# GROWTH RATE OF MARINE MICROALGAL SPECIES USING SODIUM BICARBONATE FOR BIOFUELS

A Thesis

by

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MASTER OF SCIENCE

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# ABSTRACT

With additional research on species characteristics and continued work towards cost effective production methods, algae are viewed as a possible alternative biofuel crop to current feedstocks such as corn. Current open pond production methods involve bubbling carbon dioxide ($CO_2$) gas into the media to provide a carbon source for photosynthesis, but this can be very inefficient releasing most $CO_2$ back into the atmosphere. This research began by investigating the effect of sodium bicarbonate ($NaHCO_3$) in the growth media as an alternative carbon source to bubbling $CO_2$ into the cultures. The second part examined if $NaHCO_3$ could act as a lipid trigger in higher (10.0 g/L) concentrations

The microalgae species *Dunaliella tertiolecta* (Chlorophyta), *Mayamaea spp*. (Baciallariophyta) and *Synechoccocus sp*. (Cyanophyta) were grown with 0.0 g/L, 0.5g/L, 1.0 g/L, 2.0 g/L and 5.0 g/L dissolved NaHCO3 in modified seawater (f/2) media. To investigate effects of $NaHCO_3$ on lipid accumulation, growth media cultures were divided into two "lipid phase" medias containing either 0.0g/L (non-boosted) or 10.0 g/L (boosted) NaHCO3 treatments. Culture densities were determined using spectrophotometry, which showed both all three species are able to successfully grow in media ameliorated with these high $NaHCO_3$ concentrations.

Highest growth phase culture densities occurred in $NaHCO_3$ concentrations of 2.0 g/L for *D. tertiolecta* and *Mayamaea spp*., and the 5.0 g/L treatment for *Synechoccocus sp*. Highest growth rates occurred in the 5.0 g/L $NaHCO_3$ concentration treatments for *D. tertiolecta*, *Mayamaea spp*., and *Synechoccocus sp*. (0.205 d-1 ±0.010, 0.119 d-1 ±0.004, and 0.372 d-1 ±0.003 respectively). As a lipid accumulation trigger two of the three species (*D. tertiolecta* and *Mayamaea spp*) had their highest end day oil indices in a 10.0 g/L treatment. Highest oil indices occurred in boosted 5.0 g/L Dunaliella tertiolecta and 2.0 g/L Mayamaea spp. (13136 ± 895 and 62844 ± 8080 respectively (relative units)). The results obtained indicate $NaHCO_3$ could be used as a

photosynthetic carbon source for growth in all three species and a lipid trigger for D. tertiolecta and Mayamaea spp.

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Thanks also go to my friends and colleagues in the Phytoplankton Dynamics Laboratory at Texas A&M at Galveston. You girls made me feel welcome there like a second family away from home and always helped if there was anything I ever needed. In particular from the lab I want to single out Marcella Nunez and Yuelu Jiang for all their extra help from the beginning of the project all the way to the end. To Keaton Adams and Taylor Budde, the two volunteers who helped towards the end, thank you both for the extra time you put in and spending hours doing tedious tasks with me. Also thank you to the MARB department faculty and staff for making time in the office and the courses I took at Texas A&M University at Galveston a great experience. There is also a special thanks for Louis and Jola Brown from Texas Agrilife Research for not only providing me hands on experience before starting this research, but continuing to offer advice and help throughout it.

Finally, thanks to my parents and family for encouraging me and being there when I needed, especially during stressful moments. Especially my fiancé Jess, who put up with a year and a half of being in a distance relationship with me, and my future mother in law for providing many pick up and drop offs trips to the airport for us.

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# INTRODUCTION

## Algae for Biofuel

Rising gas costs, uncertainty of future petroleum supply, and pollution concerns are leading a push for countries and private industry to develop alternatives to fossil fuels. First generation oil crops are limited due to their impact on food production, farmland requirements, and insufficient oil production to be a significant replacement (Escobar et al., 2009; Brennan & Owende, 2010). Algae are seen as one of the better replacement candidates because they avoid many of those problems. For the United States to even meet half of its oil usage just for transportation, higher yielding crops like oil palm would require approximately 24% of its existing farmland, but high oil content algal strains could use as little as 2% (Chisti, 2007). Algae do not even require the use of farmland though, so they can be grown on non-arable lands to not displace food crops. It is estimated that the United States could surpass the Department of Energy goal of having 30% of fuel come from biofuel sources by 2030 by a factor of 2.56, using only algae grown on undesirable, undeveloped land that is already available and could environmentally support industry sized growth (Quinn et al., 2012).

Using algae as a biofuel feedstock is not a new concept. Originally thought of in the 1950s (U.S. DOE, 2010), interest in algal biofuels dropped after the Department of Energy's Aquatic Species Program concluded that it was cheaper, at the time, to continue using crude oil at $20-$40 per barrel instead of providing additional funding to reach algae's estimated $40-$60 (Pienkos & Darzins, 2009). With crude oil now constantly well above $100 per barrel, algae has received renewed interest from governments and private industries to help restart research (Amer et al., 2011).

With algae under focus as a replacement for first generation oil crops and crude oil, the most important goal now is making it more economically competitive. Some improvements can target the initial stages of production that focus on growth and lipid accumulation. This can be achieved through reducing costs (e.g., growth media

materials) or developing new methods for growth that increase process efficiency, biomass, or end lipid content. One area that needs more investigating is the growth rate of marine algal species using sodium bicarbonate ($NaHCO_3$) versus gaseous $CO_2$ as the carbon source, as well as determining the effect on lipid content, types, and accumulation.

**Microalgae**

Microalgae are a diverse group of small (μm-mm sized), individual photosynthetic organisms that are able to increase quickly in biomass (Hundt & Reddy, 2011). Besides needing water and light (for photosynthesis) to grow, algae also need macro and micronutrients. Macronutrients like nitrogen and phosphorous need to be provided in large quantities as they are major requirements for growth while micronutrients like copper, iron, zinc, and vitamins need only be provided in trace amounts (Carolina, 2012). Typically most species can double their biomass every day, but some species can double every three and a half hours during their exponential growth phase (Chisti, 2007). Besides rapid growth they produce significant quantities of oil which is the required component for making biofuel. Under normal growth conditions, species on average have about a quarter of their dried biomass weight composed of lipids or triacylglycerols, but during stressed conditions when growth stops the total oil content can double in some species (Hu et al., 2008). Algal production of triacylglycerols (Gardner et al., 2012) is particularly important because it can be refined to higher energy products like biodiesel and jet fuel (Hu et al., 2008). While total lipid composition is species dependent, the large species diversity allows for producers to select strains that are most beneficial to their particular needs.

It is not uncommon for marine algal species to be able to utilize $NaHCO_3$ as a carbon source (Larsson & Axelsson, 1999). While the uptake and use of $NaHCO_3$ has been well studied, there has not been as much focus on how it effects growth and lipid formation specifically. Devgoswami et al. (2011) did examine growth rate, but only under a maximum concentration of 75mg/L and there was not a specific goal to focus on marine species. One of the more recent studies did look at two marine species and found

that using $NaHCO_3$ can lead to successful grouth and lipid formation in nitrigen depleted cultures (White et al., 2013). For commercial scale biofuel production especially, finding additional suitable marine species is important to aviod excess consumption of freshwater resources as well as looking at new lipid formation triggers.

**Using $NaHCO_3$***

Many algae are able to bring $CO_2$ and $HCO_3^-$ (bicarbonate anion) across their cell membrane to be used as the carbon source for photosynthesis (Figure 1) (Chi et al., 2011). While aqueous $CO_2$ can be directly taken up by the cells, it also gets dissolved into carbonic acid ($H_2CO_3$) [1]. Sodium bicarbonate and water also react together giving $H_2CO_3$ as part of the product [2]. The $H_2CO_3$ further breaks down into $HCO_3^-$ which is the other transportable carbon form [3]. Additionally, $HCO_3^-$ is a preferred carbon source because it is what is utilized to start the algal photosynthesis process. Any $CO_2$ brought into the cell gets held as $HCO_3^-$, and those $HCO_3^-$ molecules are what get converted back to $CO_2$ [4] to be used by rubisco during photosynthesis. With increased growth as a goal, it could be better to use $NaHCO_3$ because it keeps $HCO_3^-$ readily available in the media.

[1] Carbon dioxide and water reaction: $CO_2 + H_2O \rightarrow H_2CO_3$

[2] Sodium bicarbonate and water reaction: $NaHCO_3 + H_2O \rightarrow H_2CO_3 + OH^- + Na^+$

[3] Carbonic acid reaction: $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$

[4] Conversion back to useable $CO_2$: $HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$

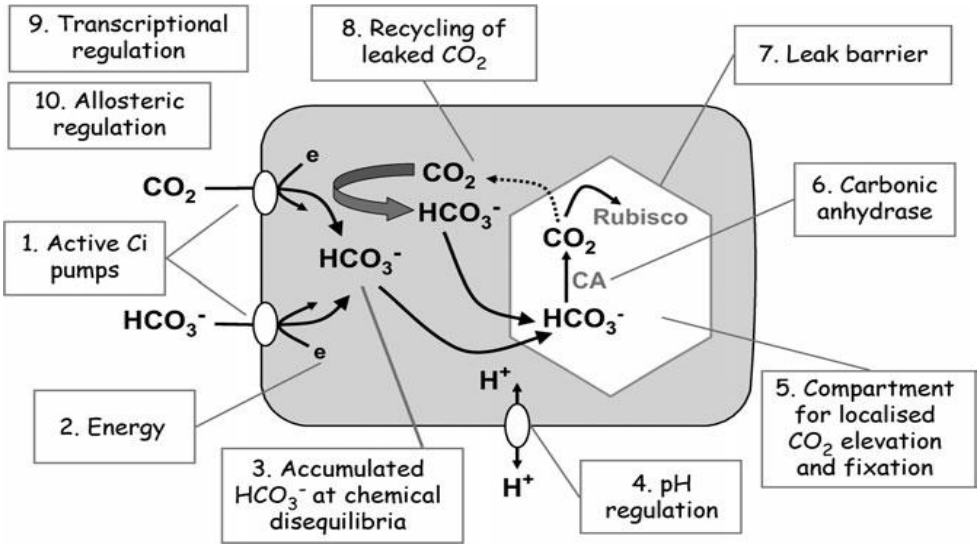


**Fig. 1** Basic operation components for bicarbonate and $CO_2$ utilization in cyanobacteria. Abbreviations: CA, carbonic anhydrase; Ci, inorganic carbon; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase.*

## Cultivation of Algae*

The two major methods for algal biofuel cultivation are open raceway ponds (Figure 2) and enclosed systems using photobioreactors (Figure 3). Using $NaHCO_3$ could provide additional benefits to both of these setups beyond simply acting as a carbon source. Raceway ponds are considered to be the most economical setup for large scale algal production, but one of the problems with pond systems is inefficient utilization of $CO_2$ (Norsker et al., 2011) that gets pumped into the system. Much of the $CO_2$ provided simply bubbles right back out due to its low solubility. The shallow depth of the raceways only provides a small exchange interface before the majority of gas escapes into the air. Sodium bicarbonate can readily dissolve into solution so losses from escaping $CO_2$ are minimized.

