Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the *chlorophyte Scenedesmus* sp. and the diatom *Phaeodactylum tricornutum*

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There is potential for algal-derived biofuel to help alleviate part of the world's dependency on petroleum based fuels. However, research must still be done on strain selec-tion, induction of triacylglycerol (TAG) accumulation, and fundamental algal metabolic studies, along with large-scale culturing techniques, harvesting, and biofuel/biomass pro-cessing. Here, we have advanced the knowledge on Scene-desmus sp. strain WC-1 by monitoring growth, pH, and TAG accumulation on a 14:10 light–dark cycle with atmospheric air or 5% CO_2 in air (v/v) aeration. Under ambient aeration, there was a loss of pH-induced TAG accumulation, presumably due to TAG consumption during the lower culture pH observed during dark hours (pH 9.4). Under 5% CO_2 aeration, the growth rate nearly doubled from 0.78 to 1.53 d^{-1}, but the pH was circumneutral (pH 6.9) and TAG accumulation was minimal. Experiments were also performed with 5% CO_2 during the exponential growth phase, which was then switched to aeration with atmospheric air when nitrate was close to depletion. These tests were run with and without the addition of 50 mM sodium bicarbonate. Cultures without added bicarbonate showed decreased growth rates with the aeration change, but there was no immediate TAG accumulation. The cultures with bicarbonate added immediately ceased cellular replication and rapid TAG accu-mulation was observed, as monitored by Nile Red fluorescence which has previously been correlated by gas chromatography to cellular TAG levels. Sodium bicarbonate addition (25 mM final concentration) was also tested with the marine diatom Phaeodactylum tricornutum strain Pt-1 and this organism also accumulated TAG.

Keywords: Triacylglycerol (TAG) · Algae · Biodiesel · Fatty acid methyl ester (FAME) · Nile Red fluorescence

Introduction

There is a concern with the effects that fossil fuel limitation will inevitably have on the global society (Bilgen et al. 2004; Brown 2006). Issues include escalating fuel prices and in-creasing atmospheric carbon concentrations from burning fos-sil fuel (Dukes 2003), along with devastating environmental spills associated with higher risk drilling operations. However, fuel derived from biomass has the potential to offset a portion of the world's petroleum dependency (Demirbas et al. 2009), especially if the fuel is derived from a microalgal feedstock. Recently, there has been a dramatic increase in interest asso-ciated with algal biomass potential (Brennan and Owende 2010; Demirbas 2010; Dismukes et al. 2008; Francisco et al. 2010; Hu et al. 2008; Mata et al. 2010; Posten and Schaub 2009; Schenk et al. 2008; Chisti 2007; Greenwell et al. 2010; Lardon et al. 2009).

Biodiesel is defined as the fatty acid methyl esters (FAME) derived from triacylglycerol (TAG) and can be synthesized from plant, animal, or microalgal biomass. FAMEs offer in-creased energy yield, compared to biologically derived ethanol,

and can be used directly in jet or diesel engines (Cunningham 2007; Hill et al. 2006). However, TAG availability will limit the use of this fuel unless non-food crop land and low quality water can be used to produce the fuel. Due to fast growth rates and physiological diversity (Hu et al. 2008), microalgae are well suited for use in growth systems that can overcome land area limitations in a variety of climates. In addition, the use of alkaline water can facilitate high dissolved inorganic carbon concentrations and potentially decrease microbial contamination (Gardner et al. 2011). However, further improvements in technology are needed to advance this field so economical industrial biodiesel production can be realized.

Historically, a major goal of the Department of Energy's Aquatic Species Program, which initially evaluated algae's potential for biodiesel production, was to identify a "lipid trigger" (Sheehan et al. 1998). This would be a set of environmental parameters or target signal(s) that would cause algae to increase TAG synthesis, but a trigger was not identified. However, nitrogen depletion has been shown to cause cellular lipid accumulation in some strains, albeit temporal TAG accumulation to reasonable concentrations can vary significantly (Gardner et al. 2011; Stephenson et al. 2010). In addition to nutrient depletion, Guckert and Cooksey first reported high pH-induced TAG accumulation (Guckert and Cooksey 1990) and Thomas and Cooksey observed TAG accumulation from delayed cell cycling by inhibiting the tricarboxylic cycle with monofluoroacetate (Guckert and Thomas 1988; Thomas 1990).

Recent results have shown that high pH and nitrate depletion caused TAG accumulation independently in a *Scenedesmus* species (Gardner et al. 2011). Further, an advantageous interaction between pH and nitrate was identified, which showed an increase in TAG per cell and a shorter culture time to realize high TAG accumulation (Gardner et al. 2011). Of additional note is the increase in TAG accumulation observed when Guckert and Cooksey added 5 mM sodium bicarbonate to cultures when nitrate was still present (Guckert and Cooksey 1990). This increase in TAG accumulation was attributed to increased pH; however, the pH studies (Gardner et al. 2011; Guckert and Cooksey 1990) were conducted with 24 h light which inherently limits dark cycle respiration.

