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Article

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

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Abstract: Microalgae are capable of synthesizing high levels of triacylglycerol (TAG) which can be used as precursor compounds for fuels and specialty chemicals. Algal TAG accumulation typically occurs when cellular cycling is delayed or arrested due to nutrient limitation, an environmental challenge (e.g., pH, light, temperature stress), or by chemical addition. This work is a continuation of previous studies detailing sodium bicarbonate-induced TAG accumulation in the alkaline chlorophyte *Scenedesmus* sp. WC-1. It was found that upon sodium bicarbonate amendment, bicarbonate is the ion responsible for TAG accumulation; a culture amendment of approximately 15 mM bicarbonate was sufficient to arrest the cellular cycle and switch the algal metabolism from high growth to a TAG accumulating state. However, the cultures were limited in dissolved inorganic carbon one day after the amendment, suggesting additional carbon supplementation was necessary. Therefore, additional abiotic and biotic experimentation was performed to evaluate in- and out-gassing of CO_2. Cultures to which 40–50 mM of sodium bicarbonate were added consumed DIC faster than CO_2 could ingas during the light hours and total photosynthetic

oxygen production was elevated as compared to cultures that did not receive supplemental inorganic carbon.

Keywords: algae; bicarbonate; biofuel; triacylglycerol (TAG); Nile Red fluorescence

1. Introduction

The industrialized world's primary infrastructure for transportation fuels and specialty chemical substrates utilizes petroleum based products. However, the basic economic principle of supply and demand has escalated petroleum prices, which is unavoidable for any important non-renewable natural resource. In short, global petroleum is not endless and the world's energy consumption is projected to increase from 524 quadrillion British thermal units (Btu) to 820 quadrillion Btu (a 56% increase) between 2010 and 2040 [1]. Further, society has become more sensitive to atmospheric CO_2 levels due to increased concern about rapid global climate change. Fossil fuels consumed globally contain 400 times the amount of annual carbon which can be fixed by the current global biota. Furthermore, fossil fuel consumption has increased annually from those levels [2]. These numbers are staggering and if current practices and trends continue, traditional energy resources will be diminished much faster than regenerated and environments will be eroded/destroyed in an effort to supply these resources [3]. However, advancement in renewable energy technologies can counteract part of this bleak scenario. Biodiesel, defined as fatty acid methyl esters (FAME) derived from plant, animal, or algal lipids, is an attractive alternative for portions of the current petroleum dependency. Algae have great potential for use in FAME production due to their inherent growth advantages, high TAG content per cell, and the potential to balance carbon levels; natural marine phytoplankton are responsible for 50% of the total global primary productivity [4]. However, as was recently pointed out by the United States National Renewable Energy Laboratory, significant technological advancements that maximize lipid content must be made to improve the near-term industrial feasibility of utilizing microalgae as a biofuel feedstock [5].

Algal TAG accumulation typically occurs when cellular cycling (the process of a parent cell splitting into daughter cells) is retarded or arrested due to a nutrient limitation [6–11], some environmental stress (e.g., pH, light, temperature stress) [7,12–14], or by chemical addition [15–17]. However, this would appear to be counterproductive for industrial algal biofuel systems, which require rapid growth rates and a wide tolerance of environmental conditions (including extreme environments) [18,19]. Therefore, one optimal growth and TAG accumulation scenario for algal biofuel production involves high biomass productivity during the exponential growth phase and sequential arresting of the cell cycle, while still maintaining a high photosynthetic rate, at the onset of the stationary phase. Most production scenarios accomplish this by timing medium nitrogen depletion with a predetermined cellular density, while controlling the pH of the system with CO_2 addition. However, we and others have recently shown that some algal strains accumulate TAG at a higher rate when bicarbonate is added to culturing systems [15,20]. Furthermore, TAG and starch accumulation in *Chlamydomonas reinhardtii* were shown to be more stable when accumulation occurred in a bicarbonate rich system [16], which offers flexibility in the harvest timing. Finally, high bicarbonate

concentrations can minimize contamination from invasive microorganisms [21], which is especially important during longer periods of stress (*i.e.*, TAG accumulation) when cellular integrity may be compromised, and predators and/or bacteria can flourish on energy-rich cell debris.

Carbon assimilation by microalgae, through carbon concentrating mechanisms (CCMs), has been extensively studied [22–28], and it is generally accepted that green algae can assimilate bicarbonate under certain environmental conditions (low CO_2 and alkaline conditions). Of particular note are carbon assimilation studies done on *Scenedesmus* strains [29–31], in which it was argued that bicarbonate is the major carbon source taken up at alkaline pH [30]. However, these studies were performed using bicarbonate concentrations of approximately 10 μM–10 mM, which are significantly less than what was shown to induce TAG accumulation in *Scenedesmus* sp. WC-1 (50 mM HCO_3^-) [15]. Due to this unconventional approach of enhancing TAG accumulation in microalgae, additional physiological research is needed to evaluate cellular responses, in terms of cell cycle inhibition, inorganic carbon utilization, and photosynthetic oxygen production, when high concentrations of bicarbonate are added to cultures.

This work reviews the current state of knowledge of *Scenedesmus* sp. WC-1 when bicarbonate is used to induce TAG accumulation. Results detailing TAG accumulation from sodium bicarbonate and sodium carbonate additions, at varying concentrations, or when combined are presented. The minimum concentration of bicarbonate required to arrest the cell cycle was determined. However, even these cultures were found to be carbon limited after 24 h. Therefore, abiotic and biotic studies were conducted to evaluate CO_2 into a given medium, dissolved inorganic carbon utilization, and photosynthetic oxygen production during high bicarbonate culturing and when *Scenedesmus* sp. WC-1 was in a bicarbonate-induced TAG accumulation state. Taken together, fundamental physiological knowledge of cellular cycling, carbon utilization, and photosynthetic oxygen production in *Scenedesmus* sp. WC-1 during bicarbonate-induced TAG accumulation is further elucidated.

