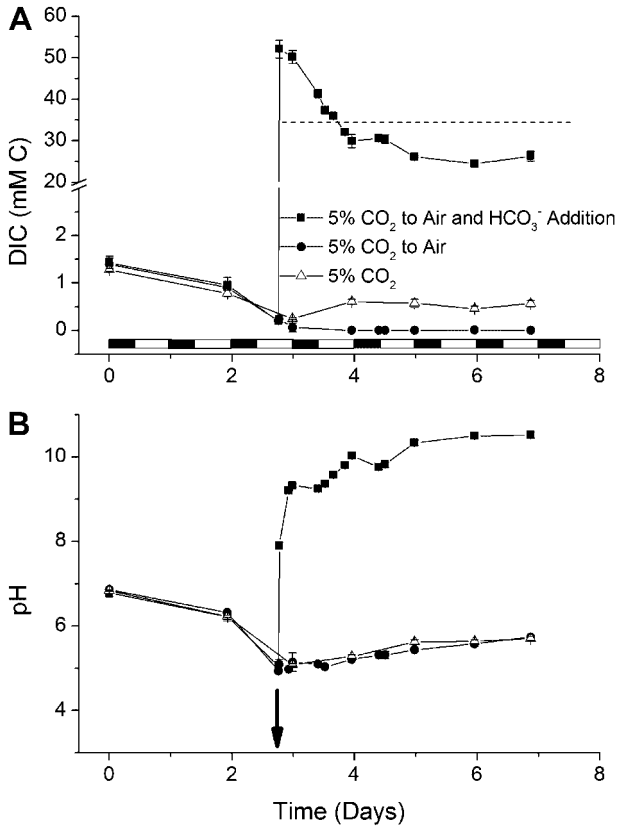

Figure 3. *C. reinhardtii* CC124 average and standard deviation of medium DIC concentration (**A**) and medium pH (**B**). Arrow indicates time of medium NH_4^+ depletion and inorganic carbon adjustment, dashed line represents geochemical modeling prediction (Visual Minteq ver 3.0) of carbon equilibrium with 50 mM sodium bicarbonate addition, and the bar represents the light and dark times of the light cycle. Note—a split scale was used on the *y*-axis of the DIC plot to better visualize the data. Growth was maintained in Sager's minimal medium illuminated with a 14:10 h L:D cycle ($n = 3$).

cellular growth, after 2.8 days, produced smaller cells that gradually grew into larger cells, as shown in Figure 2 and previously discussed. Furthermore, the pH difference between the bicarbonate amended cultures and the "no-bicarbonate" cultures could be the reason the bicarbonate cultures shed their flagella. Historically, medium pH has been used to detach *C. reinhardtii*'s flagella, however, a low pH treatment is traditionally used (Harris, 1989). Additional experimentation is needed to elucidate if pH or high DIC was the reason for *C. reinhardtii*'s flagella detachment.

Total and Specific Lipid Accumulation

To gain an accurate assessment of the accumulated lipids of *C. reinhardtii*, culture TAG properties were tracked throughout the experiments by using the Nile Red fluorescent staining method, GC analyses were performed at the end of the experiments on both extracted lipids and in situ transesterified FAMEs, and both Bodipy 505/515 and Nile Red fluorescent images were taken to visually confirm TAG accumulation. This approach allowed for monitoring neutral TAG accumulation during ammonium depletion and quantification of the final concentration of FFAs, MAGs, DAGs, TAGs, and biofuel potential in each experiment. Figure 4 shows total Nile Red fluorescence (A) and Nile Red specific fluorescence (B) for the *C. reinhardtii* experiments. Nile Red fluorescence has previously been shown to correlate with neutral TAG and has become a generally accepted screening method for analyzing TAG in algal cultures (Chen et al., 2009; Cooksey et al., 1987; da Silva et al., 2009; Elsey et al., 2007; Gardner et al., 2011, 2012; Lee et al., 1998; Liu et al., 2008; Yu et al., 2009). Prior to ammonium depletion, the cultures show low Nile Red signals. After becoming ammonium depleted (2.8 days), the Nile Red fluorescence increased in both the 5% CO_2 sparged cultures and in the bicarbonate amended cultures but remained low in the air sparged cultures where no bicarbonate was added. The low Nile Red signal observed in the air sparged cultures without added bicarbonate is presumably due to carbon limitation, as previously discussed (shown in Fig. 3).