The work presented here details the extension of our studies using pH as a control variable and is aimed at identifying strategic environmental culturing parameters, understanding TAG accumulation induced by high pH and nitrate depletion, and investigating the generality of previous pH studies (Gardner et al. 2011; Guckert and Cooksey 1990). Our work was guided by the hypotheses (1) that inhibition of the cell cycle leads to TAG accumulation and (2) high pH coupled with nitrate depletion causes increased TAG accumulation compared with controls buffered at lower pH values. To test these hypotheses, bicarbonate was added to *Scenedesmus* sp. strain WC-1 cultures to increase pH and total dissolved inorganic carbon near the time of nitrate depletion in the culture medium. This bicarbonate effect was further investigated on the marine diatom *Phaeodactylum tricornutum* strain Pt-1.

Materials and methods

Scenedesmus sp. strain WC-1 (WC-1) was isolated and confirmed unialgal as previously described (Gardner et al. 2011). WC-1 was cultured on Bold's basal medium with pH adjusted to 8.7 with KOH prior to autoclaving (Nichols and Bold 1965). *P. tricornutum* strain Pt-1 (CCMP 2561) (Pt-1) was acquired from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and accessions of *P. tricornutum* have previously been described (Martino et al. 2007). Pt-1 was cultured on ASP2 medium and 50 mM Tris buffer (Sigma-Aldrich, USA), p*K*a 7.8 (Provasoli et al. 1957). Both organisms were checked for bacterial contamination by inoculation into respective medium supplemented with 0.05% yeast extract and 0.05% glucose and incubated in the dark. Experiments were conducted in triplicate in batch culture using 70×500 mm glass tubes containing 1 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°C±1°C and 20°C±1°C, for WC-1 and Pt-1, respectively. Light (400 μmol photons m^{-2} 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 with and without 5% CO_2 (*v*/*v*) and controlled using an individual rotameter on each bioreactor (Cole-Parmer, USA). In experiments with bicarbonate addition, analytical grade sodium bicarbonate was used (Sigma-Aldrich, USA)

Cell concentrations were determined using an optical hemocytometer with a minimum of 400 cells counted per sample for statistical reliability. Light micrographs and Nile Red fluorescence images were taken using a transmitted/epifluorescence light microscope (Nikon Eclipse E800) with an Infinity 2 color camera.

Analysis of media components Medium pH was measured on samples using a standard bench top pH meter. Nitrate in Bold's basal medium was measured by ion chromatography (IC) using an IonPac AS9-HC Anion-Exchange Column (Dionex) with a 9.0-mM sodium carbonate buffer set at a flow rate of 1.0 mL min^{-1}. Detection was performed using a CD20 conductivity detector (Dionex) at 21°C, and IC data were analyzed on Dionex PeakNet 5.2 software. Due to its high salinity, nitrate in ASP2 medium was measured using a colorimetric assay based on the reaction of Czechrome NAS

reagent (Polysciences Inc., USA) with nitrate ions. In brief, 1 mL of culture was centrifuged at 16,000×*g* for 15 min. The supernatant liquid was then collected for nitrate quantification. Sample (0.1 mL) was gently mixed with 1 mL reagent solution prepared as described by the manufacturer (Polysciences Inc., USA) and incubated 20 min at room temperature. The absorbance was read at 570 nm, and nitrate concentration of the sample was calculated using a nitrate standard curve.

Lipid analysis Cellular TAG accumulation was estimated using the Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one; Sigma-Aldrich, USA) fluorescence method (Cooksey et al. 1987), which has been correlated with cellular TAG concentrations (Chen et al. 2009; Cooksey et al. 1987; Elsey et al. 2007; Lee et al. 1998). TAG accumulation over time was measured by removing 1 mL aliquots from cultures and assayed directly with Nile Red (4 μL from 250 μL/mL in acetone) or by diluting with 4 mL ultrapure H_2O or salt water for WC-1 and Pt-1, respectively, before assaying directly with Nile Red (20 μL from 250 μL 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} and 3×10^6 cells mL^{-1} for WC-1 and Pt-1, respectively. Total Nile Red fluorescence was quantified on a microplate reader (Bio-Tek, USA) utilizing 480/580 nm excitation/emission filters. 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. It has been shown (Cooksey et al. 1987) that the Nile Red intensity shifts for different algal strains over time, and this was recently reconfirmed (Elsey et al. 2007). Measurement times of 60 min and 3 min after staining were optimal for WC-1 and Pt-1, respectively.

Results

To expand our understanding of TAG accumulation in light–dark cycled cultures, WC-1 was grown with a 14:10 light–dark cycle. Figure 1 shows cell growth (a), total Nile Red fluorescence (b), and medium pH (c) for WC-1 grown under ambient aeration with unbuffered Bold's basal medium. The unbuffered culture maintained exponential growth up to 7 d and exhibited a 0.8 d^{-1} maximum specific growth rate (21 h doubling time). The total Nile Red fluorescence showed no significant lipid accumulation through 10 d, after which the fluorescent signal increased to a maximum (16–18 d). Nitrate became depleted at 9 d, and the increase in fluorescence after 10 d is attributed to nitrate depletion. The culture