2. Materials and Methods

2.1. Organism, Culturing, and Analysis

Scenedesmus sp. strain WC-1 (WC-1) was isolated from an alkaline (pH 8.7) stream in Yellowstone National Park, USA and confirmed unialgal as previously described [12]. WC-1 was cultured using Bold's basal medium with the pH titrated to 8.7 with KOH prior to autoclaving [32]. Experimental cultures were confirmed axenic by inoculation into medium supplemented with 0.05% glucose and yeast extract followed by dark incubation. Experiments were conducted in biological triplicate batch airlift tubular reactors with aeration, temperature, light cycle (L/D), and light flux being as previously described [15].

Sodium, bicarbonate, and carbonate ion effects were screened by culturing WC-1 on a 5% CO_2 gas sparge for 3.8 days (0.04 mM NO_3^- remaining from 2.94 mM initial concentration), at which time the gas sparge was changed to atmospheric CO_2 (0.04% *v*/*v*) and cultures were amended with 25 mM or 50 mM sodium bicarbonate, 25 mM sodium carbonate, 25 mM both sodium bicarbonate and carbonate, 25 mM sodium bicarbonate with 50 mM N-cyclohexyl-3-aminopropanesulfonic acid buffer

(CAPS, pKa 10.3), or 25 mM sodium carbonate with 50 mM CAPS buffer. TAG accumulation was assessed using Nile Red fluorescence after a total culturing time of 10.8 days.

The minimal concentration of bicarbonate needed to arrest cellular replication was determined by culturing WC-1 on a 5% CO_2 gas sparge for 2.92 days (1.07 mM NO_3^- remaining from 2.94 mM initial concentration), at which time the gas sparge was changed to atmospheric CO_2 (0.04% *v*/*v*) and cultures were amended with 0 mM, 5 mM, 10 mM, and 15 mM sodium bicarbonate or 15 mM sodium chloride. Cell concentration, dissolved inorganic carbon, pH, and Nile Red fluorescence were monitored as described below.

Abiotic and biotic changes in pH and dissolved inorganic carbon during bicarbonate amendment were determined by 40 mM bicarbonate addition (at 2.95 days with 0.85 mM NO_3^- remaining from 2.94 mM initial concentration) to WC-1 or 50 mM bicarbonate addition to medium without algae. In addition, 50 mM N-cyclohexyl-2-aminoethanesulfonic acid buffer (CHES, pKa 9.3) was added to medium without algae, and KOH as needed from a 1 M KOH stock to maintain a pH value around 9.2. Dissolved inorganic carbon and pH were monitored as described below.

To assess changes in photosynthetically derived dissolved oxygen concentrations during bicarbonate amendment, cultures of WC-1 were grown on a 5% CO_2 gas sparge for 3.8 days (0.03 mM NO_3^- remaining from 2.94 mM initial concentration), at which time the gas sparge was changed to atmospheric CO_2 (0.04% *v*/*v*) and cultures were amended with 50 mM sodium bicarbonate. Dissolved oxygen and pH were monitored as described below.

Algal concentrations were determined using an optical hemacytometer and no less than 400 cells were counted for statistical reliability. Medium pH was measured on culture samples using a standard bench top pH meter. Medium nitrate concentrations were measured by ion chromatography using an IonPac AS9-HC anion-exchange column (Dionex, Sunnyvale, CA, USA) with a 9.0 mM sodium carbonate solution set at a flow rate of 1.0 mL min^{-1}. Detection was performed using a CD20 conductivity detector (Dionex) at 21 °C and samples were quantified by peak area correlation to a calibrated standard curve. Medium phosphate and sulfate were checked, using the ion chromatography protocol previously described, at the end of the experiments to confirm that replete culturing conditions existed throughout the experiments.

2.2. Triacylglycerol Analysis

Algal TAG accumulation was estimated using the Nile Red (9-diethylamino-5H-benzo(α)phenoxazine-5-one) (Sigma-Aldrich, St. Louis, MO, USA) fluorescence method [33]. Briefly, 1 mL culture aliquots were assayed directly with Nile Red (4 µL from 250 µg/mL in acetone) or by diluting with 4 mL ultrapure H_2O before assaying directly with Nile Red (20 µL from 250 µg/mL in acetone). To maintain linearity of the Nile Red assay, dilution was required when population counts exceeded 10^7 cell mL^{-1}. Total Nile Red fluorescence was quantified on a microplate reader (Bio-Tek, Winooski, VT, USA) utilizing 480/580 nm excitation/emission filters with a baseline sensitivity set at 75 to maximize signal-to-noise ratio and accommodate fluorescent changes over 10,000 arbitrary units (A.u.). The Nile Red fluorescence method has become a standard method to evaluate TAG in both academia and industry [11,33–38] and has previously been confirmed by gas chromatography to correlate with intracellular TAG concentrations in WC-1 (R^2 = 0.95) [12].

2.3. Dissolved Inorganic Carbon and Dissolved Oxygen Analysis

Medium dissolved inorganic carbon (DIC, sum of CO_2, HCO_3^-, and CO_3^{2-}), was measured using previously described protocols [16]. Briefly, 8 mL of culture was filtered (0.2 µm pore size) and the supernatant solution was analyzed with a Skalar Formacs TOC/TN analyzer (using a Skalar LAS-160 autosampler, Skalar, Breda, The Netherlands). DIC quantification was conducted using peak area correlation to a standard curve from bicarbonate and carbonate mixture standards (Sigma-Aldrich).