The rate of fluorescence increase was highest in the 5% CO_2 sparged cultures, but only increased for 1.2 days. The rate of fluorescence increase in the bicarbonate added cultures was slower, but increased over the next 3.2 days. One of the 5% CO_2 sparged cultures seemed to exhibit a lower Nile Red fluorescence during TAG accumulation (Fig. 4A insert), and explains the large variation shown in the triplicate standard deviation for that system. It is unclear why this culture did not exhibit as high of a Nile Red signal, given that it did not show significant variation in the cell density, ammonium utilization, DIC, or pH.

Nile Red specific fluorescence is calculated by normalizing the total Nile Red fluorescence with 10,000 cells. It offers insight into the amount of TAG per cell, and/or the metabolic state of the cultures (Gardner et al., 2011, 2012).

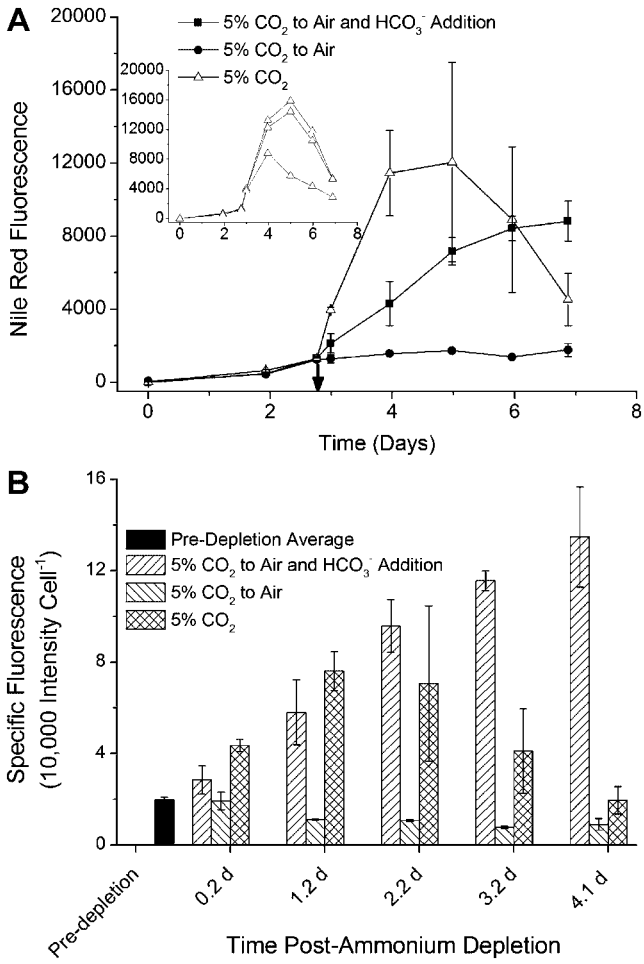

Figure 4. *C. reinhardtii* CC124 average and standard deviation of total Nile Red fluorescence (**A**), insert is the Nile Red fluorescence of the three cultures constituting the average for the cultures maintained on 5% CO_2, and Nile Red specific fluorescence at and post-NH_4^+ depletion (**B**). Arrow indicates time of medium NH_4^+ depletion and inorganic carbon adjustment. Growth was maintained in Sager's minimal medium illuminated with a 14:10 h L:D cycle ($n = 3$).