The buffering capacity of $NaHCO_3$ can be beneficial to both open and closed production systems. Using strains tolerate to high pH and $NaHCO_3$ concentrations in open ponds could potentially reduce contamination from other less tolerant species (Chi

* Reproduced with permission from "Advances in understanding the cyanobacterial CO2-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants" by.Price GD, Badger MR, Woodger FJ, & Long BM, 2008. *Journal of Experimental Botany*, 59 (7): 1441-1461, Copyright [2008] Oxford University Press

et al., 2011). The buffering properties also help keep the pH from turning basic during intense growth periods of high photosynthetic activity, caused by cell intake of $H^+$ [4] from the media to offset $OH^-$ formation when $HCO_3^-$ converts to $CO_2$ (Chi et al., 2011). The buffering property can also be beneficial to photobioreactor growth setups. Since this production method is a closed system, it is important to have additional safeguards to prevent the pH of the media from changing rapidly. For either setup however, the high water solubility of $NaHCO_3$ provides an additional cost saving benefit. Transportation of $NaHCO_3$ is more economical than capturing, compressing, and transporting $CO_2$ gas over long distances (Chi et al., 2011). Using the aqueous or solid salt form of $NaHCO_3$ eliminates the need for storing compressed $CO_2$ gas.

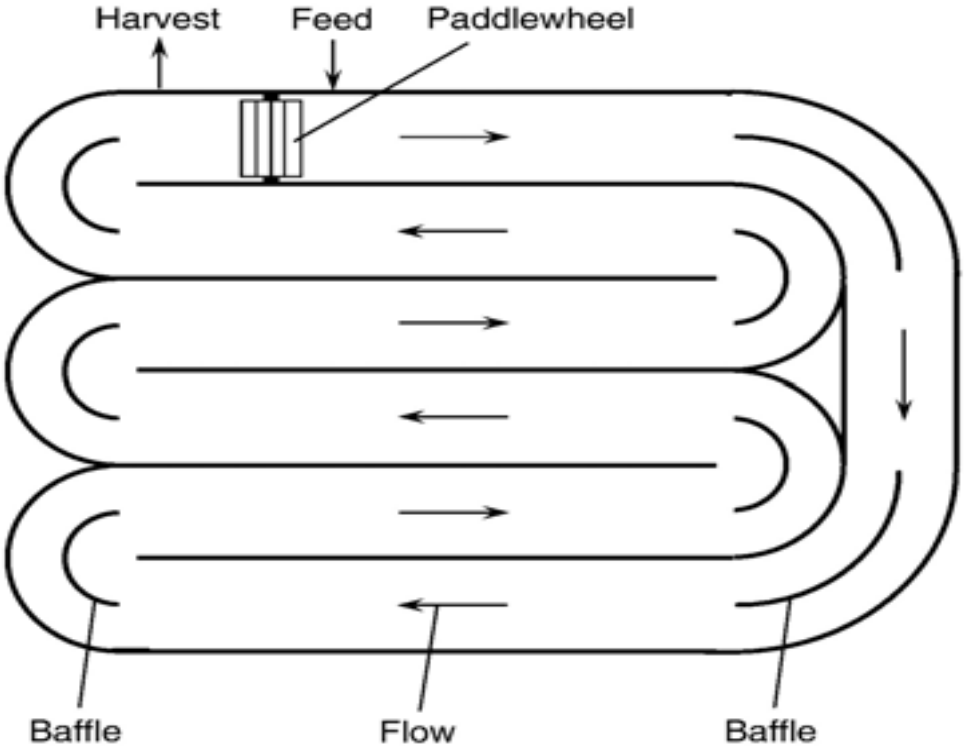


**Fig. 2** An example of an open pond raceway. The raceway has a unidirectional flow that is generated by a paddlewheel in this figure. Raceway ponds are open systems to that can be affected by the surrounding environment, but have the advantage of being simple to maintain and operate. Reproduced from Chisti (2007)*

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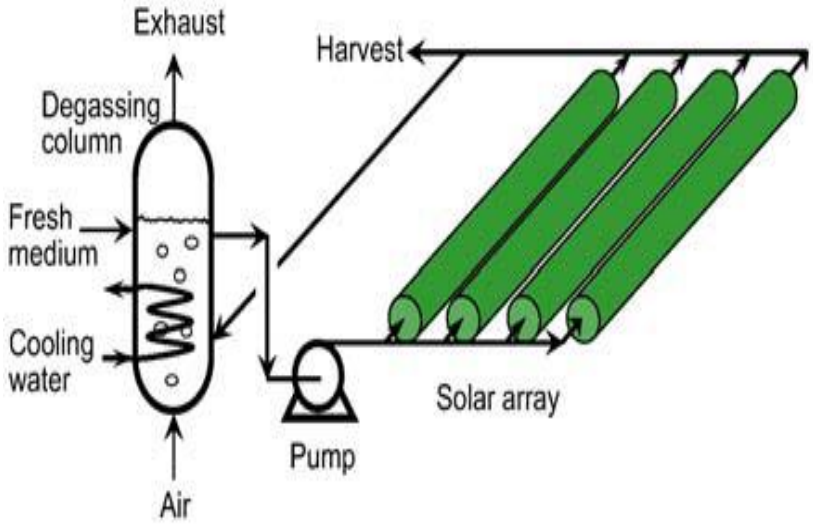


**Fig. 3** Outline of an algal photobioreactor setup. Algae grown in a closed system for reduced contamination and additional media control. The disadvantage though is initial investment and upkeep costs more and conditions need to be monitored more frequently than raceway ponds. Reproduced from Chisti (2007)*

## Objectives

- Measure growth characteristics of *Dunaliella tertiolecta* UTEX LB999 (Chlorophyta; green algae), *Mayamaea spp.*TAMU-10AM (Bacillariophyta; diatom), and *Synechoccocus sp.* CCMP 1379 (Cyanophyta; cyanobacteria) using different concentrations of sodium bicarbonate
- Determine if $NaHCO_3$ can act as a lipid formation trigger
- Measure lipid accumulation during growth and lipid phases
- Determine which sodium bicarbonate concentration has best growth rate and lipid formation for each species

## Hypothesis

H1: Sodium bicarbonate can increase growth rate and lipid production in *D. tertiolecta*, *Synechoccocus* sp., and *Mayamaea spp*. relative to zero additional carbon.

H2: Sodium bicarbonate can act as a lipid accumulation trigger if added in higher concentrations than found in the growth media.

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# METHODS

## Growth Phase

Three marine algal species *Synecoccocus* sp., *Dunaliella tertiolecta*, and *Mayamaea spp*. were cultured for this experiment. The algal strains were grown in one liter bottles and shaken each day to prevent sedimentation of the cells and to help facilitate gas exchange. During the growth phase each species was grown in Gulf of Mexico seawater (pH ~8.2) that had been modified to f/2 medium (Guillard & Ryther, 1962; Guillard, 1975). The seawater sourced for this experiment originated in the Gulf of Mexico and was provided through NOAA's National Marine Fisheries Service (NMFS) facility located in Galveston, TX. Using filtered and sterilized seawater visibly showed less precipitation of $NaHCO_3$ than enriched artificial seawater medium (ESAW) which was why the natural seawater was chosen. To ensure sterility the seawater was double filtered and then autoclaved before being made into f/2 media. Since this was natural seawater minute amounts of naturally dissolved carbon could have been another source of a small amount of growth in the control media, though this would have also been available to every other treatment as well.

Nutrients, trace metals and vitamins were added in the concentrations recommended for the f/2 recipe. To this we also added varying amounts of additional $NaHCO_3$ (0.5, 1.0, 2.0, and 5.0 mg/L). The control treatment (no added $NaHCO_3$) had background occurring dissolved inorganic carbon. The seawater was sterilized prior to use by double filtration (filtered two times through a 0.45 micron filter) followed by autoclaving before modification to f/2 nutrients, trace metals, and vitamins. A stock solution containing 50.0g/L dissolved $NaHCO_3$ in distilled water was prepared; the required $NaHCO_3$ was then added to f/2 medium to reach the desired concentration. All five $NaHCO_3$ concentrations were prepared in triplicates, with the bottles incubated at 19º C in a light controlled chamber with lights cycling on and off every 12 hours to approximate day and night and 130-150 µmoles photons $m^{-2}$ $s^{-1}$. The first part of the

experiment was collecting data for the ―growth phase" until the cultures reached their stationary phase in approximately 7-10 days.

**Lipid Phase**

The second part of the experiment was examining the effects of a sodium bicarbonate ―boost" on lipid formation in the cultures. Each of the cultures was given either fresh f/2 media containing no $NaHCO_3$ or fresh f/2 media with a boosted 10.0g/L dissolved $NaHCO_3$. The latter treatment will be referred to as ―boosted" throughout the remainder of the thesis.

| Original $NaHCO_3$ Conc. | | Part 2 $NaHCO_3$ Conc. |
|---|---|---|
| 0.0 g/L | → | 0.0 and 10.0 g/L |
| 0.5 g/L | → | 0.5 and 10.0 g/L |
| 1.0 g/L | → | 1.0 and 10.0 g/L |
| 2.0 g/L | → | 2.0 and 10.0 g/L |
| 5.0 g/L | → | 5.0 and 10.0 g/L |

Sample collection and testing was conducted at approximately the same time each day throughout the lipid phase (for a total of approximately 5 days). If a culture crashed due to the new $NaHCO_3$ concentrations, then the measurements for that bottle were stopped early.

**Data Analysis**

Throughout both parts of the experiment, samples were measured daily to determine changes in growth and lipid accumulation. Optical density (OD) measurements were taken using a Shimadzu UV/VIS 2501PC spectrophotometer at the 750, 680, 650, and 600 nm wavelengths using 3ml of undiluted, suspended samples in cuvettes.

Oil indices (used to estimate lipid content) were measured using a separate 3ml sample of cells stained with 9µl Nile Red (final concentration in acetone, 0.75 $\mu g/ml^{-1}$) (Cooksey, 1987). These samples were then run through a Shimadzu RF-5301PC

spectrofluorophotometer using a 490 nm excitation and 500-750 nm emission wavelengths. Actual oil indices were calculated in Microsoft Excel though a macro that combined the spectrofluorophotometer and spectrophotometer data (in house).

Photosynthetic efficiency ($F_v/F_m$) was recorded using a fluorescence induction and relaxation florometer (FIRe) (Satlantic Inc.) using blue light emissions for *D. tertiolecta* and *Mayamaea spp*. and blue/green light emissions for *Synechoccocus* sp., with all samples acclimated to 30 minutes darkness prior to the measurements. Samples processed for analysis using the software FIRePro (Satlantic Inc.).

pH was measured to track any buffering benefits provided by using $NaHCO_3$ using an Accumet AB15 pH meter calibrated prior to use. All samples were discarded in a container of bleach after all measurements were completed on a given day.

Ash free dry weight (AFDW) sampling involved pre-weighed and pre-combusted 47mm GF/F filters to vacuum filter 40 ml samples from each culture. Under vacuum, each sample was washed with 0.1 N HCl and 0.5 M ammonium formate (Zhu & Lee, 1997). The vacuum was then turned off and each filter was placed in pre-weighed and pre-combusted boats to dry at 95ºC. After drying, the boats/filters were weighed and then combusted at 500ºC for 4 hours before measuring the final weight of the crucible and filter. AFDW was calculated using the obtained weights and sample volume.

At the end of each growth and lipid phase, additional samples were collected. Samples to measure protein content were saved by freezing 2mL culture in falcon tubes for future testing using a Thermo Scientific Pierce BCA (Product 23227) kit and directions. For measurement recording the standards and samples were run through a Shimadzu UV/VIS 2501PC spectrophotometer measuring absorbance at 562nm. The measurements of the standards and results following the kits directions were initiation put against a standard curve to obtain an $R^2$ value to ensure the process did not have to be retried. The sample results were then into a different Microsoft Excel spreadsheet containing prepared formulas, along with AFDW g/L results to calculate protein (in mg/g of algae).