Dissolved oxygen (DO) and pH were monitored continuously using a Sartorius Biostat B Plus. The pH and DO (both Sartorius, Bohemia, NY, USA) electrodes were fitted into the top of the tubular airlift reactors prior to autoclaving. A 2-point DO calibration was conducted by sparging the airlift reactors with nitrogen and a pure oxygen gas prior to culture inoculation.

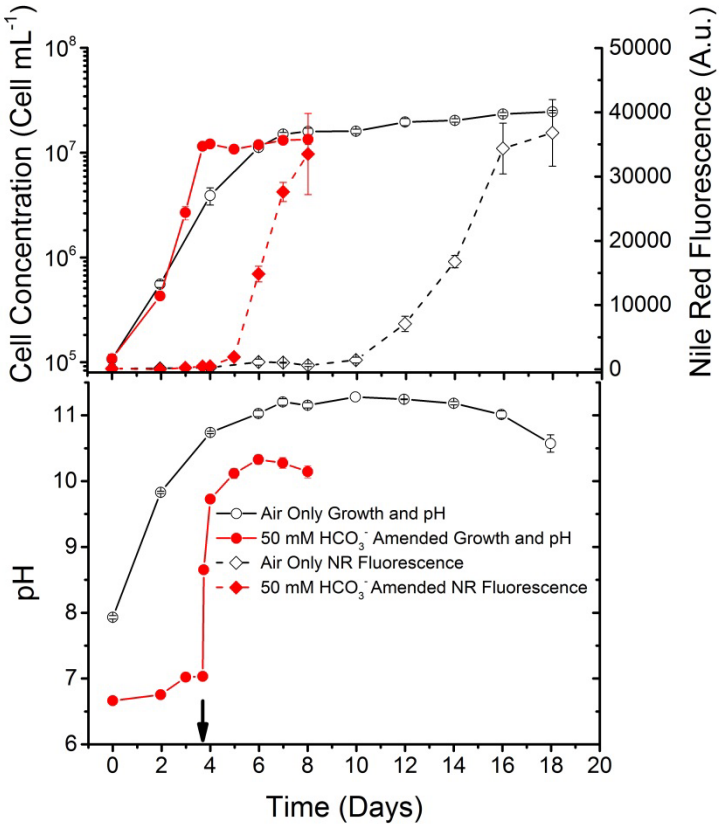
3. Results and Discussion

Previous research utilizing WC-1 evaluated the effects of pH, N-stress, combination of pH and N-stress, high CO_2 (5% *v*/*v*), and the addition of 50 mM sodium bicarbonate on the algal cellular cycle and on TAG accumulation [12,15]. Figure 1, adapted from previously published work [15], summarizes the use of elevated CO_2 and sodium bicarbonate to increase WC-1 growth rate and "trigger" TAG accumulation, as compared to cultures that only received inorganic carbon from an atmospheric air sparge. The main points to consider from this work are the following:

(1) Cultures which received elevated CO_2 became nitrate deplete at approximately 3.7 days as compared to the cultures sparged with air, which became deplete at approximately 9 days;
(2) After the gas sparge was switched to atmospheric air and 50 mM sodium bicarbonate was added, there was a cessation of the cellular cycle and an increase in the TAG accumulation rate which resulted in a total culturing time of 8 days, to reach equivalent TAG accumulation, as compared to 16 days required for the other cultures. It should be noted that cultures which received elevated CO_2 (5% *v*/*v*) after nitrate became depleted reached a fluorescence level of only 3200 A.u. (data from [15]);
(3) The added sodium bicarbonate buffered the cultures so that the pH was approximately one pH unit below the cultures which were only grown on air. This is important because the pKa for bicarbonate-carbonate equilibrium is pH 10.3 and at pH 11.3 the majority of the inorganic carbon in the system is carbonate, which is potentially unavailable for carbon fixation.

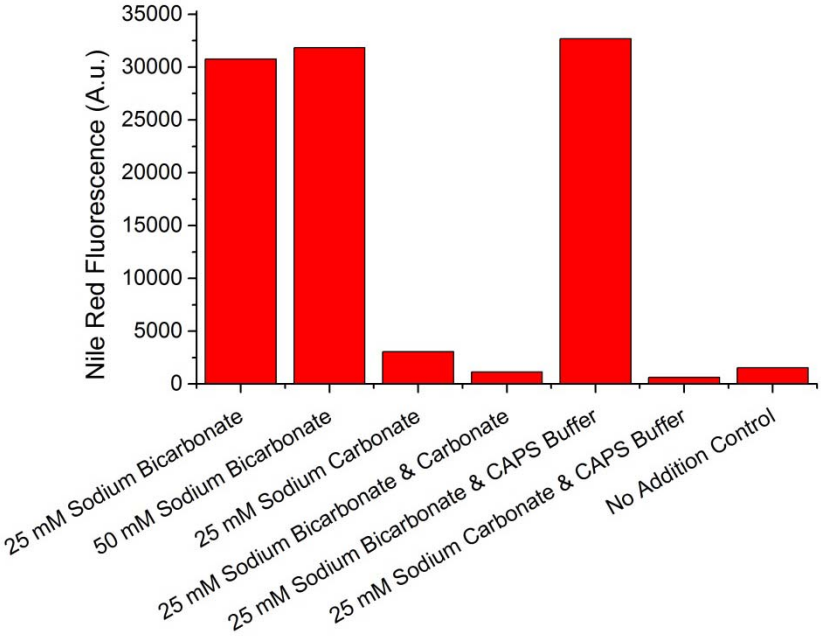
Thus, the use of sodium bicarbonate as an alternative inorganic carbon source and a TAG accumulation "trigger" has the potential to aid industrial algal biofuel production. However, further fundamental investigation is warranted to determine which ion (sodium, bicarbonate, or carbonate) is responsible for the algal metabolic changes. In addition, the minimal amount of sodium bicarbonate needed to arrest cellular replication and induce TAG accumulation must be identified. Finally, inorganic carbon use and photosynthetic properties associated with elevated sodium bicarbonate use must be investigated.