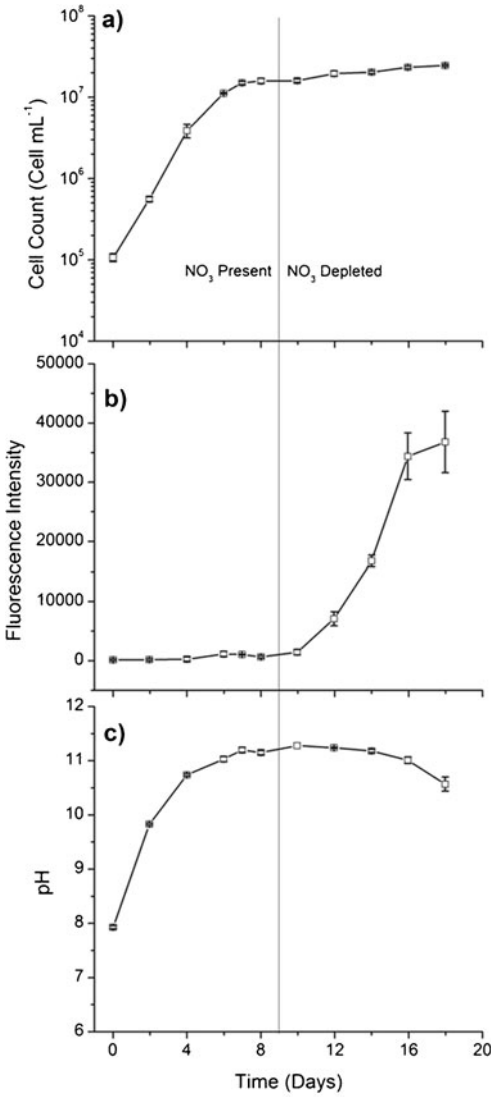

Fig. 1 *Scenedesmus* WC-1 batch growth average and standard deviation of cellular density (**a**), total Nile Red fluorescence intensity (**b**), and pH (**c**) under ambient aeration. *Line* indicates time of medium nitrate depletion. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (n=3)

pH increased during exponential growth up to a maximum of pH 11.3. After the nitrate was depleted, there was a decrease in medium pH which can be attributed to an increase of carbonic acid in the medium presumably caused by a change from growth to a TAG accumulation state of the microalgal culture.

To gain an accurate assessment of pH and lipid changes during the diel light cycle, the culture was monitored over a 24-h period during late-exponential growth. Figure 2 shows that both Nile Red fluorescence and culture pH were at elevated levels just as the light cycle ended and the dark cycle began at 0 h. The pH and fluorescence decreased until the cycle was shifted from dark to light 10 h later, when the illumination caused an increase in both pH and Nile Red fluorescence.

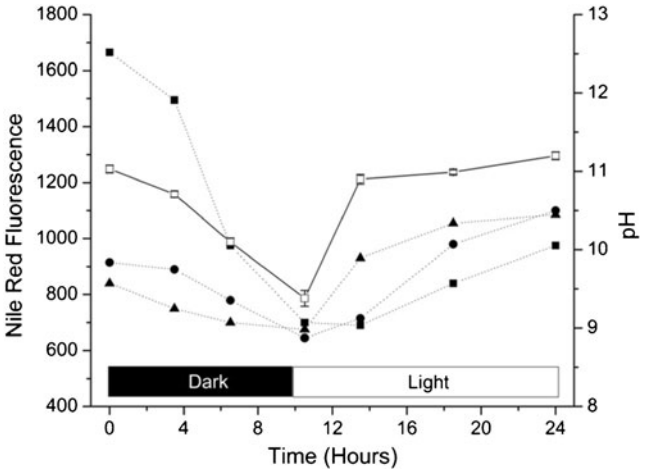

Fig. 2 *Scenedesmus* WC-1 Nile Red fluorescence of three biological replicates (*filled circle*, *filled square*, and *filled triangle*) and average medium pH (*square*), with one standard deviation, monitored over 24 hours during late exponential growth (7–8 d) and prior to medium nitrate depletion in unbuffered Bold's basal medium under ambient aeration batch culturing. *Bar* indicates light and dark times during the 14:10 L/D cycle (n=3)

We sought to increase the rate of growth and lipid content for WC-1 by increasing the CO_2 concentration from ambient air (0.04%) to 5%. Figure 3 shows the growth (a), total Nile Red fluorescence (b), and medium pH (c) during 5% CO_2 aeration. With the added CO_2, the culture grew exponentially to 5 d with a specific growth rate of 1.5 d^{-1} (10.9 h doubling time), as compared to 0.8 d^{-1} (21 h doubling time) without CO_2 added. The medium pH stayed near constant at 6.9±0.15 throughout the experiment and the nitrate was depleted at 4 d. After 4 d, the Nile Red fluorescence shifted to a slightly higher value. However, the total fluorescence was much smaller compared to cultures grown with ambient air. After 10 d, cultures in 5% CO_2 showed a decrease in total fluorescence, indicating cellular TAG was being utilized or degraded.

Cultures were also grown with 5% CO_2, until nitrate depletion was imminent. At that time, the aeration was shifted from 5% CO_2 to ambient air to investigate cellular TAG accumulation, with and without the addition of bicarbonate (50 mM). Figure 3 shows the growth (a), total Nile Red fluorescence (b), and medium pH (c) of WC-1, with and without a 50-mM bicarbonate addition when aeration is shifted from 5% CO_2 to ambient aeration at the time of nitrate depletion. Before nitrate depletion, both cultures grew exponentially with a specific growth rate of 2.1 d^{-1} (8.1 h doubling time). Nitrate was depleted at 4 d for both cultures. The aeration was switched and bicarbonate was added at 3.7 d when there was 3.4 mg L^{-1} nitrate remaining (200 mg L^{-1} nitrate initially), and the addition stopped cellular replication immediately. With nitrate depletion, the bicarbonate-free control shifted to a slower growth rate and continued to double 1.6 times.