# RESULTS

The growth characteristics and composition of *D. tertiolecta*, *Mayamaea spp*., and *Synechoccocus* sp. were recorded throughout their growth and lipid phases. All results are presented as means plus or minus the standard deviations (S.D).

**Growth Phase**

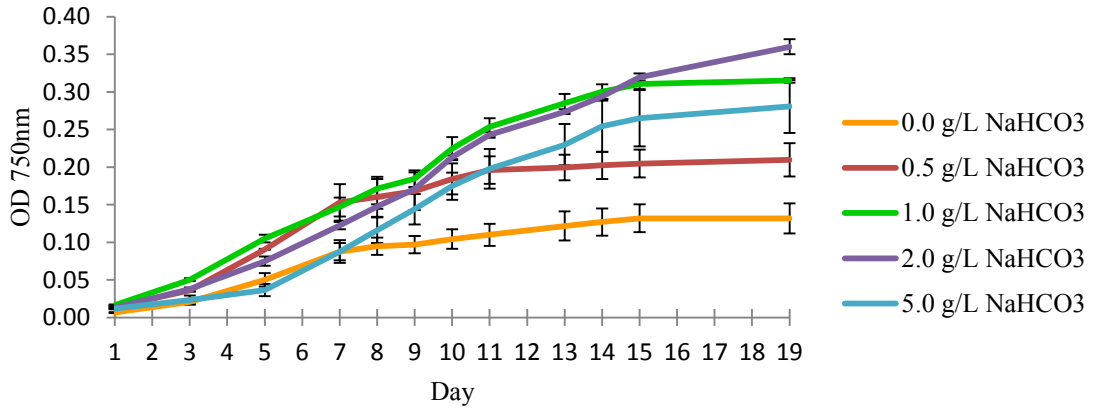
Based on optical density the end of the growth phase both *D. tertiolecta* and *Mayamaea spp*. had highest culture densities in 2.0 g/L $NaHCO_3$ concentrations, while *Synechoccocus* sp. was highest in 5.0 g/L $NaHCO_3$ concentrations (Figure 4a, b, and c). The 1.0 and 2.0 g/L $NaHCO_3$ concentrations had greater optical densities for *D. tertiolecta* than any other concentration (Figure 4a). For the *Synechoccocus* sp., the optical density was highest in the 5.0 g/l treatment with both the 1.0 and 2.0 g/L $NaHCO_3$ concentrations (Figure 4c); their highest ODs were about 12.5 and 33.5 percent higher. *Mayamaea spp*.'s optical density was similar in all cultures with added $NaHCO_3$; these optical densities were all higher than that present in the control – no $NaHCO_3$ added (Fig. 4b).

Highest growth rates during the growth phase for *D. tertiolecta* (days 3-15), *Mayamaea spp*., (days 5-14) and *Synechoccocus* sp. (days 2-8) occurred in the 5.0 g/L $NaHCO_3$ concentration treatments, with rates of 0.205 $d^{-1}$ ±0.010, 0.119 $d^{-1}$ ±0.004, and 0.372 $d^{-1}$ ±0.003 respectively (Figures 5a,b, and c). *Mayamaea spp*.'s and *Synechoccocus sp*. growth rates were higher than the control, but still within ±1 standard deviation of it. Unlike the OD results which clearly showed the 0.0 g/L control groups lagging behind the other concentrations by the half-way point of each growth phase, the average growth rates indicate the controls might not be the slowest growing group for *D. tertiolecta*. Both 0.5 and 1.0 g/L concentrations for *D. tertiolecta* were similar to the 0.0 g/L concentration, while only the 1.0 g/L *Mayamaea spp*. and 0.5 g/L *Synechoccocus* treatments were similar or lower than their respective 0.0 g/L treatment.

The photosynthetic efficiency (Fv/Fm) was measured during growth of *D. tertiolecta*, *Mayamaea spp*., and *Synechoccocus* sp. (Fig. 6a, b, c). The Fv/Fm measurement provides a general health indication of the algae by how efficient photosynthesis was occurring. At the final growth phase day Fv/Fm were not different between $NaHCO_3$ treatments. Final Fv/Fm values were within ±1 standard deviation for treatments of *D. tertiolecta* and *Mayamaea spp.* during the growth phase. The highest Fv/Fm was observed in the *Mayamaea spp.,* with this species having the most efficient photosyntheis with an average of all treatments of Fv/Fm of 0.579 ± 0.009. The 5.0 g/L treatment for *D. tertiolecta* had a final Fv/Fm of 0.423 ±0.026 which was within ±1 standard deviation of lower treatments. For *Synechoccocus sp*. (Figure 6c), Fv/Fm values showed a lower overall photosynthetic efficiency, with the highest value at the end date of the growth phase being only about 0.281 ±.008 in the 2.0 g/L concentration and the lowest Fv/Fm of 0.190 ± .008 in the 0.5 g/L $NaHCO_3$ treatment.

For all three species use of $NaHCO_3$ showed signs of acting as a buffer to help reduce the change in pH over the growth phase (Figure 7a, b, and c). This was particularly obvious when examining our findings for *Synechoccocus sp*. grown with and without $NaHCO_3$ (Figure 7c). In treatments with the $NaHCO_3$, the pHs were variable but close to 8.3 ± 0.2 throughout the growth phase which was similar to the seawater pH of 8.2, while the treatment without $NaHCO_3$ was around 7.9 over the most of the days after inoculation. The pH trend for *D. tertiolecta* and *Mayamaea spp.* were more variable with no trend related to the $NaHCO_3$ treatment or to the control (with no added $NaHCO_3$). While the ending pHs for *Mayamaea spp.* (figure 7b) did not show a treatment falling within a standard deviation of the starting pH, the 5.0 g/L concentration clearly was more stable over the growth phase period. For *D. tertiolecta* (figure 4a) the 1.0 g/L showed the most stabile pH over the growth phase. Even though the 2.0 and 5.0 g/L were not quite as stable as the 1.0 g/L concentration and had a higher ending pH, they did maintain a more stable pH over the majority of the growth phase as well especially when compared against the two lowest $NaHCO_3$ concentrations.

a

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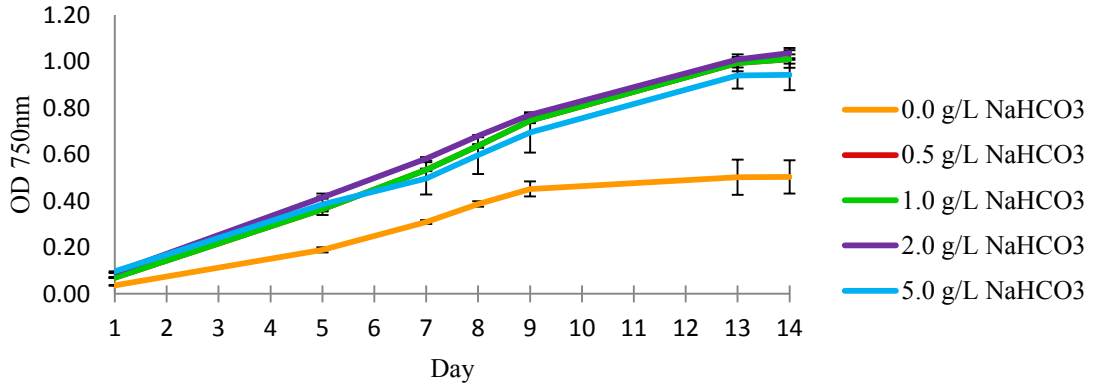


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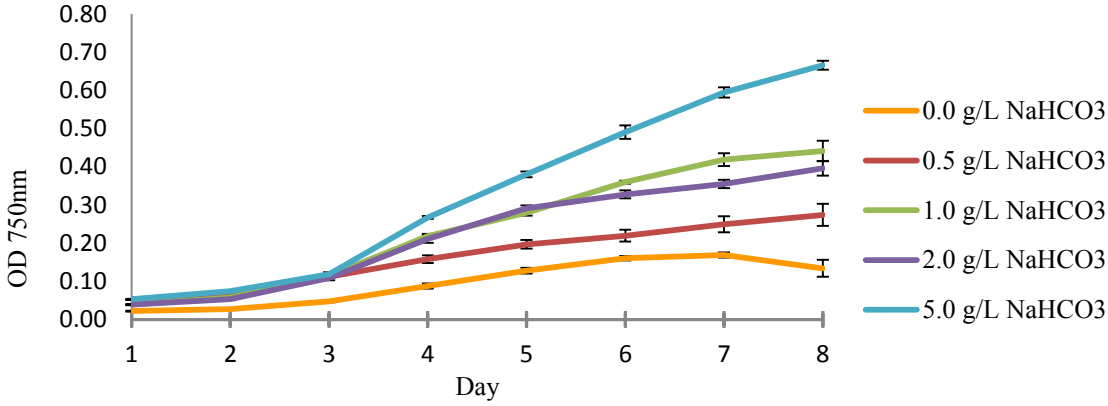



**Fig 4.** Optical density of (a) *D. tertiolecta* , (b) *Mayamaea spp.* and (c) *Synechoccocus* sp. during growth phase.