Figure 1. *Scenedesmus* sp. WC-1 mean and standard deviation of cellular concentration, total Nile Red fluorescence, and pH of cultures aerated with ambient air and cultures which were sparged with 5% CO_2 (*v*/*v*, in air) until near nitrate depletion, at which time they were amended with 50 mM sodium bicarbonate and switched to ambient air. Arrow indicates time of the gas sparge change and bicarbonate addition. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (*n* = 3). Figure adapted from [15].



To assess which ion was responsible for the cessation of the cell cycle and subsequent TAG accumulation, WC-1 was screened for TAG accumulation when sodium bicarbonate and sodium carbonate were added individually or in combination, and when combined with 50 mM CAPS buffer (pKa 10.3). CAPS buffer was added in an effort to minimize changes of the culture pH. All additions resulted in a cessation of the cellular cycle, and Nile Red fluorescence values (*i.e.*, TAG accumulation) taken at the end of the experiments are shown in Figure 2. All cultures which received bicarbonate or bicarbonate in addition to the CAPS buffer accumulated TAG. All cultures which received carbonate did not accumulate TAG, either when added individually or in combination with bicarbonate or CAPS buffer. The CAPS buffer was able to maintain the pH within a difference of 0.5 units between the cultures that received bicarbonate and carbonate, thus ion speciation differences were minimized. Comparison of the 25 mM sodium carbonate and the 50 mM sodium bicarbonate offers evidence that that bicarbonate ion is responsible for TAG accumulation and not the sodium ion. Sodium was present in equimolar concentration in these two treatments but only the bicarbonate system accumulated TAG. Additional evidence that bicarbonate, and not sodium, was influencing TAG accumulation is discussed below.

Figure 2. Nile Red fluorescence responses of *Scenedesmus* sp. WC-1 cultures that were amended with sodium bicarbonate, sodium carbonate, and combinations of the two with and without 50 mM CAPS buffer (pKa 10.3) (values are for the conclusion of the experiments). Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle.



To begin optimizing algal systems for bicarbonate-induced TAG accumulation, the minimum amount of bicarbonate needed to change the cellular metabolism from high growth to fast TAG accumulation, must be identified. Figure 3 shows the growth characteristics of WC-1 when 0 mM, 5 mM, 10 mM, and 15 mM bicarbonate were added to WC-1 cultures. Additionally, the effect of sodium chloride addition was demonstrated to further verify the sodium ion is not responsible for the cessation of the cell cycle. All cultures were sparged with 5% CO_2 until near medium nitrate depletion, at which time the sparge was changed to atmospheric air and the salts were added. Cultures that received less than 10 mM bicarbonate and 15 mM sodium chloride continued to divide, producing new cells. The cultures that received 10 mM bicarbonate showed a delayed cell cycle, and the cultures that received 15 mM bicarbonate exhibited an arrested cell cycle.

Nile Red fluorescence was monitored over the course of the experiment as it is a rapid indication of TAG accumulation in WC-1 [12]. Figure 4 shows the specific Nile Red fluorescence of WC-1 when 0 mM, 5 mM, 10 mM, and 15 mM bicarbonate, or 15 mM sodium chloride, were added to the cultures. Specific Nile Red fluorescence was calculated based on Equation (1), and represents the total Nile Red fluorescence normalized to the cell number. This number allows comparison of the average TAG content of each algal cell, and a large number represents a high TAG content:

$$sNR = 10{,}000 * NR * N_c^{-1} \quad (1)$$

where *sNR* is the specific Nile Red (A.u. cell^{-1}), *NR* is the total Nile Red signal of the algal culture (A.u. mL^{-1}), and N_C is cellular concentration (cell mL^{-1}). An arbitrary scaling factor of 10,000 has previously been used to shift results to 0–100 [12,15,16].

Figure 3. *Scenedesmus* sp. WC-1 mean growth and standard deviation of cultures which were sparged with 5% CO_2 (*v*/*v*) in air and switched to ambient air, at which time they were amended with 0, 5, 10, and 15 mM sodium bicarbonate, along with 15 mM sodium chloride as a control amendment. The arrow indicates time of the gas sparge change and salt amendments. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (*n* = 3).