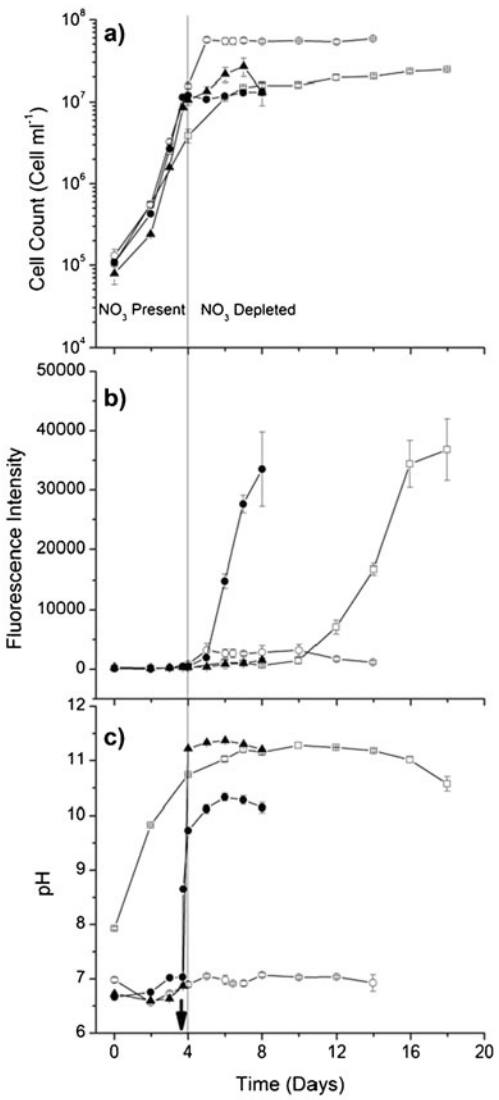

Fig. 3 *Scenedesmus* WC-1 average and standard deviation of cellular density (**a**), total Nile Red fluorescence intensity (**b**), and pH (**c**) of culture aerated with ambient air (*square*), 5% CO_2 in air (*circle*), and 5% CO_2 in air that is changed to ambient air near nitrate depletion, both with (*filled circle*) and without (*filled triangle*) a 50-mM bicarbonate addition. *Arrow* indicates time of bicarbonate addition and *line* indicates time of medium nitrate depletion. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (n=3). Note ambient aeration results are replotted from Fig. 1 for the purpose of comparison

Both cultures maintained a pH of 6.75±0.14 until the time of bicarbonate addition, after which the bicarbonate addition culture and the bicarbonate-free control shifted to a maximal pH of 10.4 and 11.4, respectively. Bicarbonate acted as a buffer which held pH lower than the bicarbonate-free control cultures. For the bicarbonate addition culture, the total Nile Red fluorescence began to increase at 5 d and reached 33,500 units 3 days later. In contrast, the fluorescence for the control culture (without bicarbonate) increased to only 1,500 units.

The addition of bicarbonate at the time of nitrate depletion clearly changed the metabolic activity of the culture. Immediately after bicarbonate addition, WC-1 stopped cellular replication and began to accumulate TAG. The timing of this effect was further investigated by comparing effects of bicarbonate addition before nitrate depletion and after the medium was nitrate depleted when cultures were in stationary phase. Figure 4 shows the cellular growth and total Nile Red fluorescence of unbuffered WC-1 when bicarbonate was added before nitrate depletion and at 6.4 d when nitrate was depleted. Cultures grew exponentially under 5% CO_2 aeration with a 1.9 d^{-1} specific growth rate and a doubling time of 8.7 h. Cultures became nitrate depleted at 4 d. It can be seen that cultures to which bicarbonate was added at 3.7 d (pre-nitrate depletion) showed an immediate cessation of cellular replication. Furthermore, these cultures accumulated TAG from 5 to 8 d. There was a difference in nitrate concentration at the time of bicarbonate addition, due to differences in inoculum concentration, which may have affected the final TAG concentration at 8 d. These results suggest an optimal time or nitrate concentration for bicarbonate addition.

When the cultures were maintained on 5% CO_2 until after nitrate depletion at 4 d, the Nile Red fluorescence increased to 3,500 units at 5 d presumably due to nitrate depletion. This initial increase is comparable to the fluorescence increase observed in the 5% CO_2 control of Fig. 3. At 6.4 d, aeration was adjusted to ambient air (low CO_2) and bicarbonate was added (50 mM final concentration). When bicarbonate was added, the culture pH immediately shifted to pH 9.1 and the culture reached a maximum pH of 10.0 over the remainder of the experiment (data not shown). Nile Red fluorescence of these cultures decreased to baseline (450 units) indicating that accumulated lipids were consumed and no significant TAG