The Nile Red specific fluorescence increased in both the 5% CO_2 sparged cultures and the bicarbonate amended cultures over 2.2 days after ammonium depletion. However, the Nile Red specific fluorescence decreased in the 5% CO_2 sparged cultures after this time. The bicarbonate amended cultures continued to increase after ammonium depletion throughout the end of the experiment. By the end of the 7th day of culturing, the bicarbonate added cultures exhibited a significantly higher TAG per cell as compared to the other systems monitored, although for a short time the 5% CO_2 sparged cultures displayed higher TAG accumulation per cell (discussed below).

Final Nile Red fluorescence properties for the experiments are given in Table I. At the end of the experiments, Nile Red fluorescence and Nile Red specific fluorescence in the bicarbonate added, the 5% CO_2 sparged, and the air sparged cultures without bicarbonate added was highest to lowest,

Table II. Comparisons of percent (w/w) extractable neutral constituents of biofuel and total biofuel potential of *C. reinhardtii* cultured in 5% CO_2, air, or air with 50 mM HCO_3^- during ammonia depletion (combined extraction or in situ transesterification of triplicate cultures).

Aeration	Free fatty acid (%)[a]	Mono-glyceride (%)[a]	Di-glyceride (%)[a]	Tri-glyceride (%)[a]	Sum of extracted (%)	Total biofuel potential (%)[b]
5% CO_2	3.06	0.34	6.46	4.58	14.44	21.08
5% $CO_2 \rightarrow$ air	2.61	0.25	3.39	2.07	8.32	15.59
5% $CO_2 \rightarrow$ air + HCO_3^-	1.94	0.33	3.21	7.59	13.07	19.61

[a]From bead beating combined with organic solvent extraction.
[b]Total FAMEs from direct in situ transesterification.

respectively. This trend can be compared to the fluorescence images of Figure 2, where both fluorescent stains Bodipy 505/515 and Nile Red, that are commonly used to assess TAG vacuoles in algal cells, were used to visually confirm the trend in Nile Red fluorescence values (Bertozzini et al., 2011; Chen et al., 2009; Cooksey et al., 1987; Cooper et al., 2010; Work et al., 2010). Furthermore, the *C. reinhardtii* biomass was extracted for quantitative analysis of biofuel precursors and results are given in Table II. The Nile Red fluorescence correlates directly with % TAG, $r^2 = 0.998$, but not with % FFA, MAG, DAG, or biofuel potential. In addition, comparing the Nile Red fluorescence trend over time (Fig. 4) with the % FFA, MAG, DAG, and TAG (Table II), it can be seen that for the 5% CO_2 cultures TAGs accumulated and then likely degraded into FFAs and DAGs. This deconvolutes the discrepancy between the Nile Red fluorescence being less in the 5% CO_2 sparged cultures, as compared to the bicarbonate amended cultures, at the end of the experiments but having a higher sum of biofuel precursors, and explains the higher Nile Red signal observed at 5 days in the 5% CO_2 sparged cultures (Fig. 4). These observations would suggest that TAG was degraded for cellular energy requirements. This is supported by the cultures maintaining motility and observations of starch/chlorophyll data discussed below.

The difference between the total biofuel potential and the sum of extracted biofuel precursors represents the polar lipid contribution to biofuel capability. Fatty acids contained in polar molecules (e.g., phospholipids) have been shown to contribute to biofuel potential (Wahlen et al., 2011). The difference between the biofuel potential and the sum of extractable precursors is $6.8 \pm 0.4\%$ for all of the *C. reinhardtii* experiments. Thus, the polar lipid influence on biofuel potential is similar for each inorganic carbon substrate tested. Furthermore, Table III details the composition of the FAMEs produced during in situ transesterification. Table III also shows similar profiles between the *C. reinhardtii* experiments, albeit, subtle differences where observed in the degree of saturation of the C16 palmitic acid methyl ester.