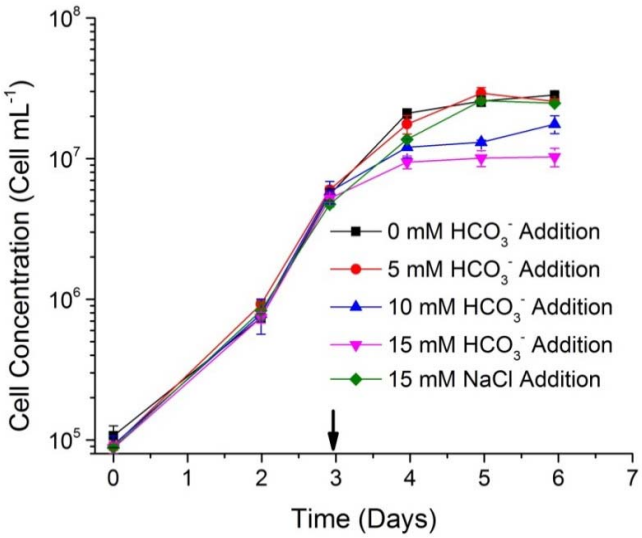
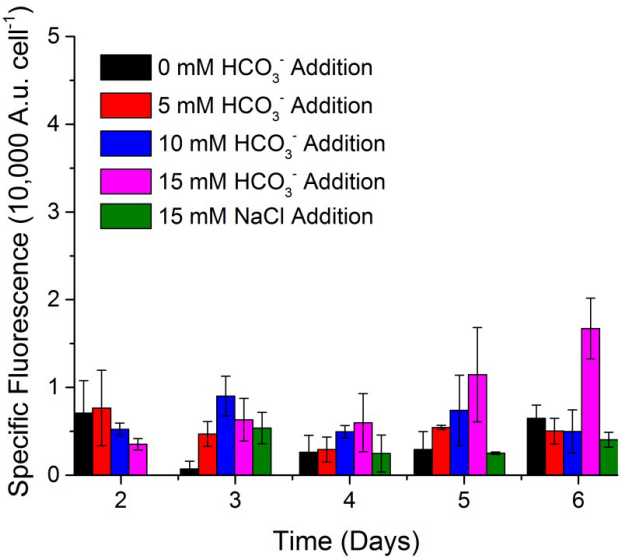


Figure 4. Mean and standard deviation of *Scenedesmus* sp. WC-1 specific Nile Red fluorescence in cultures which were sparged with 5% CO_2 (*v*/*v*) in air and switched to ambient air, at which time they were amended with 0, 5, 10, and 15 mM sodium bicarbonate, along with 15 mM sodium chloride as a control amendment. The gas sparge was changed and salt amendments were added at 3 days. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (*n* = 3).

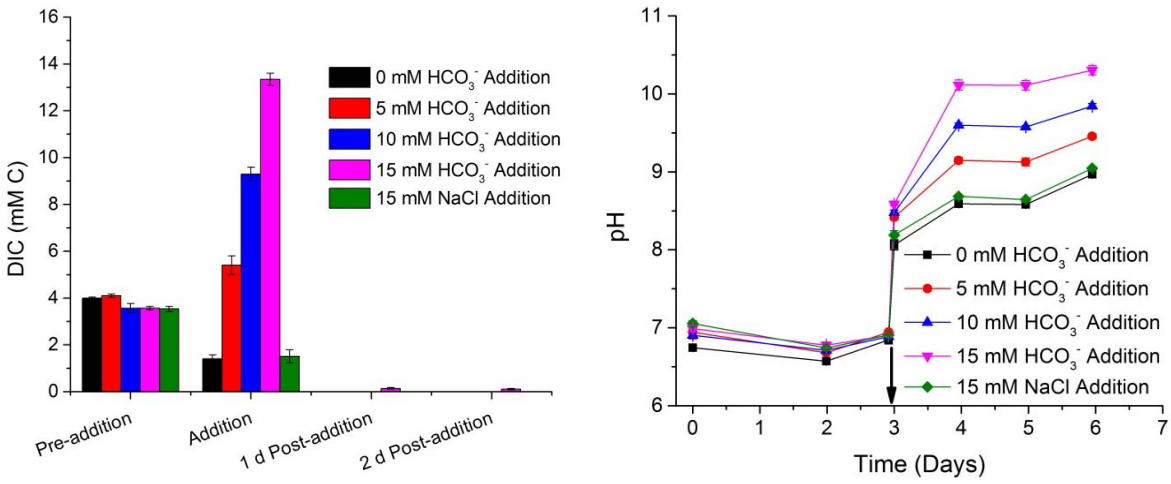


As shown in Figure 4, only the cultures which received a 15 mM bicarbonate addition began to accumulate TAG (6 days) after the gas sparge was shifted from 5% CO_2 to air and the addition of bicarbonate at 3 days. However, this addition resulted only in a specific Nile Red fluorescence of

1.7 ± 0.4 A.u. cell^{-1} which is far below the previously observed specific Nile Red result of 24.8 ± 2.2 A.u. cell^{-1} (error is one standard deviation, $n = 3$) when 50 mM bicarbonate was used to induce TAG accumulation (with identical culturing conditions) [15]. This suggests that while 15 mM bicarbonate was sufficient to stop the algal cell cycle, it was not sufficient to maximize TAG accumulation during the arrested cell cycle.

To advance our understanding of the total dissolved inorganic carbon (DIC, sum of the dissolved CO_2, bicarbonate, and carbonate) was monitored before the gas sparge change and bicarbonate additions, as well as two days post addition. As shown in Figure 5, prior to the gas sparge change, when all cultures were receiving 5% CO_2, the DIC was 3.75 ± 0.3 mM as carbon in all the cultures. Directly after the gas sparge change to atmospheric CO_2 concentrations and the bicarbonate additions, the 0 mM, 5 mM, 10 mM, and 15 mM bicarbonate additions had 1.4, 5.4, 9.3, and 13.3 mM C, respectively. The 15 mM sodium chloride addition had 1.5 mM C and can be compared to the 0 mM bicarbonate experiment as the addition of sodium chloride did not add any extra inorganic carbon. Due to the rapid sampling directly after the gas sparge change (<3 min), the 0 mM bicarbonate additions and the 15 mM sodium chloride additions still had CO_2 remaining from the 5% CO_2 sparge. It is expected that the extra CO_2 did not remain in these systems for long after the measurements at the time of gas change and salt additions. The most striking finding is that the DIC in all the systems was completely utilized 1 day after the additions; effectively demonstrating the rapid carbon utilization and consumption capacity of this microalga.