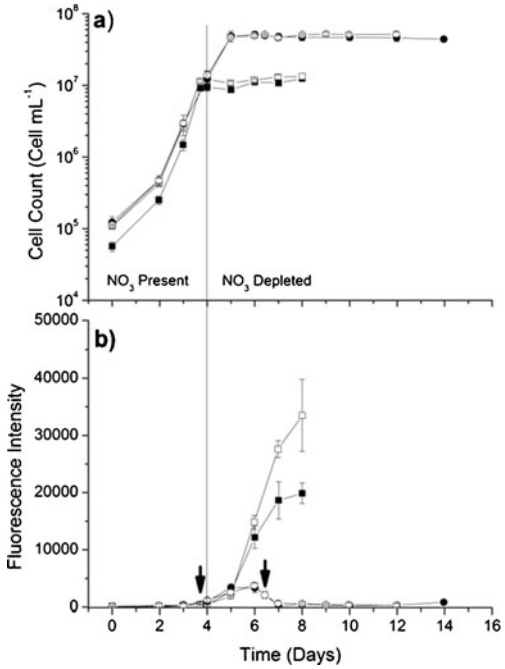

Fig. 4 *Scenedesmus* WC-1 average and standard deviation of cellular density (**a**) and total Nile Red fluorescence intensity (**b**) of culture aerated with 5% CO_2 in air that is switched to ambient air and 50 mM bicarbonate addition pre-nitrate depletion (*filled square* and *square*) or post-nitrate depletion (*filled circle* and *circle*). *Arrows* indicate time of bicarbonate addition and *line* indicates time of medium nitrate depletion. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (n=3)

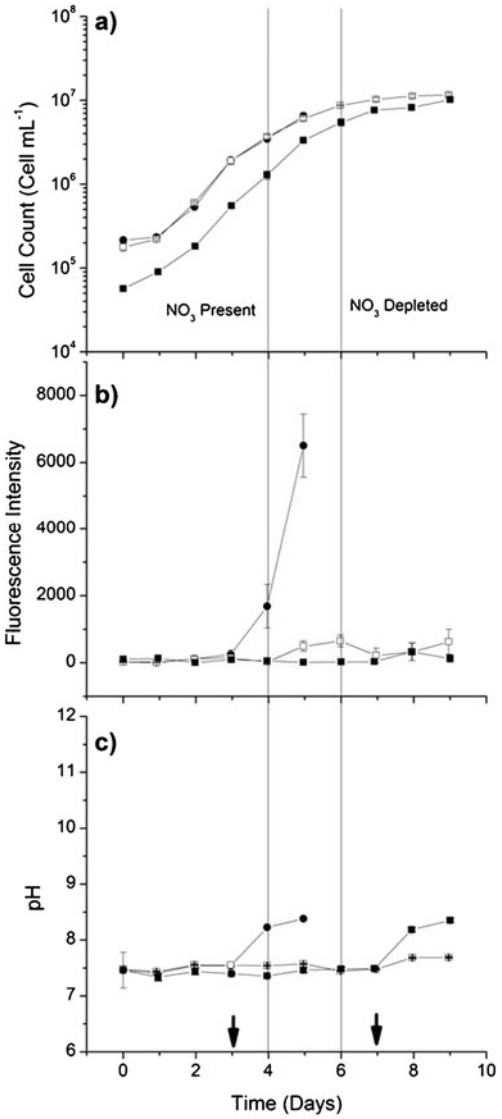

Fig. 5 *Phaeodactylum tricornutum* Pt-1 batch growth average and standard deviation of cellular density (**a**), total Nile Red fluorescence intensity (**b**), and pH (**c**) of culture aerated with ambient air and 25 mM bicarbonate addition pre-nitrate depletion (*filled circle*), added post-nitrate depletion (*filled square*), and no addition (*square*). *Arrow* indicates time of bicarbonate addition and *line* indicates time of medium nitrate depletion (4 d for *filled circle* and *square*, 6 d for *filled square*). Growth was maintained in 50 mM Tris (p*K*a 7.8) buffered ASP2 medium illuminated with a 14:10 L/D cycle (n=3)

accumulation was observed throughout the remainder of the experiments.

To ascertain whether bicarbonate addition would give similar results in other algae, the marine diatom *P. tricornutum* Pt-1 was studied in a similar manner to WC-1. However, the final concentration of bicarbonate addition was decreased from 50 mM to 25 mM, and 50 mM Tris buffer (p*K*a 7.8) was incorporated to minimize pH shifts because as the pH approaches 9.0, the constituents of the ASP2 medium precipitate, which could potentially confound results. Furthermore, ambient aeration was maintained throughout incubation because 5% CO_2 caused a decrease in culture pH which significantly inhibited cellular growth of Pt-1 (data not shown). Figure 5 shows cellular growth (a), total Nile Red fluorescence (b), and medium pH (c), of Pt-1, with and without 25 mM bicarbonate addition, both pre- and post-nitrate depletion. All cultures grew exponentially with a 0.9 d^{-1} specific growth rate (18.4 h doubling time). Nitrate became depleted at 4 d for the control and pre-nitrate bicarbonate triggered cultures, while the post-nitrate depletion triggered cultures became nitrate depleted at 6 d (2 d time difference due to the lower initial inoculum concentration). Bicarbonate was added at 3 d and 7 d for the pre- and post-nitrate depleted cultures, respectively. Similar to WC-1, Nile Red fluorescence increased when bicarbonate was added while the medium still had nitrate, but did not increase when no nitrate was available. However, unlike WC-1, cell replication of Pt-1 was not stopped by the addition of bicarbonate. Previous experimentation with Pt-1 indicated that Nile Red fluorescence decreased after 2 d of TAG accumulation, therefore the culture was harvested on the second day after bicarbonate addition (i.e., 5 d).