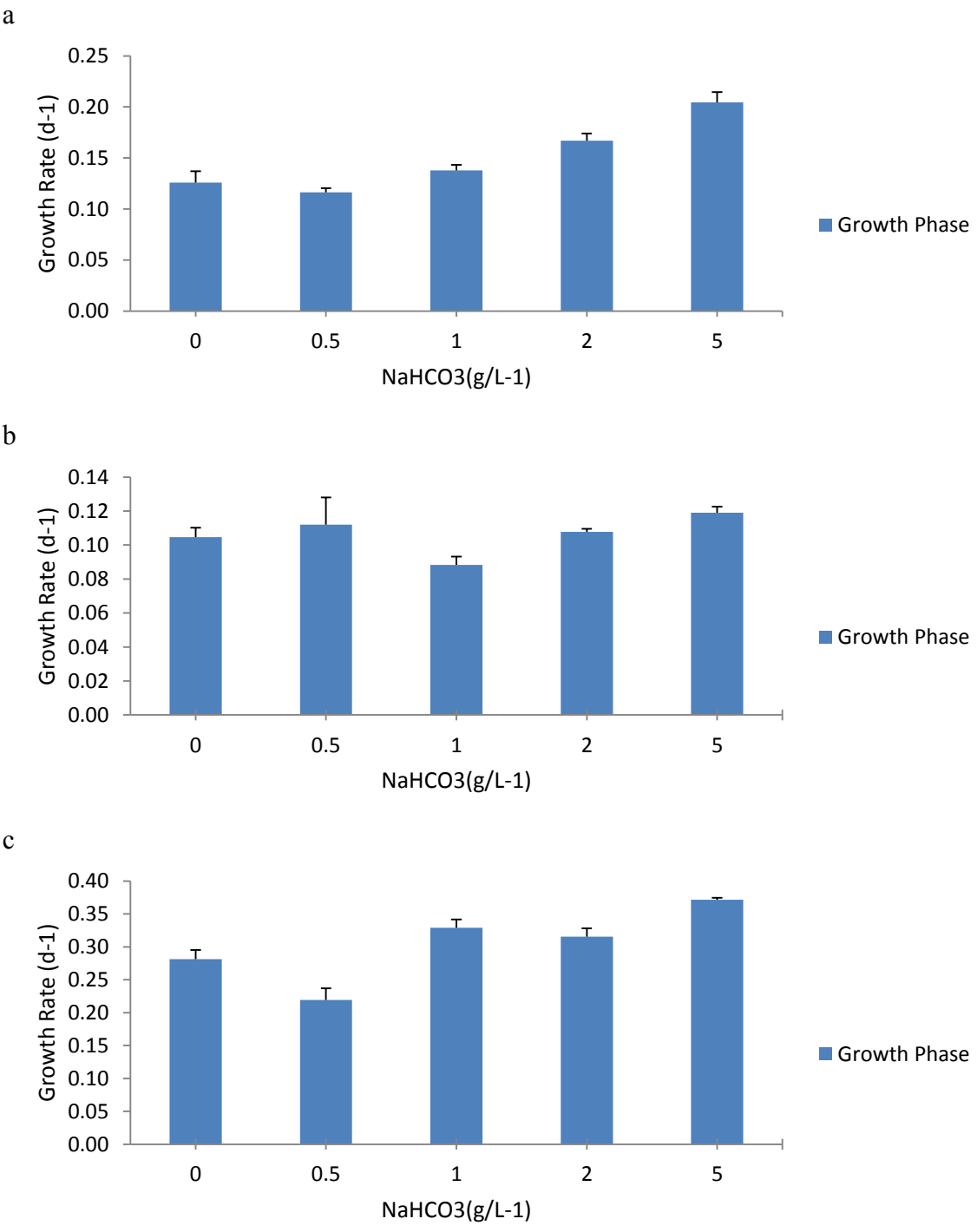



**Fig 5.** Growth rates of (a) *D. tertiolecta*, (b) *Mayamaea spp*., and (c) *Synechoccocus* sp. during growth phase

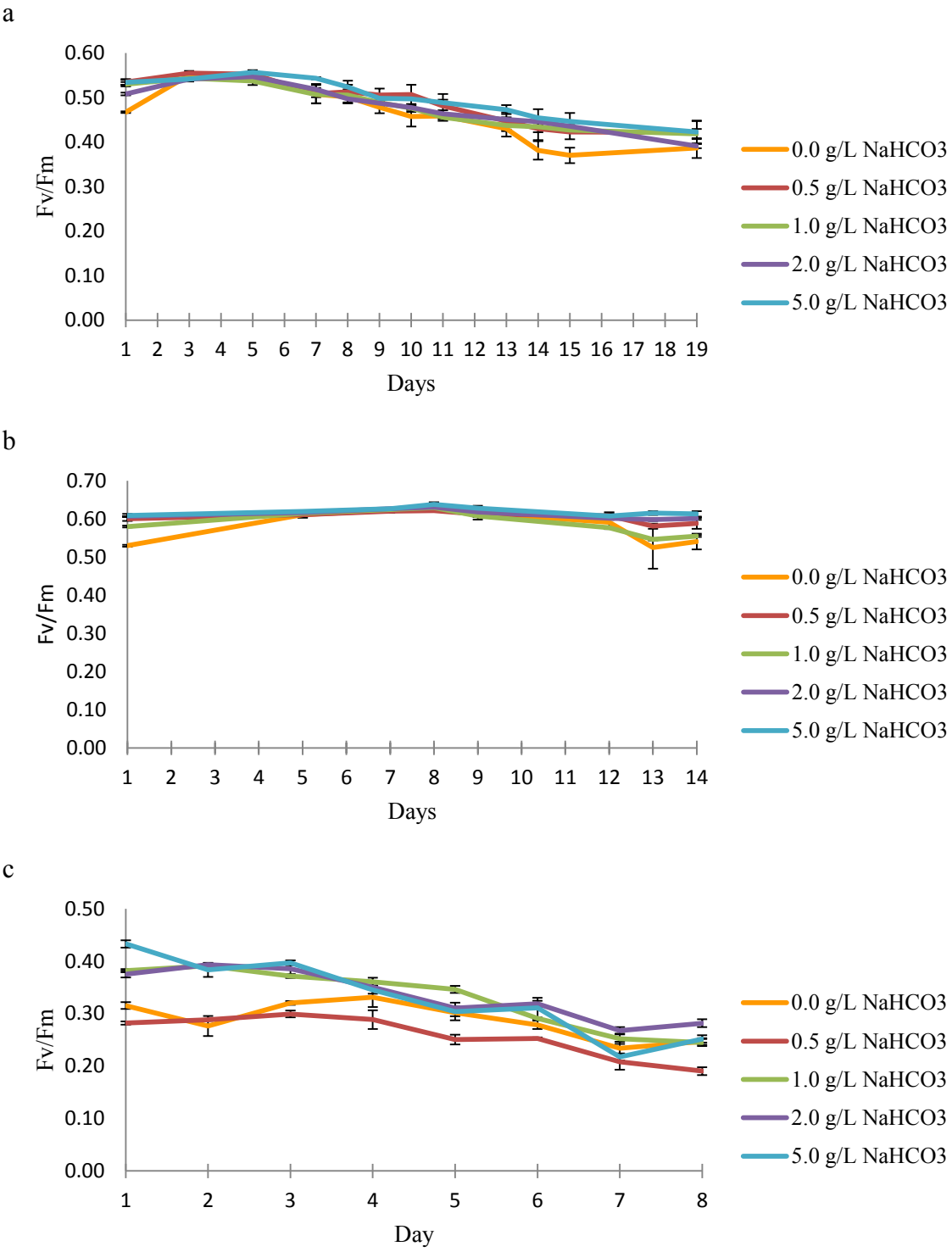



**Fig 6.** Fv/Fm of (a) *D. tertiolecta*, (b) *Mayamaea spp.* and (c) *Synechoccocus* sp. during growth phase using FIRe

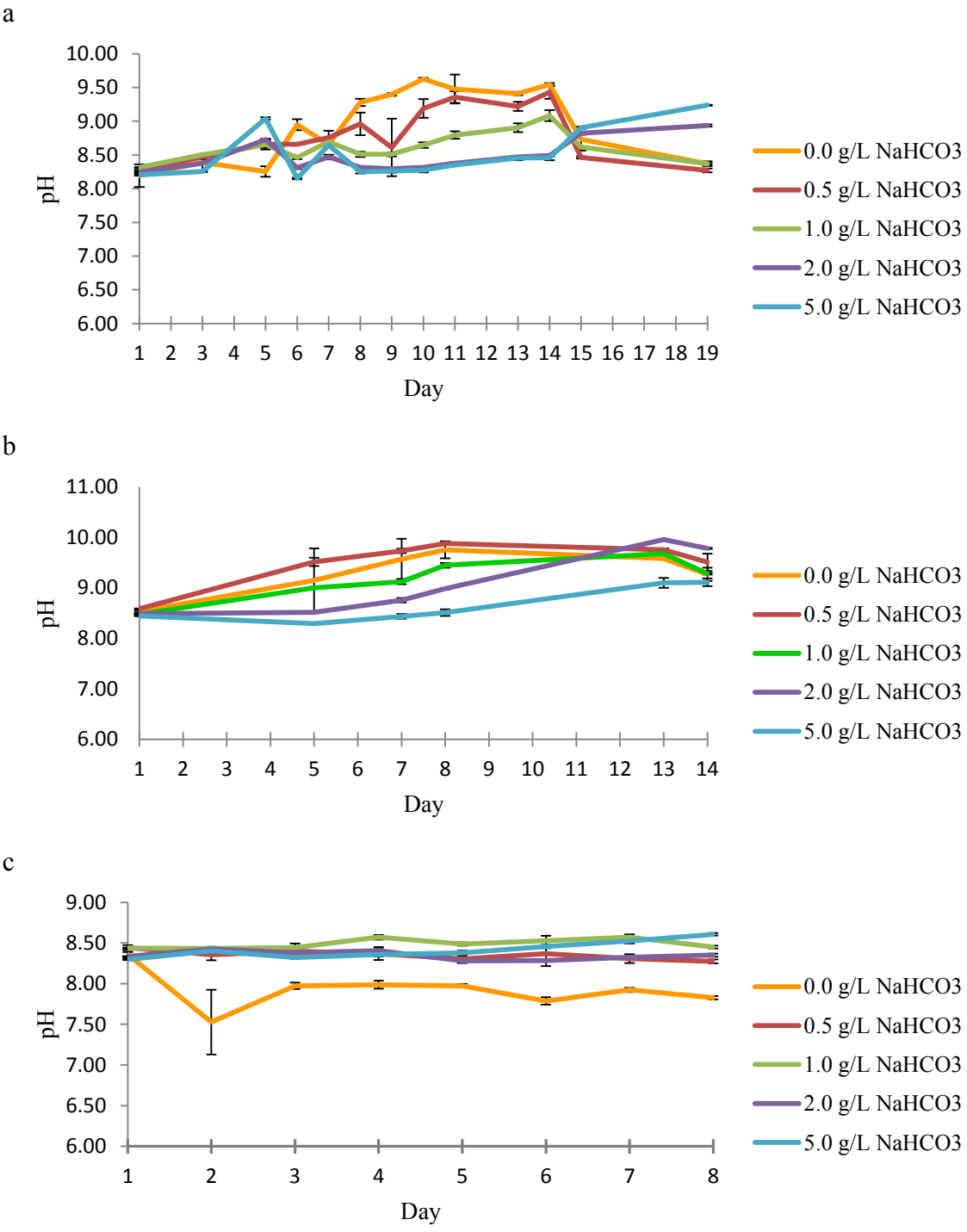
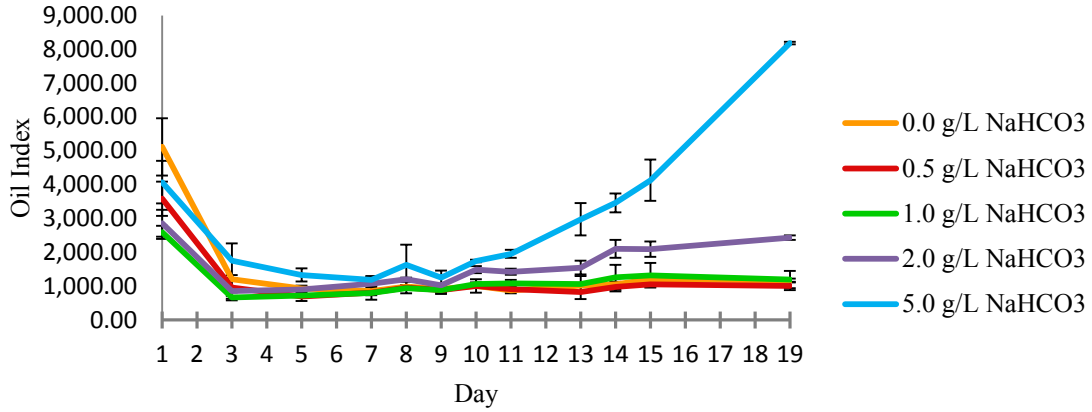