Figure 5. Mean and standard deviation of DIC (**a**) and culture pH (**b**) for *Scenedesmus* WC-1 cultures which were sparged with 5% CO_2 in air and then switched to ambient air, at which time they were amended with 0, 5, 10, and 15 mM sodium bicarbonate, along with 15 mM sodium chloride as a control amendment. Arrow indicates time of the gas sparge change and salt amendments. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle ($n = 3$).



(**a**) (**b**)

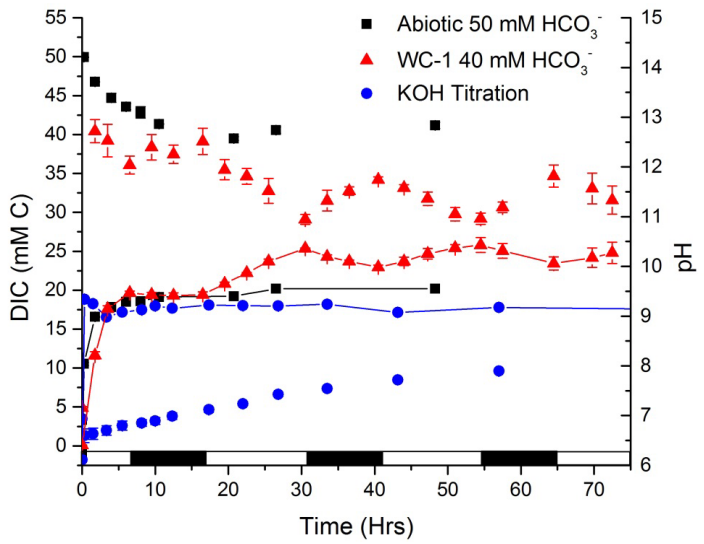
In addition to the DIC information shown on Figure 5, the culture pH was monitored over the course of the experiments (Figure 5b). Before the gas change and salt additions, the pH remained at approximately pH 7 due to the acidification of the culture medium by the 5% CO_2. At the time of gas

change and salt addition, there was a rapid increase in culture pH from the bicarbonate additions and the off gassing of CO_2. After which, and over the next 24 h (10:14 D/L cycle beginning at 3 days and ending at 4 days), the pH increased and was correlated with the bicarbonate addition. This was due to increased carbon utilization and hence increased photosynthetic activity for each respective bicarbonate load. However, after 4 days the pH stabilized due to the establishment of a steady-state between CO_2 supply (ingassing into the culture) and CO_2 consumption by the algal cells. It should be noted that during the dark hours CO_2 was net ingassing from the atmospheric air being supplied to the systems, and that in-gassing CO_2 will cause a decrease in culture pH; thus providing DIC for light dependent carbon fixation (*i.e.*, Calvin-Benson cycle).

When culturing on a diel cycle (L/D cycle), the addition of bicarbonate to induce TAG accumulation causes immediate changes in cell cycling, culture pH, buffering capacity, and the external DIC available to the alga for carbon fixation. While most of these parameters readily change throughout a typical algal culturing experiment (e.g., photosynthetically driven pH increase over time or external DIC decrease in availability as cultures become increasingly dense), the addition of bicarbonate causes rapid changes and complicates equilibrium prediction. Therefore, additional experimentation was pursued to elucidate CO_2 in- and out-gassing kinetics, culture pH buffering, and photosynthetic oxygen production when excess bicarbonate (over that needed to arrest cellular cycling) was added to induce TAG accumulation. Figure 6 shows DIC and pH measurements over time for two abiotic experiments, one to monitor offgassing of CO_2 during bicarbonate addition and the other to monitor DIC ingassing during pH titration, and these are compared to a bicarbonate addition scenario in WC-1 cultures. An abiotic addition of 50 mM bicarbonate changed the medium pH from pH 6.8 to 8.3 which corresponds to almost 100% of the DIC being present as bicarbonate. Over the next 10 h, 10 mM C offgassed as CO_2 and the medium pH increased to pH 9.3. Conversely, when 50 mM CHES buffer and KOH were added to maintain the pH (KOH was added as needed) at approximately pH 9.3, carbon ingassed over 60 h to an equilibrium concentration of 11 mM C. Thus, there was approximately 30 mM more carbon in the system that received the bicarbonate amendment; also, equilibrium was reached in 10 h compared to 60 h for the pH titrated system.