Discussion

Light–dark cycling effect on pH-induced TAG accumulation in Scenedesmus WC-1 Previous studies have detailed that alkaline pH can cause TAG accumulation, and these studies showed that pH and nitrate depletion are independent stress responses that can be utilized alone or in combination (Gardner et al. 2011; Guckert and Cooksey 1990). However, results were obtained from cultures grown with 24 h light, which prevents dark cycle respiration while continuously maintaining photosynthesis. As previously observed with 24 h light illumination studies (Gardner et al. 2011; Guckert and Cooksey 1990), there was an expected pH-induced TAG accumulation caused when culture pH increased above pH 10. However, with 14:10 light cycling this accumulation was not observed during exponential growth (4–9 d of Fig. 1). To understand this lack of TAG accumulation, pH and Nile Red fluorescence were measured over a 24-hour period during late-exponential growth and prior to nitrate depletion (Fig. 2). The pH decrease during the dark and increase in the light can be attributed to carbonic acid accumulation and photosynthesis, respectively (Shiraiwa et al. 1993). The decrease in Nile Red fluorescence correlates to dark cycle respiration utilizing TAG for cellular energy, and TAG accumulation was observed during light hours. This trend of cellular TAG accumulation and utilization has previously been observed in *Chlorella* CHLOR-1, and our results corroborate the hypothesis that TAG accumulates during light exposure followed by utilization in the dark (Thomas 1990).

Culture pH (Fig. 1c) was measured at approximately the same diel sampling time (just prior to the dark hours each day), and the medium pH decreased to 9.4 during the dark

Table 1 Comparison of average and standard deviation in culture growth and lipid production properties of WC-1 and Pt-1 cultured in unbuffered Bold's basal and 50 mM Tris buffered ASP2 media, respectively, during 14:10 light–dark cycling (n=3)

Organism	Aeration	Time of HCO_3 addition (d)	Time of harvest (d)	Final cell density ($\times 10^7$ cells mL^{-1})	Dry weight (gL^{-1}; DCW)[a]	Final total Nile Red fluorescence ($\times 10^3$ units)	Final specific Nile Red intensity $cell^{-1}$ [b]
Scenedesmus WC-1	Air	N/A	18.0	2.46±0.06	1.1±0.1	36.8±5.2	15.0±2.3
	5% CO_2	N/A	14.0	5.87±0.15	2.7±0.1	1.2±0.2	0.2±0.1
	5% CO_2→Air	N/A	8.0	2.69±0.69[e]	0.8±0.1	1.0±0.4 [e]	0.4±0.1[e]
	5% CO_2→Air	3.7 [c]	8.0	1.34±0.15	1.1±0.1	33.5±6.3	24.8±2.2
	5% CO_2→Air	6.4 [d]	12.0	5.18±0.41	2.1±0.0	0.3±0.3	0.1±0.1
Phaeodactylum Pt-1	Air	N/A	9.0	1.17±0.04	0.2±0.0	0.6±0.4	0.3±0.2
	Air	3.0 [c]	5.0	0.65±0.01	0.1±0.0	6.5±0.9	9.9±0.8
	Air	7.0 [d]	9.0	1.02±0.05	0.1±0.0	0.1±0.1	0.1±0.1

N/A not applicable

[a] Dry cell weight (DCW) determined gravimetrically with filtered samples dried at 60°C

[b] Calculated by fluorescence signal/cell density × 10,000 (scaling factor)

[c] Pre-nitrate depletion

[d] Post-nitrate depletion

[e] 7 d reported due foaming at 8 d (time of harvest)

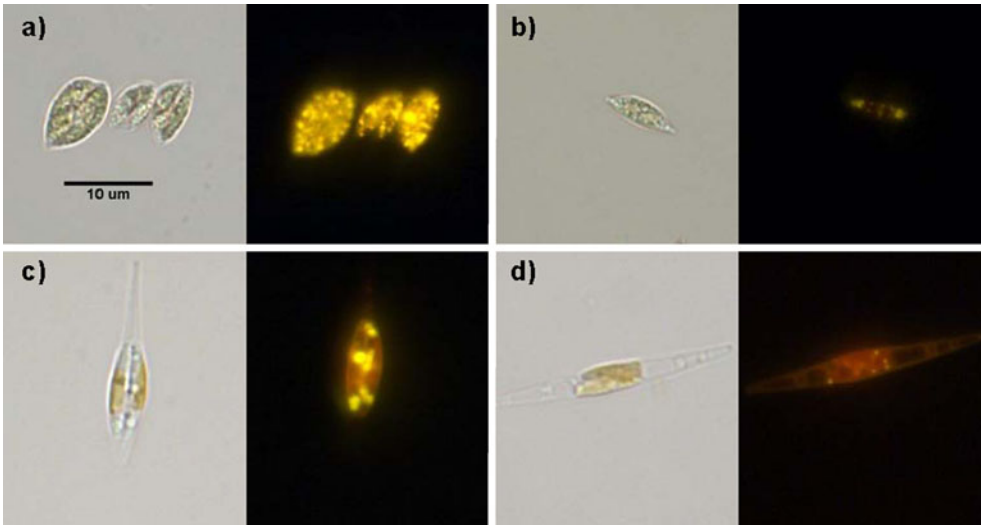

Fig. 6 Transmitted micrographs and Nile Red epifluorescent images of *Scenedesmus* WC-1 (*top*) and *Phaeodactylum tricornutum* Pt-1 (*bottom*) when bicarbonate was added before nitrate depletion (**a**, **c**) and when added after nitrate depletion (**b**, **d**). *Cells imaged* represent average cells for each respective culture and all micrographs are at the same magnification