Total and Specific Starch Accumulation

The *C. reinhardtii* wild type has been shown to accumulate starch during N-depleted conditions (Ball et al., 1990), and because starch is a direct competitor to TAG as a carbon storage molecule, it was monitored throughout ammonium depletion. Figure 5 shows total starch (A) and specific starch (B) for the *C. reinhardtii* experiments beginning near ammonium depletion. All cultures had low levels of starch upon ammonium depletion (2.8 days); however, the 5% CO_2 sparged cultures rapidly increased in starch concentration within 0.2 day. After which the starch concentration decreased rapidly to $\sim 200\,\mu g\,mL^{-1}$ and slowly decreased throughout the remainder of the experiment. The cultures to which bicarbonate was added accumulated starch, reaching $\sim 1050\,\mu g\,mL^{-1}$ at 5 days, which was higher than the 5% CO_2 sparged cultures; however, maximum starch concentration may have been missed in the 5% CO_2 sparged cultures because of the fast rate of accumulation/degradation and limited sampling during that time. After peak starch was realized, the bicarbonate added cultures slowly decreased in starch concentration to $\sim 750\,\mu g\,mL^{-1}$. The cultures to which no bicarbonate was added and was sparged with air did not accumulate starch throughout the experiment which is further evidence of the carbon limitation previously discussed.

Specific starch concentration is calculated by normalizing the total starch with 100,000 cells, and the value offers

Table III. Comparisons of percent composition of in situ transesterified FAMEs from *C. reinhardtii* cultured with 5% CO_2, air, or air with 50 mM HCO_3^- during ammonia depletion (combined transesterification of triplicate cultures).

FAME	5% $CO_2 \rightarrow$ air + HCO_3^- (%)	5% $CO_2 \rightarrow$ air (%)	5% CO_2 (%)
C14:0	0.5	0.3	0.4
C16:0	25.6	21.4	27.9
C16:1	3.5	2.1	3.0
C16:2-3[a]	11.3	15.9	12.4
C18:0	2.8	2.6	2.9
C18:1-3[b]	49.9	50.0	46.5
C20:0-2[c]	0.4	1.3	0.3
C24:0	N/D	N/D	N/D
C26:0	0.3	N/D	0.4
Other	5.6	6.3	6.2

N/D, not detected.
[a]C16:2 and C16:3 taken together.
[b]C18:1, C18:2, and C18:3 taken together.
[c]C20:1, C20:2, and C20:3 taken together.

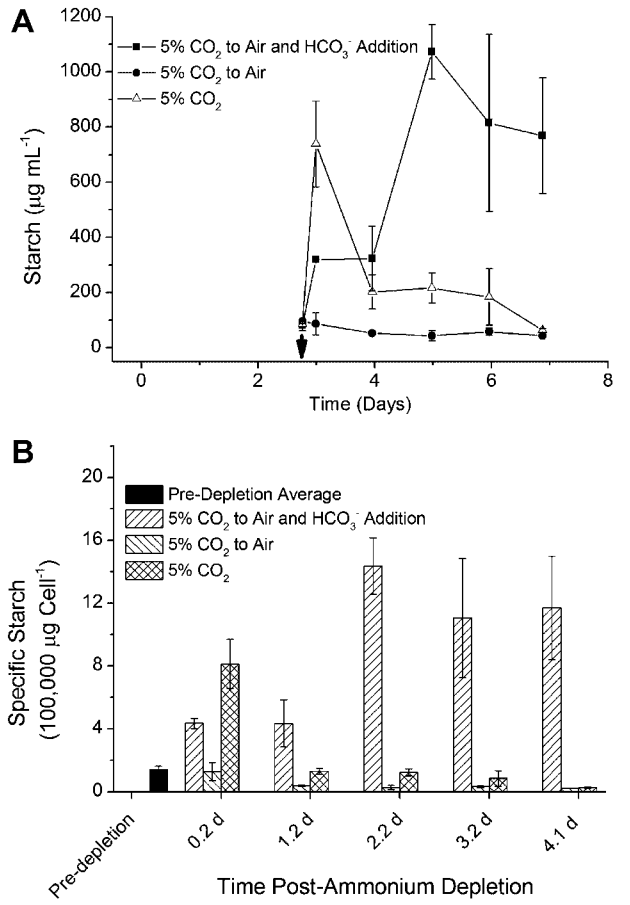

Figure 5. *C. reinhardtii* CC124 average and standard deviation of total starch (μg mL^{-1}) (**A**) and specific starch (**B**), both at and post-NH_4^+ depletion. Arrow indicates time of medium NH_4^+ depletion and inorganic carbon adjustment. Growth was maintained in Sager's minimal medium illuminated with a 14:10 h L:D cycle ($n = 3$).