**Fig 7.** pH of (a) *D. tertiolecta*, (b) *Mayamaea spp.*, and (c) *Synechoccocus* sp. during growth phase

a

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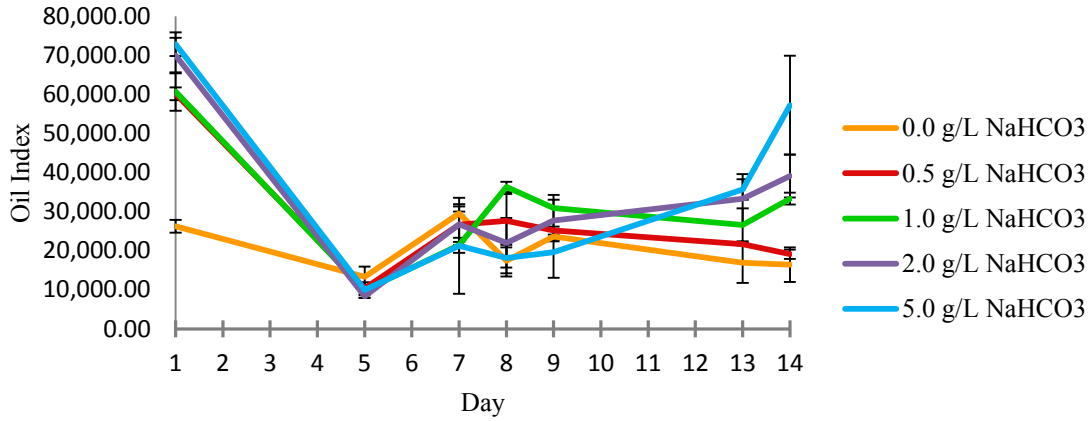


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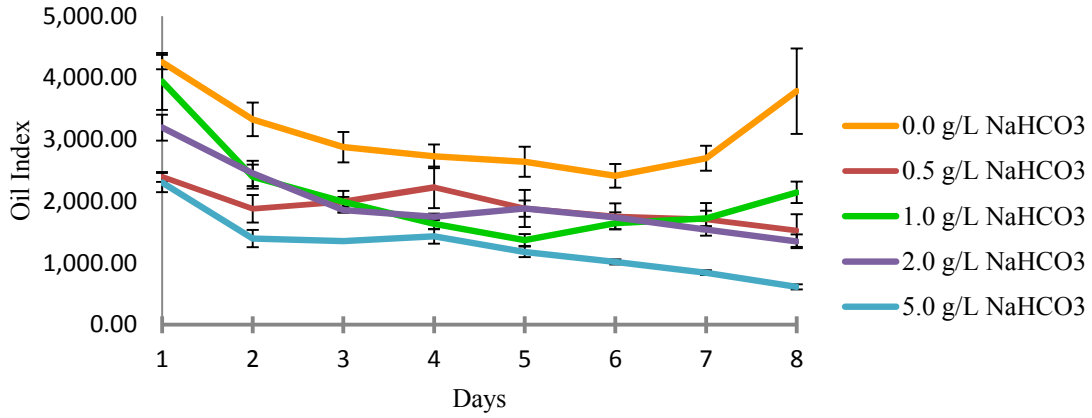


**Fig 8.** Average oil index of (a) *D. tertiolecta*, (b) *Mayamaea spp.*, and (c) *Synechoccocus* sp. during growth phase

Both *D. tertiolecta* and *Mayamaea spp*. (Figures 8a and b) showed a similar trend in oil content throughout the growth phase, with oil indices dropping immediately after inoculation and then leveling off for a period of time corresponding to the exponential growth phase of the cultures (see Fig. 1 above), and then increasing again as cultures entered stationary phase. The average oil indices during the plateau phase (relative units) was 1,100 ±100 in *D. tertiolecta*. However after 18 days the oil index was ~ 2.1 and 7 times higher in the 2.0 and 5.0 g/L treatments respectively for *D. tertiolecta* (Figure 8a) while the oil index did not change from the control for the 0.5 and 1.0 g/L treatment.

The range of oil indices during the plateau phase was 17,423 to 36,317; these oil indices were higher than what was observed in *D. tertiolecta* (Figures 8a and b). In *Mayamaea spp*., it was observed that by day 19 the oil index was about 2.2 times higher than the control in the 1.0 g/L and 2.0 g/L treatments, while the oil index for 0.5 g/L did not change from the control treatment (Figure 8b). While the 5.0 g/L treatment had a larger error, it was still greater than one standard deviation from the 2.0 g/L treatment, and its oil index was almost 3.5 times higher than the control.

*Synechoccocus* sp. started with a similar trend in the growth phase (Figure 8c), but most concentrations finished almost leveled off or still slightly decreasing at the end date. At day 6 when the treatments either plateaued or were slightly decreasing the oil indices ranged from about 1,017 to 2,411. Only the control and 2.0 g/L concentration had stated to increase again by the final day, but the 2.0 g/L concentration was still lower even with the standard deviation of the control's oil end oil index average of 3783 ± 692. Not just the 2.0 g/L concentration, but all test concentrations were well below (greater than ±1 standard deviation among the trails) the control oil index by the final day. The closest to the control group was the 1.0 g/L concentration which was about 1.8 times lower, while the definitively lowest oil index was the 5.0 g/L concentration at almost 6.2 times lower than the control. There was greater variability in the oil indices measured for *Synechoccocus* sp., ranging from 1,017 to 2,411for the 0.0 to 5.0 g/L treatments.

**Lipid Phase**

During the lipid phase, growth and internal composition of every growth phase trial treatment was measured under two conditions, either by getting fresh media with no $NaHCO_3$ or fresh media with a boosted concentration of 10.0 g/L $NaHCO_3$. For the lipid phase, the control group was 0.0 → 0.0 g/L concentration in the non-boosted media group.

The growth phase media concentrations of 0.5 g/L for *D. tertiolecta*, 0.0 g/L for *Mayamaea spp.* (Figures 9a,b and 9c,d respectively), and 5.0 g/L for *Synechoccocus* sp. (Figures 9e, f) were the only cases where no additional $NaHCO_3$ (non-boosted) had a higher optical density than their boosted $NaHCO_3$ counterparts. For *D. tertiolecta*, the only non-boosted media treatments to be higher than the control beyond one standard deviation were the 2.0 g/L and 5.0 g/L concentrations (Figure 9a). All *D. tertiolecta*'s boosted treatments except the 0.5 g/L concentration were greater than one standard deviation higher than the control (Figure 9b). For *Mayamaea spp*. every concentration except the boosted 0.0 g/L trial was greater than one standard deviation higher than the control OD750 reading at 0.524 (Figures 9c and 9d). *Synechoccocus* sp. had every concentration end with OD750s that were higher than the control reading average at 0.248 (Figures 9e and 9f), even when including a standard deviation.

The overall highest average optical density for *D. tertiolecta* was 0.543 in the boosted 2.0 g/L $NaHCO_3$ (Figures 9a and 9b), higher than any other concentration tested for the species even if counting the range of one standard deviation for each treatment. The highest *Mayamaea spp*. OD750 averages were in the boosted 2.0 g/L and 5.0g/L concentrations (Figures 9c and 9d) with readings of 0.853 and 0.839 respectively. Though the 2.0g/L was higher, there was not a difference between the two since they were within one standard deviation of each other. *Synechoccocus* sp. was highest in the boosted 0.5g/L $NaHCO_3$ with a final OD750 average of 0.957, but it fell within the standard deviation of ] 2.0 g/L and 1.0g/L treatments, the respective 2nd and 3rd highest overall (Figures 9e and 9f).

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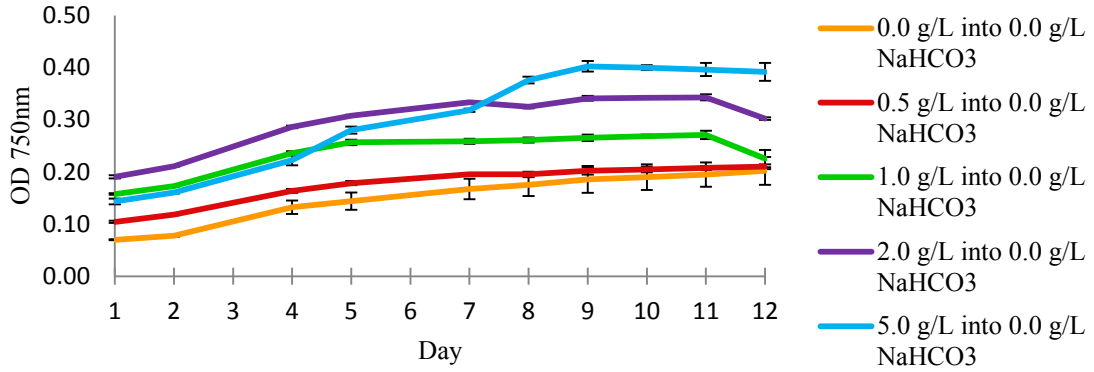


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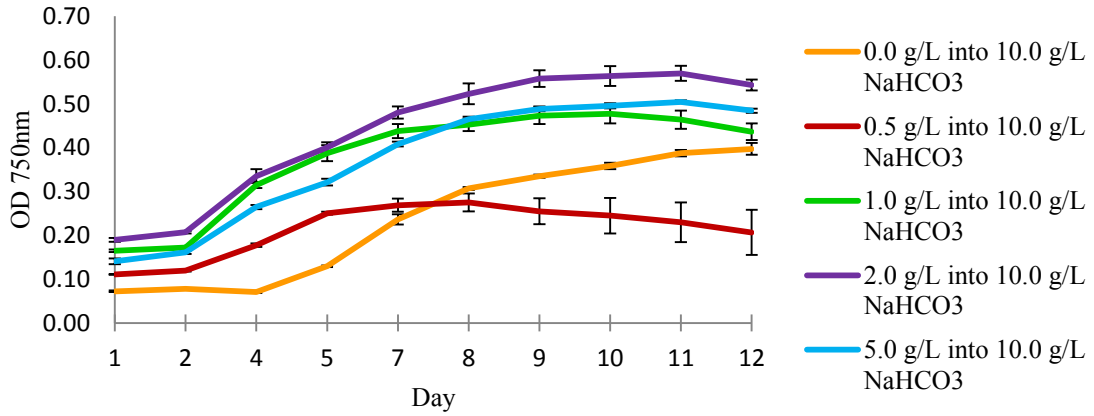


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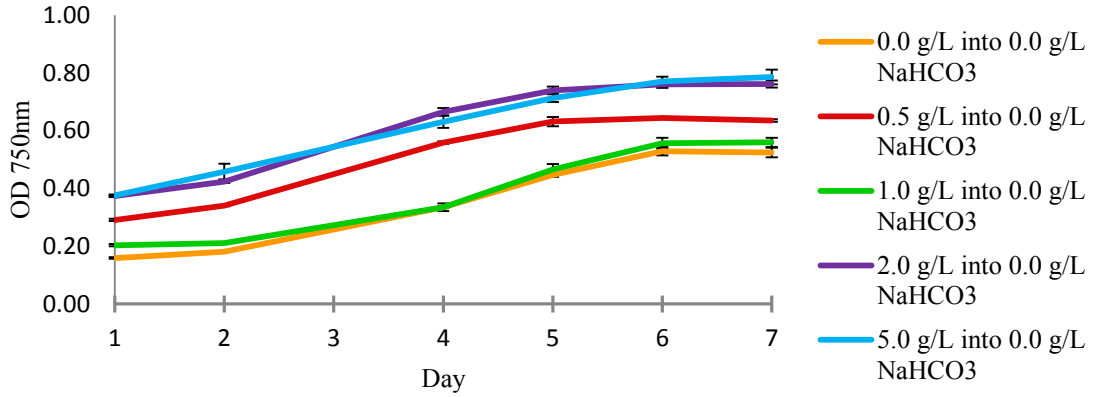
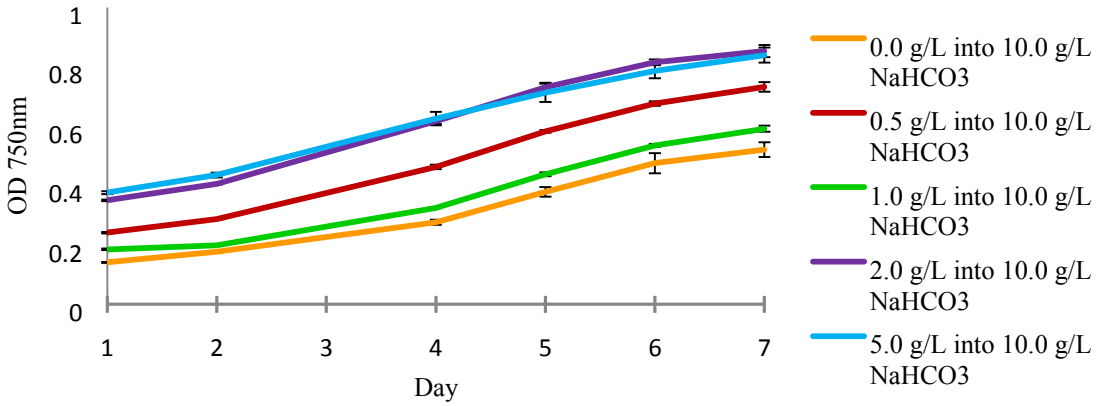