hours (Fig. 2). Previous studies of WC-1 have shown that CHES-buffered cultures (p*K*a 9.3) did not exhibit pH-induced TAG accumulation at pH 9.4 (Gardner et al. 2011). Therefore, this lower pH during the dark hours is likely why WC-1 did not accumulate TAG during the late exponential phase of growth, even though pH reached 11.3 in the light cycle. It is important to state that Nile Red fluorescence depends on the TAG content of the cells and not on the pH of the medium. It has been observed that cell viability is preserved using the Nile Red staining method (Cooksey et al. 1987), and it is expected that these intact cells maintain a constant internal pH independent of the medium pH. Further, Nile Red fluorescence was correlated directly with GC analysis of the extracted lipid and was independent of medium pH, R^2=0.95 for WC-1 (Gardner et al. 2011).

Growth kinetics of Scenedesmus WC-1 under 5% CO_2 aeration It was apparent from Fig. 3 that higher CO_2 concentrations increased the growth rate of WC-1 (twofold); however, the increased CO_2 caused the culture pH to remain low. The low pH at the time of nitrate depletion has been previously shown to correlate with low TAG accumulation in WC-1 (Gardner et al. 2011). This led us to envision a significantly improved growth and TAG production scenario, where aeration with 5% CO_2 was utilized during exponential growth, but just prior to nitrate depletion the culture was shifted to air-only aeration, to allow photosynthesis to naturally increase culture pH. Further, we hypothesized that maintaining a high dissolved inorganic carbon concentration would improve TAG production (i.e., increased available carbon at elevated pH). This led to the addition of sodium bicarbonate as a dissolved inorganic carbon source when changing from 5% CO_2 to ambient air.

Scenedesmus WC-1 growth and TAG accumulation with 5% CO_2 shifted to ambient air, with and without bicarbonate addition Comparison of the bicarbonate supplemented culture with the ambient aerated culture (Fig. 3), shows maximum total Nile Red fluorescence was reached in only 8 d instead of 16–18 d. Using high CO_2 during the initial exponential growth contributed to the decreased culturing time needed to reach nitrate depletion. In addition, when bicarbonate was added, the rate of TAG accumulation was twice as fast, which contributed to the decreased culturing time for maximum TAG accumulation. Table 1 summarizes biomass and TAG yields for WC-1 under the different culture conditions. Of particular note, WC-1 reached high total Nile Red fluorescence when grown on air only, similar to cultures with bicarbonate added at nitrate depletion. However, in the air-only culture, there were almost twice as many cells and they required 10 days longer to accumulate maximum TAG. Thus, with bicarbonate addition, there was more TAG per cell in the culture as shown in Table 1 by the specific Nile Red fluorescence. A final Nile Red specific fluorescence of 24.8 correlates to 15.2% TAG based on previous correlation data, Equation 1 and R^2=0.95 (Gardner et al. 2011).

$$\%\text{TAG} = (\text{Nile Red specific fluorescence} - 1.104)1.56^{-1} \quad (1)$$

Comparison of bicarbonate addition in WC-1 and Pt-1 Analysis of the results from WC-1 and Pt-1 indicate that the addition of bicarbonate can stimulate TAG accumulation in both Chlorophytes and diatomaceous algae, and there is possibly a nitrogen dependency for this stimulation. In essence, the results strongly suggest that a well-timed bicarbonate addition acts as a "trigger" for TAG accumulation.

Figure 6 shows optical micrographs and Nile Red epifluorescence images of both WC-1 and Pt-1 when bicarbonate was added pre- or post-nitrate depletion. In the Nile Red epifluorescence images, yellow shows the lipid bodies that have accumulated within the cells. Clearly, the images corroborate the experimental observation of bicarbonate inducing

TAG accumulation when added just prior to nitrate depletion. Furthermore, Nile Red 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). Specifically, previous work done with marine diatoms *Amphora coffeaeformis* and *Navicula* sp., which are similar to Pt-1, showed strong correlations between Nile Red fluorescence and gas chromatography analysis of neutral lipids (i.e., TAG) which substantiates our use of Nile Red to monitor cellular TAG accumulation (Cooksey et al. 1987).

Adding bicarbonate was shown to trigger TAG accumulation in the green alga WC-1 and the diatom Pt-1. However, significant differences were observed in the specific Nile Red fluorescence (Table 1). Further, cellular replication in WC-1 was arrested by bicarbonate addition, while Pt-1 replication was not stopped. The differences in specific Nile Red fluorescence can be attributed to different strain specific lipid properties. The greater question is, since both organisms increased TAG accumulation, why did one organism stop replicating while the other did not? The micrographs in Fig. 6a indicate that WC-1 cells stop replicating just before cell division, which was similar to the delayed cell cycling observed when pH-induced TAG accumulation was shown (Gardner et al. 2011; Guckert and Cooksey 1990). This is further evidence that TAG accumulation is the net result of TAG synthesis and utilization, and that inhibiting the cell cycle allows TAG to accumulate. However, the addition of bicarbonate seemed to do more than just delay cell cycling in WC-1, it arrested replication altogether.