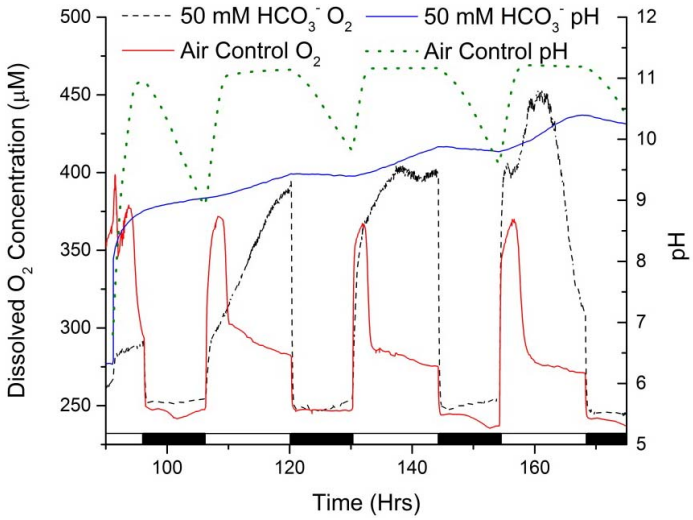
In abiotic experiments equilibrium conditions are easily predicted; however, once a photosynthetic alga (*i.e.*, a sink term) is introduced to the system, a steady state condition is often approached, rather than the system reaching a state of equilibrium. Steady state conditions are difficult to predict, and may be significantly different from equilibrium, which is often assumed incorrectly. To visualize DIC and pH changes in a biotic system, 40 mM bicarbonate was added to WC-1 that was cultured on a 14:10 L/D cycle (Figure 6). Fifty mM C was targeted for direct comparison to the abiotic system that was amended with bicarbonate. While this concentration was missed, similar trends in DIC in- or out-gassing and pH values were still observed. The amendment was made with 6.5 h remaining in the light cycle and there was a decrease of approximately 5 mM C due to offgassing of CO_2 and/or inorganic carbon fixation.

Figure 6. Bold's basal medium DIC (symbol only) and pH (symbol with line) when 50 mM $NaHCO_3$ was added, when 50 mM CHES buffer (pKa 9.3) and KOH was added to titrate the pH to 9.3 (abiotic experiments, n = 1), or mean and standard deviation when 40 mM $NaHCO_3$ was added to cultures of *Scenedesmus* WC-1 to induce TAG accumulation (biotic experiments, n = 3). Time axis begins at onset of abiotic experimentation or time of the gas sparge change and bicarbonate addition for the biotic experiment that was illuminated with a 14:10 L/D cycle (bottom bar).



The pH of the cultures increased to pH 9.4. During the following 10 h dark cycle (starting at 6.5 h), the pH and DIC concentration remained statistically unchanged at 9.4 and 37 mM C, respectively. After the initial dark cycle there was a clear trend during the remaining light and dark cycles (beginning at 16.5 h) where the DIC concentration decreased during the light periods and then increased during the dark hours. Conversely, the pH in the system increased during the light hours and decreased in the dark. These trends indicate that during the light hours, DIC was consumed by the alga at a faster rate than CO_2 could ingas, otherwise the carbon concentration would be dictated by carbon dioxide and bicarbonate equilibrium and there would not have been an increase in DIC during the dark hours as the system tried to regain equilibrium DIC concentrations (with no photosynthesis taking place). Additionally, the DIC increased at a rate of 0.19 mM C h^{-1} and 0.49 mM C h^{-1} for the abiotic pH titrated system and for the cultures of WC-1 during the dark, respectively. The increased DIC ingassing rate of the biotic system can be explained by the increased alkalinity from the bicarbonate addition and the higher pH due to photosynthesis; however, it must be noted that respiration and other CO_2 producing metabolic processes may have increased the DIC concentration in the dark. Although, this would consume significant amounts of oxygen and that was not observed in similar experiments on WC-1 where dissolved oxygen concentrations were monitored over L/D cycles (Figure 7 and discussed below).

Figure 7. Dissolved oxygen (DO) concentration and medium pH of *Scenedesmus* WC-1 cultures which were sparged with 5% CO_2 (*v*/*v*) in air until near nitrate depletion, at which time (92 h) they were switched to ambient air and amended with 50 mM sodium bicarbonate. Comparison is made to cultures that did not receive the bicarbonate amendment. Growth was maintained in unbuffered Bold's basal medium illuminated with a 14:10 L/D cycle (bottom bar) (representative individual pH and DO profiles for triplicate culturing, $n = 3$).



To further evaluate carbon fixation, photosynthetic oxygen production, and oxygenic respiration during the dark hours when bicarbonate is used to induce TAG accumulation in WC-1, experiments were conducted with data logging dissolved oxygen (DO) and pH probes. Figure 7 shows representative pH and DO profiles for WC-1 cultures that were grown on 5% CO_2 until near nitrate depletion (92 h of culturing), at which time the gas sparge was switched to atmospheric air and half of the cultures were amended with 50 mM sodium bicarbonate. Directly after the gas sparge shift, the cultures to which bicarbonate was added increased to pH 8.3, and these cultures continued to increase in pH until the end of the light cycle (pH 9.2). The cultures that did not receive a bicarbonate amendment increased from pH 6.8 to pH 11 at the conclusion of the light cycle. After which, there was periodic decrease and sequential increase of the pH correlated directly to the light/dark cycle. The pH oscillated between pH 9.0 and pH 11.2 for the remainder of the experiment. The cultures to which bicarbonate was added also exhibited an increase in pH during the light hours and a sequential decrease during the dark hours, but the pH oscillation was significantly dampened due to the buffering capacity of the bicarbonate. The pH profile for the cultures to which 50 mM bicarbonate was added is nearly identical to the pH trend observed when 40 mM bicarbonate was added and the DIC was monitored over time (Figure 6).