**Fig 9.** Lipid phase OD750 for (a) non-boosted and (b) boosted *D. tertiolecta*, (c) non-boosted and (d) boosted *Mayamaea spp.*, and (e) non-boosted and (f) boosted *Synechococcus sp*

d

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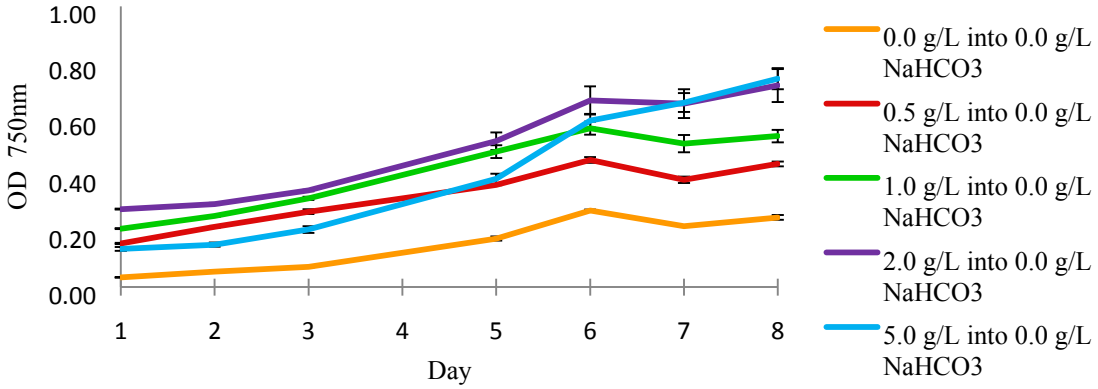


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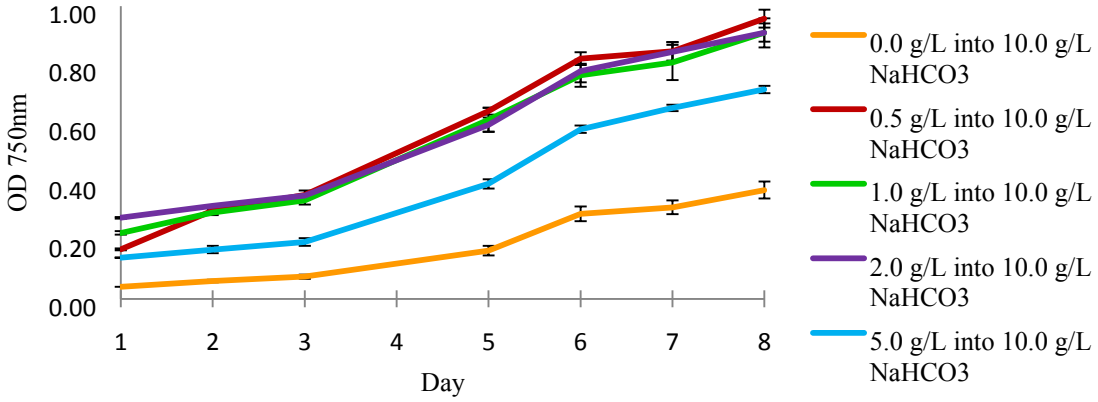
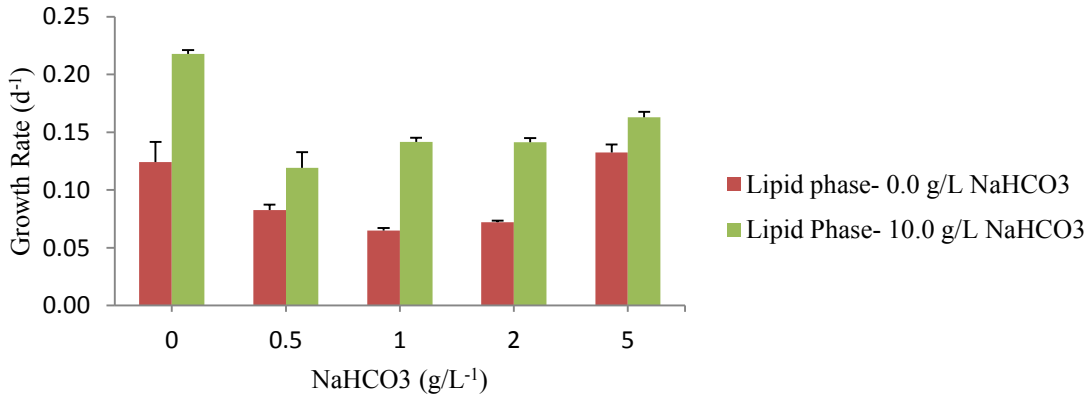



**Fig 9.** Continued

a

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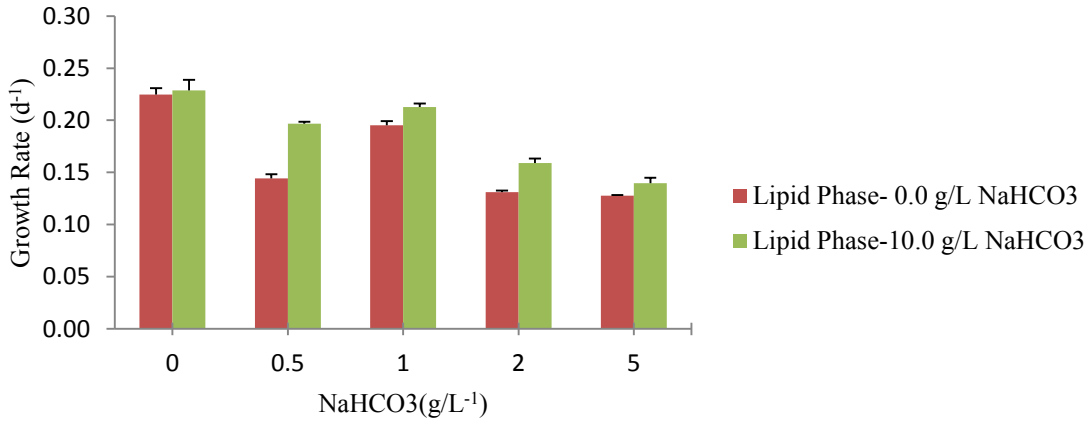


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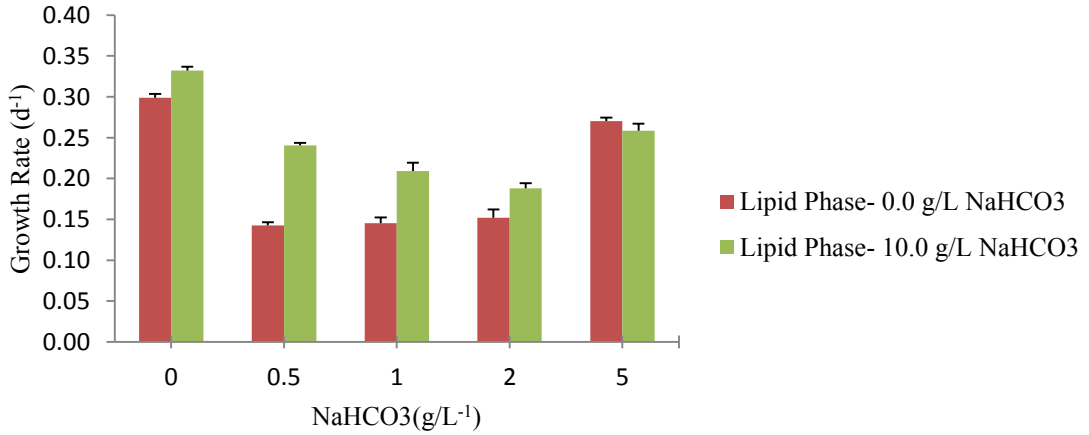



**Fig 10.** Growth rates of (a) *D. tertiolecta*, (b) *Mayamaea spp*., and (c) *Synechoccocus* sp. during lipid phase

The highest growth rate for *D. tertiolecta* during the lipid phase was found in the boosted 0.0 g/L $NaHCO_3$ (Figure 10a) with a rate of 0.218 $d^{-1} \pm 0.003$ (standard deviation). At the end of the lipid phase all of the boosted treatments showed a higher final average growth rate than their non-boosted counterparts, even counting the range of one standard deviation for each treatment. *Mayamaea spp.* had its highest growth rate in the boosted 0.0 g/L treatment (Figure 10b) at 0.229 $d^{-1} \pm 0.010$, although it fell within the standard deviation of the control treatment which had a growth rate of 0.225 $d^{-1} \pm$ 0.006. Similar to what was observed the *D. tertiolecta* treatments, it can clearly be seen that the boosted *Mayamaea spp.* treatments had higher growth rates than their non-boosted counterparts even with the standard deviation of each treatment. The highest growth rate for *Synechoccocus sp*. was in the boosted 0.0 g/L treatment (Figure 10c) with a final average growth rate of 0.332 $d^{-1} \pm 0.005$. While most of the boosted treatments showed higher growth rates than their non-boosted counterparts, the 5.0 g/L boosted treatment was the only one to be lower than the 5.0g/L non-boosted treatment although the difference between them was within one of their respective standard deviations. The boosted 5.0 g/L had a growth rate of 0.259 $d^{-1} \pm 0.009$ while the non-boosted treatment's growth rate was 0.270 $d^{-1} \pm 0.004$.