Further work is needed to elucidate the exact mechanism that the bicarbonate addition induces in microalgae. Work should focus on microalgal cellular metabolism changes as well as monitor inorganic carbon speciation and utilization. For example, it is questionable whether the effect of pH change can be differentiated from the effect of bicarbonate ions, in relation to stopping cell cycling and inducing TAG accumulation. Previous culturing work with WC-1 in the pH ranges reported in this study showed that (Gardner et al. 2011) growth was minimally inhibited by the different pH values, however pH-induced TAG accumulated at pH values greater than pH 10. This may have been due to a limitation by available dissolved inorganic carbon or some other consequential ion effect. Therefore, additional work monitoring dissolved inorganic carbon during bicarbonate addition, along with pH measurement, may offer insight into carbon speciation and utilization during TAG accumulation. Additional measurements on pigment concentrations and photosynthetic capacity will also offer insight into the culture's ability to fix carbon.

Recently, there have been a number of reviews focused on inorganic carbon use and carbon concentrating mechanisms in microalgae (Colman et al. 2002; Giordano et al. 2005; Kaplan and Reinhold 1999; Moroney and Somanchi 1999; Moroney and Ynalvez 2007; Raven 2010). Of specific interest are studies on *Chlorella* (Beardall 1981; Beardall and Raven 1981; Bozzo et al. 2000; Matsuda and Colman 1995; Rotatore and Colman 1991), *Scenedesmus* (Palmqvist et al. 1988; Radmer and Ollinger 1980; Thielmann et al. 1990), and marine diatoms (Reinfelder et al. 2004; Tortell et al. 1997), but the most studied carbon concentrating system is that of the model chlorophyte *Chlamydomonas reinhardtii* (Ghoshal and Goyal 2001; Goyal and Tolbert 1990; Moroney et al. 1987; Moroney and Somanchi 1999; Moroney and Tolbert 1985; Palmqvist et al. 1988). It has been proposed that when algae, especially green algae, are moved from high CO_2 conditions (1–5%, *v*/*v*) to low CO_2 conditions (atmospheric, 0.04%, *v*/*v*), there are a number of carbonic anhydrases and bicarbonate specific transporters that are synthesized within a short amount of time (up to 6 hrs). These anhydrases and transporters work in concert to shuttle inorganic carbon across the periplasmic membrane, through the cytosol, across the chloroplast membrane, to convert it to CO_2 in the direct vicinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). In our experiments, switching from 5% CO_2 to ambient air for aeration followed a similar procedure, so it is possible that WC-1 synthesized similar carbonic anhydrases and transport proteins. However, through the use of timely bicarbonate addition, we have possibly affected the carbon concentrating process by overloading the cultures with bicarbonate. To our knowledge, no one has studied the effect of excess bicarbonate on the induction of the enzymes involved in carbon concentrating mechanisms. Future work will be needed to clearly understand if carbon concentrating mechanisms are involved in the bicarbonate triggered TAG accumulation processes presented here. Additionally, it has been debated whether diatoms use a C_3- or C_4-like mechanism to concentrate carbon (Reinfelder et al. 2004). The C_4-like mechanism utilizes the direct carboxylation of glycolytic intermediates to oxaloacetate which, after transportation events, can then be decarboxylated to concentrate CO_2 in the vicinity of RUBISCO. While there has been no direct evidence for C_4-like metabolism in *Phaeodactylum*, differences in carbon concentrating mechanisms between the green and diatom algae may be the reason for differences we observed in WC-1 and Pt-1 replicating after bicarbonate addition, but again, further work is needed to identify the specific metabolic process that may occur in different phototrophic microorganisms.

In conclusion, growth and TAG accumulation was measured in *Scenedesmus* sp. strain WC-1 and *P. tricornutum* strain Pt-1 during 14:10 light–dark cycling to further understand how pH and bicarbonate can be coupled with nitrogen depletion to "trigger" TAG accumulation. There was a loss of pH-induced TAG accumulation in the WC-1 cultures during light–dark

cycling due to the pH drop in the dark hours of the cycle. Furthermore, when grown on 5% CO_2 (*v*/*v*) the growth rate doubled, however the culture pH remained low which led to minimal TAG accumulation. Thus, 5% CO_2 was utilized during the exponential phase and aeration was switched to ambient air at or just prior to nitrate depletion. TAG accumulation was not observed presumably due to lower pH during the dark hours.WC-1 cultures given 5% CO_2 during exponential phase switched to ambient air with bicarbonate added (50 mM) at nitrate depletion caused cellular replication to cease and TAG accumulation rates to immediately increase significantly. The bicarbonate addition led to the same level of TAG accumulation as that observed with air grown cells, but in half the time. A similar TAG triggering response was observed when bicarbonate (25 mM) was added to Pt-1 as compared with WC-1; however, the cells continued to replicate unlike WC-1. Bicarbonate addition was tried during stationary phase, after nitrate depletion, for both WC-1 and Pt-1 and a complete loss of accumulated TAG was observed.

The ability of a bicarbonate addition to stop replication in a chlorophyte and not in a diatom suggests significant differences in the metabolic pathways employed by these two algae in response to nutrient balance and resource allocation. Using bicarbonate as a triggering mechanism may potentially improve the commercial use of algae as a biofuel resource; however, further experimentation is needed to better understand the metabolic responses of these organisms to the bicarbonate addition to optimize biofuel production.

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