Most interesting (Figure 7) are the trends in the DO and the difference between cultures amended with bicarbonate and those that were not amended. The cultures which did not receive bicarbonate initially had a higher photosynthetic oxygen production rate. However, during the start of each of the following light cycles, there was a short period of high oxygen production for approximately 4 h, after which the oxygen production rate drastically decreased. This correlates potentially with two factors: (1) the pH in these systems approaching pH 11 which effectively drives the DIC equilibrium towards carbonate, which minimizes carbon bioavailablity for carbon fixation; and (2) complete consumption

of available DIC which had accrued during the dark as the system ingassed CO_2. The decreased DO concentration after 4 h, in each light cycle, was caused by carbon limitation; which in turn limited photosynthesis and hence DO production. The cultures to which bicarbonate was added increased in oxygen production over each sequential light cycle following the initial amendment. This could be due to increased biomass concentration (as opposed to cell number), or a higher chlorophyll per cell content; however, it must be noted that WC-1 did not increase in cell concentration after the bicarbonate amendment (Figure 1). Another possible explanation is the onset of the operation of carbon concentrating mechanisms (CCMs) which employ carbonic anhydrase and bicarbonate transport proteins to effectively shuttle bicarbonate to the ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) enzyme located in the pyrenoid of the chloroplast [24–27]. Besides the fundamental importance of increasing the carbon flux into microalgae, many studies have been done on the induction of algae's CCM by shifting from high CO_2 (typically 1%–5% *v*/*v*) to atmospheric levels (0.04%) and they report 2–10 light hours are needed to synthesize the metabolic machinery necessary to concentrate inorganic carbon [29–31,39–43]. Further experimentation is needed to elucidate definitively the observed increase in photosynthetic oxygen production over time in the cultures which received additional bicarbonate. However, it should be noted that the total amount of photosynthetically-driven oxygen production was much greater for the cultures which received bicarbonate as compared to those that did not receive an amendment, and it is these cultures that accumulated more TAG. As a final observation, in the bicarbonate amended cultures there was no noticeable increase in DO consumption during the dark hours, which would be indicative of increased respiration and CO_2 production. This argues that the increase in DIC observed in the cultures of WC-1 during the dark hours was due to CO_2 ingassing and not from accelerated respiration (Figure 6).

The use of sodium bicarbonate to enhance TAG accumulation has been demonstrated in both freshwater and marine chlorophytes as well as diatoms [15,16,20,44,45], and its use offers inherent advantages. Gaseous CO_2 is costly to store and transport, whereas bicarbonate salts can easily be transported to algal facilities and stored until needed. Further, gas sparging systems are inefficient at delivering CO_2 into the culture medium in shallow raceways or ponds, with the majority of the CO_2 off-gassing into the atmosphere [46]. However, at elevated pH, loss of inorganic carbon is drastically reduced if inorganic carbon is added. Additionally, with increasing concerns about greenhouse gases, algal systems are being proposed to sequester CO_2 from coal-fired power plants and other CO_2 point sources. If the emitted CO_2 is directly transferred to an algal growth facility, it may have to be stored during the night requiring extra capital cost for compressors and storage tanks. An alternative approach would be to temporarily sequester the CO_2 as bicarbonate which has the aforementioned storage and transport advantages [47]. However, use of bicarbonate in this manner is new; thus further fundamental understanding and optimization is needed to determine effects on algal physiology, and evaluate cost comparisons.

4. Conclusions

Assimilation of bicarbonate by microalgae at concentrations similar to that in seawater has historically been well studied. However, the effects of high concentrations of bicarbonate on algal physiology and TAG accumulation have only recently been considered. A culture amended with high

concentrations of bicarbonate near N-depletion in the growth medium caused a cessation of this green alga's cellular cycle and induced enhanced TAG accumulation. This technique to "trigger" TAG accumulation increased the DIC concentration of the medium and buffered the culture in a pH range where bicarbonate was bioavailable for carbon fixation.

This study advances the fundamental physiological knowledge of bicarbonate-induced TAG accumulation in the alkaline strain WC-1 by screening bicarbonate and carbonate sodium salts to identify the ion responsible for inducing TAG accumulation. The minimum amount of bicarbonate needed to arrest the cell cycle was determined. It was demonstrated that additional carbon is beneficial (above the minimal concentration to arrest cellular cycling) to enhance TAG accumulation, and the effects of high concentrations of bicarbonate on DIC assimilation and photosynthetic oxygen production were examined. It was found that the bicarbonate ion was responsible, directly or indirectly, for induced TAG accumulation. It was also found that a 15 mM bicarbonate addition was sufficient to arrest the cell cycle in WC-1; however, TAG accumulation was significantly lower than previously observed. This was attributed to cultures of WC-1 becoming limited for inorganic carbon within 24 h after a bicarbonate amendment of 15 mM and additional carbon is needed for timely TAG accumulation, as has been observed with 25 mM and 50 mM bicarbonate amendments. Therefore, elevated bicarbonate amendments, over the minimum amount need to arrest the cell cycle, were used to monitor DIC consumption and photosynthetically driven oxygen production. DIC was biologically consumed faster than CO_2 could ingas and the photosynthetically driven increase in pH, along with the pH decrease from ingassing CO_2 during the dark hours, was buffered due to the bicarbonate-carbonate buffering properties. It is speculated that the buffering characteristics of these amended cultures contributed to total TAG accumulation by minimizing TAG consumption during the dark hours, as TAG accumulation is the net result of TAG synthesis minus utilization. Furthermore, during the light hours after amendment, the total amount of photosynthetic activity (assessed via oxygen production) was much greater for the cultures which received bicarbonate than those that did not receive an inorganic carbon amendment. It was these cultures that were induced for TAG accumulation.

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Conflicts of Interest

While this work does not directly address the bicarbonate trigger itself, in the interest of full disclosure on related work and a potential perceived COI, a patent has been filed by Montana State

University entitled, "Bicarbonate Trigger for Inducing Lipid Accumulation in Algal Systems," with inventors Keith E. Cooksey, Robert D. Gardner, Brent M. Peyton, *PCT/US Patent Application* 2011/053238, *Publication Number* WO2012040698 A3, Filed 26 September 2011.

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