insight into the amount of starch per cell. As shown in Figure 5B, 0.2 days after ammonium depletion, the 5% CO_2 sparged cultures had the highest specific starch. However, the starch appeared to rapidly degrade over the next day and the starch content per cell continued to decrease throughout the remainder of the experiment. The bicarbonate amended cultures accumulated starch for 2.2 days after ammonium depletion and then starch remained high throughout the remainder of the experiment, with a possible decrease after 2.2 days (albeit no statistical difference between 2.2 and 4.1 days after ammonium depletion). Final measured starch concentrations are given in Table I. Starch is clearly remaining in the bicarbonate added cultures compared to the "no-bicarbonate" cultures.

Algal carbon reallocation from starch into lipid or a switch in metabolic pathways to form lipid in preference to starch during nutrient limitation has been hypothesized in the past (Roessler, 1990), but specific mechanisms were not studied at the time. Additional evidence has been gathered using advanced spectroscopic analysis (Giordano et al., 2001; Murdock and Wetzel, 2009), and it was recently pointed out that additional experimentation in support of this hypothesis is needed (Merchant et al., 2011). Comparison of the total, and specific, starch versus Nile Red fluorescence (Figs. 5 and 4, respectively) provides additional evidence for this hypothesis. In the cultures, to which 5% CO_2 was sparged and possibly in the cultures to which bicarbonate was amended, starch accumulated to maximum values and then decreased as TAG accumulated (Nile Red signal) to a maximum value. However, the bicarbonate amended cultures maintained high starch accumulation as maximum TAG was realized. Essentially, bicarbonate caused the cultures to maximize carbon storage metabolites in the form of starch and biofuel precursors.

Chlorophyll State During Lipid Accumulation

To ascertain the underlying health of the *C. reinhardtii* cultures during ammonium depletion and metabolite accumulation, chlorophyll concentrations were monitored. Figure 6 shows chlorophyll *a* (A), chlorophyll *b* (B), and total chlorophyll (C) for the experiments reported. During exponential growth and up to 4 days there are no discernible differences in chlorophyll concentrations between the different cultures. After which there was a decrease through 7 days for all the cultures. However, the rate of decrease was highest to lowest in the cultures to which 5% CO_2 was sparged, bicarbonate was added, and air sparged without added bicarbonate, respectively.

The decreased chlorophyll concentrations toward the end of the experiment can be compared with the decrease in DIC utilization rate (Fig. 3) observed in the cultures to which bicarbonate was added, suggesting a slowing of DIC utilization. Further, the cultures to which 5% CO_2 was sparged had the lowest amount of chlorophyll at the end of the experiment, and was degraded in both TAG and starch (Table I and Figs. 4 and 5, respectively), suggesting non-optimal health. Further, at the end of the experiment the cultures to which air was sparged without added bicarbonate had the highest amount of remaining chlorophyll, but never accumulated TAG or starch, an indication of carbon limitation (Fig. 3).