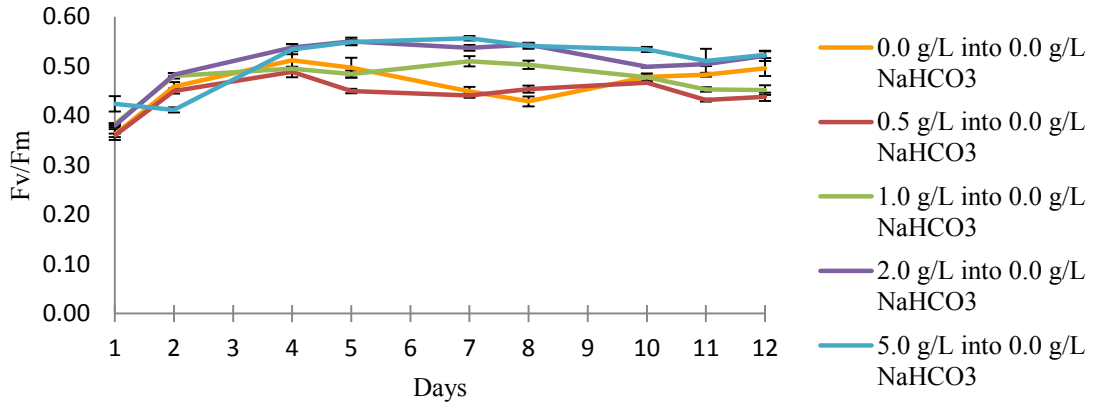
At the end of the lipid phase, Fv/Fm values for each species across all their concentrations were about 0.475, 0.6, and 0.6 for *D. tertiolecta*, *Mayamaea spp.* and *Synechococcus sp*. respectively (Figures 11a-f). Looking at the figures, all three species showed fairly consistent Fv/Fm efficiency values across each concentration trial throughout the lipid phase, but *Mayamaea spp.* trials clearly lower variation toward the final days while maintaining low standard deviations across all treatments. For *D. tertiolecta,* the only two treatments with higher Fv/Fm than the control were the non-boosted 5.0 g/L and boosted 0.0 g/L concentrations (Figures 11a and 11b). Highest overall ending average Fv/Fm was in the non-boosted 5.0 g/L concentration with an Fv/Fm of 0.523 ± 0.007. For *Mayamaea spp.* the boosted concentration and the non-boosted 5.0 g/L treatment had higher Fv/Fm values than the control (Figures 11c and 11d). While it is difficult to observe in the figures, highest ending average was the

boosted 0.5 g/L treatment with an Fv/Fm of 0.623 ± 0.002 but it was not higher than the boosted 2.0 g/L treatment with a Fv/Fm of 0.622 ± 0.001 when factoring in ±1 standard deviation. For *Synechococcus sp*. the only treatment that did not have a higher (± 1 standard deviation) ending Fv/Fm value than the control was the non-boosted 1.0 g/L $NaHCO_3$ concentration. The highest ending day average for this species was non-boosted 5.0 g/L $NaHCO_3$ with an Fv/Fm of 0.651 ± 0.006, but it was within the standard deviation of the second highest average found in boosted 0.0 g/L media.

Effects of $NaHCO_3$ on pH indicate it has some buffering capacity on all three species, especially when comparing the pH of boosted (10.0 g/L $NaHCO_3$) vs non-boosted trials (0.0 g/L $NaHCO_3$) (Figures 12a-f). When looking at the pH trend over the lipid phase there is clearly a trend with the boosted trials (Figures 12b, d, and f) of having either less day to day variation than the non-boosted treatments (Figures 12a, c, and e). For *Mayamaea spp.*, every boosted treatment had lower pHs than every non-boosted treatment, even with ±1 standard deviation. For *Synechococcus sp.* all boosted treatments except for 1.0 g/L ended with final average pH's lower than their non-boosted counterparts. While the final average pH values for *D. tertiolecta* were about the same for boosted vs. non-boosted treatments, the pH over the entire lipid phase did not change as much day to day in the boosted treatments.

The oil indices for *D. tertiolecta* (Figures 13a and 13b) show that every treatment had higher oil indices beyond ±1 standard deviation of the control group. The four highest oil indices were all found in boosted media, 5.0 g/L, 2.0 g/L, 0.5 g/L, and 1.0 g/L from highest to lowest, these were all higher than the corresponding non-boosted treatment. Even though the non-boosted 5.0g/L treatment was the 5$^{th}$ highest average 5204 ±628, it was within the standard deviation of the boosted 0.0g/L and non-boosted 1.0g/L (4576 ±216 and 4352 ±564 respectively).

a

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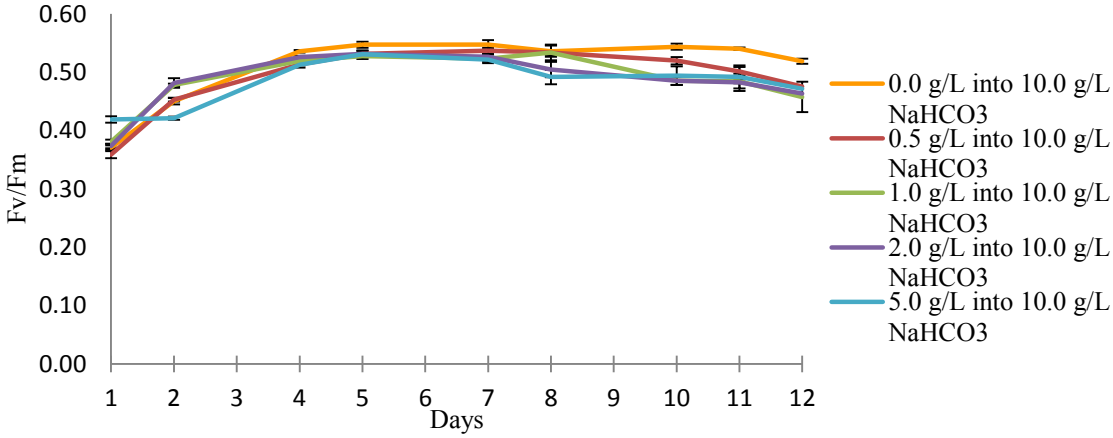


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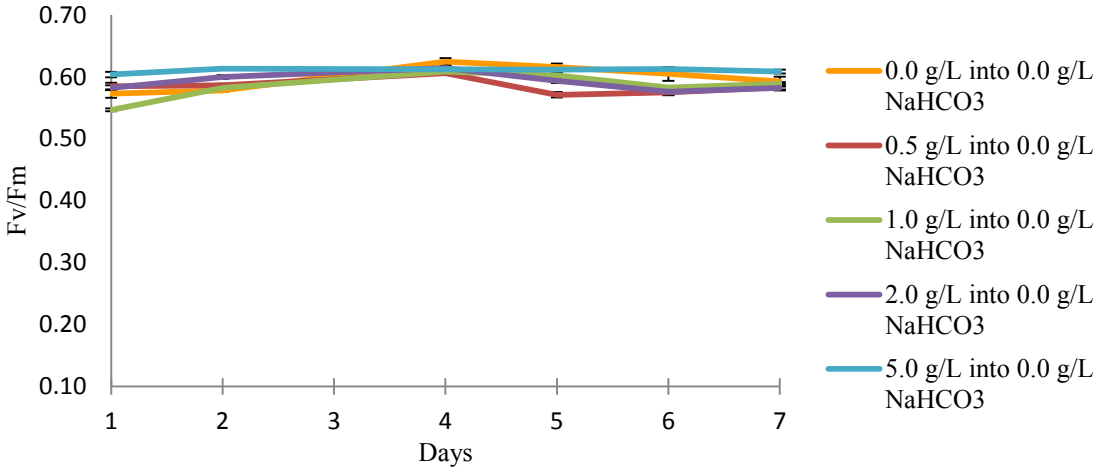
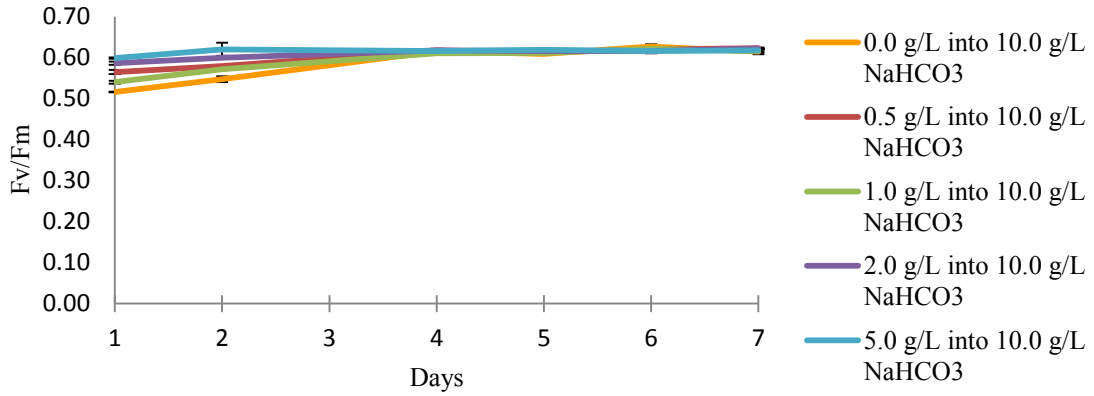



**Fig 11.** Lipid phase Fv/Fm results for (a) non-boosted and (b) boosted *D. tertiolecta*, (c) non-boosted and (d) boosted *Mayamaea spp.*, and (e) non-boosted and (f) boosted *Synechococcus sp*

d

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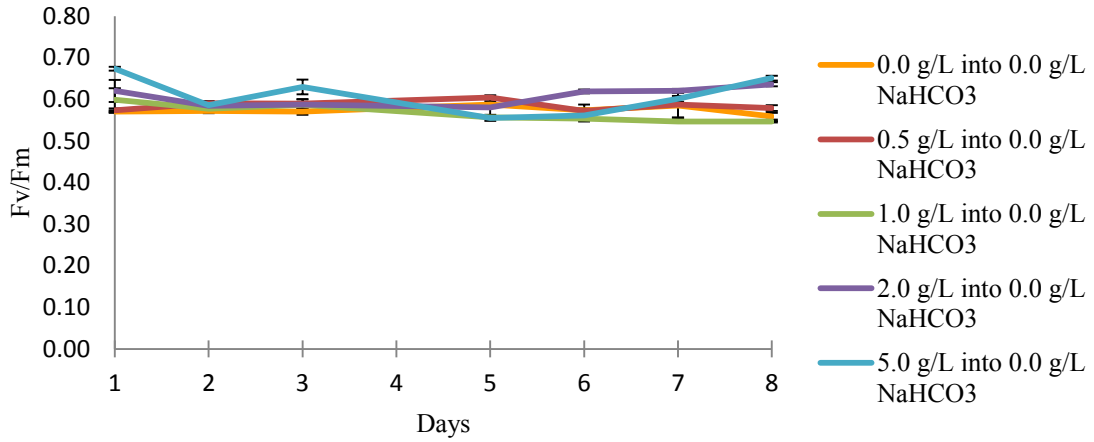


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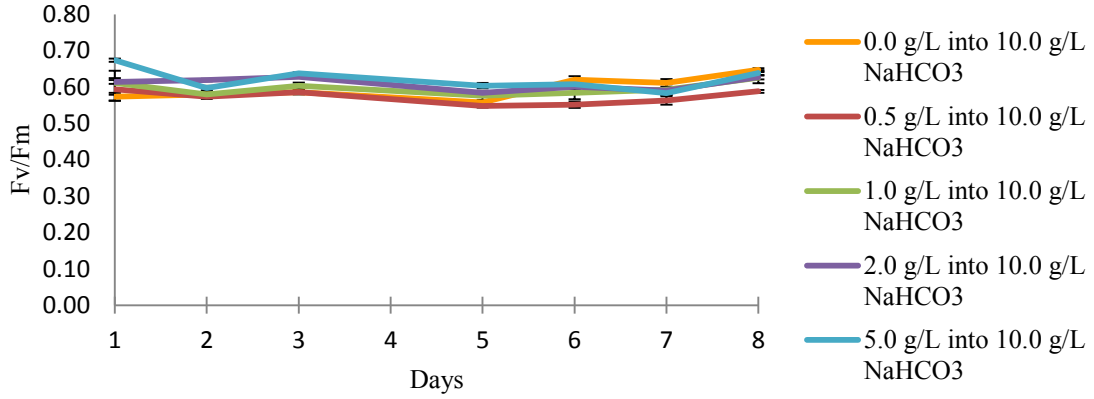