Conclusions

C. reinhardtii grew rapidly with a 5% CO_2 in air sparge until ammonium in the medium was depleted. During this time there was a characteristic decrease in pH, which is consistent for microalgal growth on ammonium and high CO_2 levels (Eustance, 2011; Fuggi et al., 1981). During ammonium depletion, *C. reinhardtii* accumulated TAG and starch as carbon storage compounds when a 5% CO_2 gas-sparge or bicarbonate was used as inorganic carbon sources. The 5% CO_2 sparged cultures, after ammonium depletion, quickly accumulated starch which was then likely reallocated

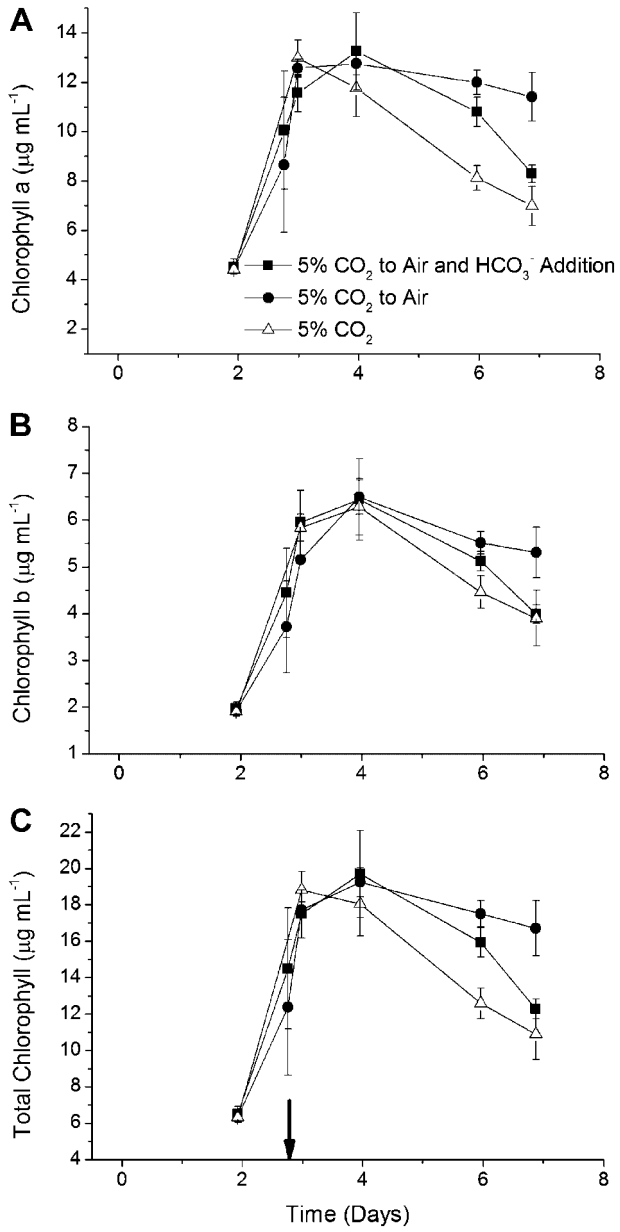

Figure 6. *C. reinhardtii* CC124 average and standard deviation of chlorophyll *a* (**A**), chlorophyll *b* (**B**), and total chlorophyll (**C**). Arrow indicates time of medium NH_4^+ depletion and inorganic carbon adjustment. Growth was maintained in Sager's minimal medium illuminated with a 14:10 h L:D cycle ($n = 3$).

to TAG. Further, the highest TAG accumulation was observed when sparged with 5% CO_2; however, the TAG began to degrade into FFA and DAG within a few days. While the 50 mM bicarbonate amended cultures accumulated starch and TAG at a slightly slower rate, starch reallocation and stable carbon storage was observed. The low CO_2 cultures exhibited carbon limitation and slow metabolic growth with minimal TAG or starch accumulation.

The highest biofuel potential was observed when high CO_2 was used as the carbon substrate. However, these conditions were not as stable, in comparison with bicarbonate as the inorganic carbon source, evident by the fast accumulation followed by somewhat rapid degradation of both TAG and starch. Additionally, the bicarbonate amended cultures exhibited more carbon storage, due to the high starch remaining at the end of the experiments. This suggests industrial use of algae to produce biofuel could benefit from using bicarbonate during nutrient deplete conditions to boost output, especially if the industrial strain does not have the capabilities to produce starch, such as *C. reinhardtii* starchless mutants (Li et al., 2010; Wang et al., 2009; Work et al., 2010).

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