**Fig 11.** Continued

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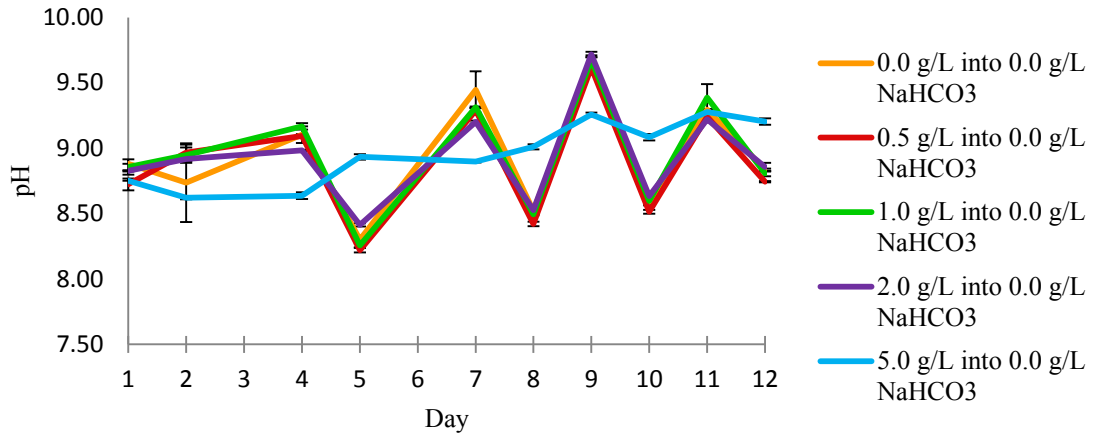


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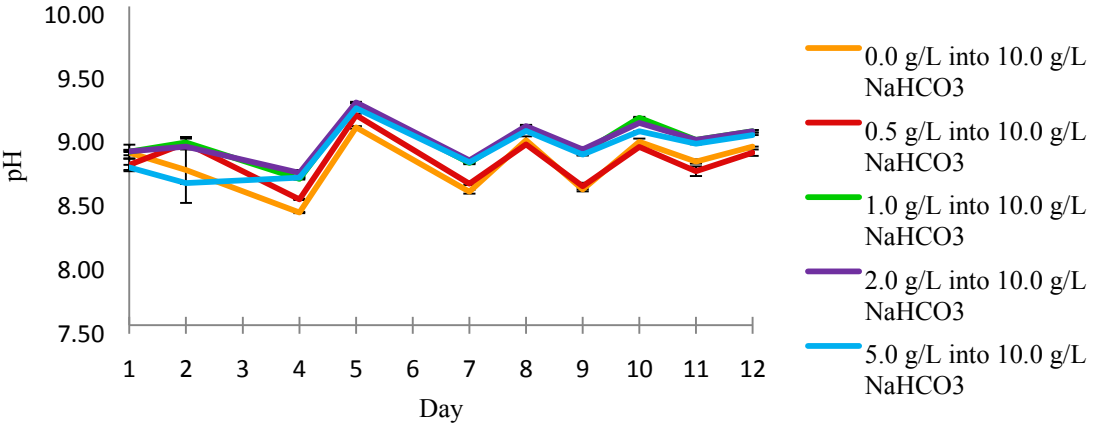


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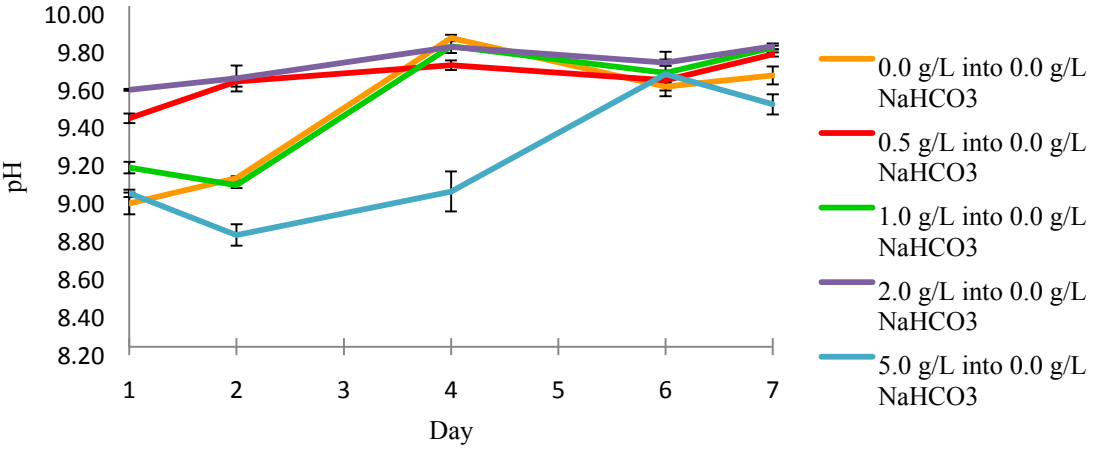
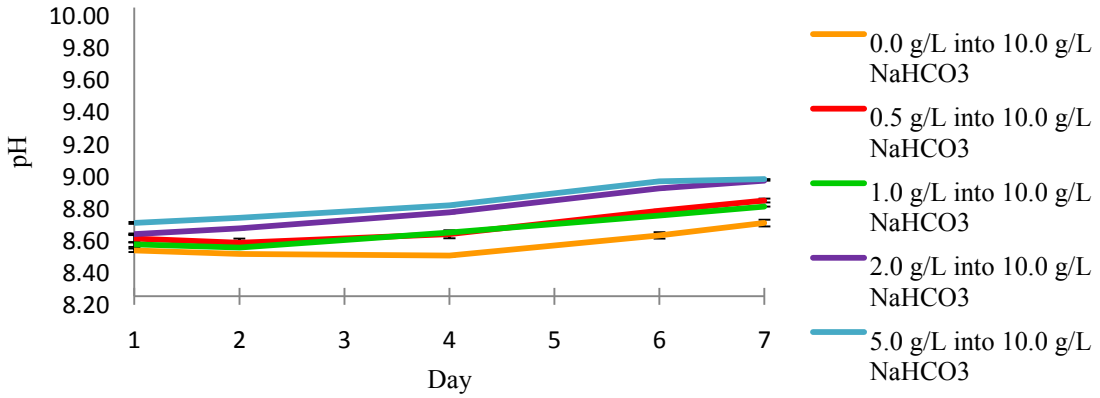



**Fig 12.** Lipid phase Fv/Fm results for (a) non-boosted and (b) boosted *D. tertiolecta*, (c) non-boosted and (d) boosted *Mayamaea spp.*, and (e) non-boosted and (f) boosted *Synechococcus sp*

d

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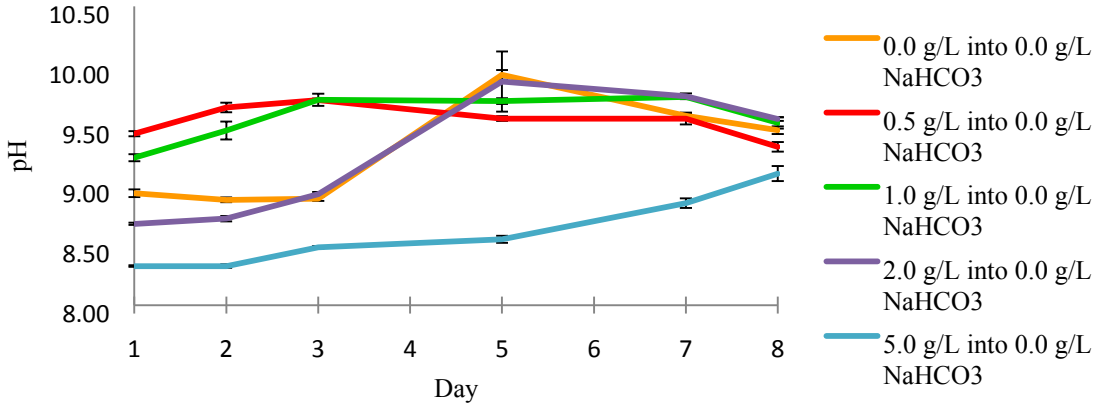


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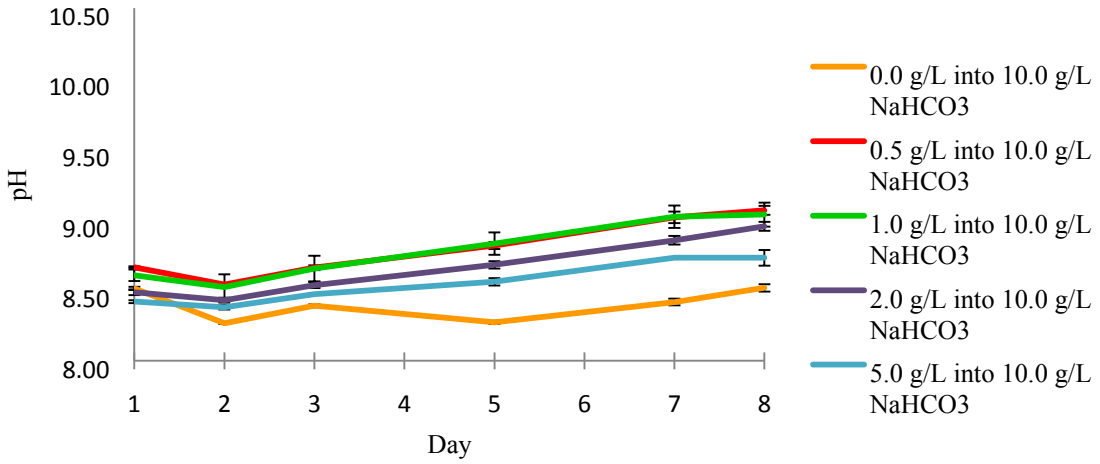


**Fig 12.** Continued

The boosted *D. tertiolecta* 5.0 g/L treatment clearly had the highest oil index though with an average of 13136 ± 895 (relative units). That makes it 1.43 times higher than the next highest (which was the 2.0 g/L treatment with 9207 ±1249) and 7.6 times higher than the control (1729 ± 240). *Mayamaea spp*. (Figure 13 c and d) had its highest oil index in the boosted 2.0 g/L $NaHCO_3$ treatment (Figure 13d). Its final oil index average was 62844 ± 8080, and even with the high deviation, it was still greater than the next highest treatments even at the upper end of their standard deviations.

While both *D. tertiolecta* and *Mayamaea spp*. clearly had their highest oil indices in the boosted media, *Synechoccocus* sp. showed a completely different trend. For this species every boosted treatment was lower than the control (Figures 13e and 13f). Figures 13e and 13f also show that each boosted treatment had a lower oil index than their non-boosted counterpart. The highest treatment was the control, 0.0 g/L non-boosted media with an oil index of 3840 ± 212, higher than any other treatment ( greater than ±1 standard deviation).

When the final day oil indices for the lipid phase treatments of to be placed side by side for each species (Figure 14 a, b, and c) it is clear to tell if boosted or non-media typically resulted in a higher or lower oil index value.

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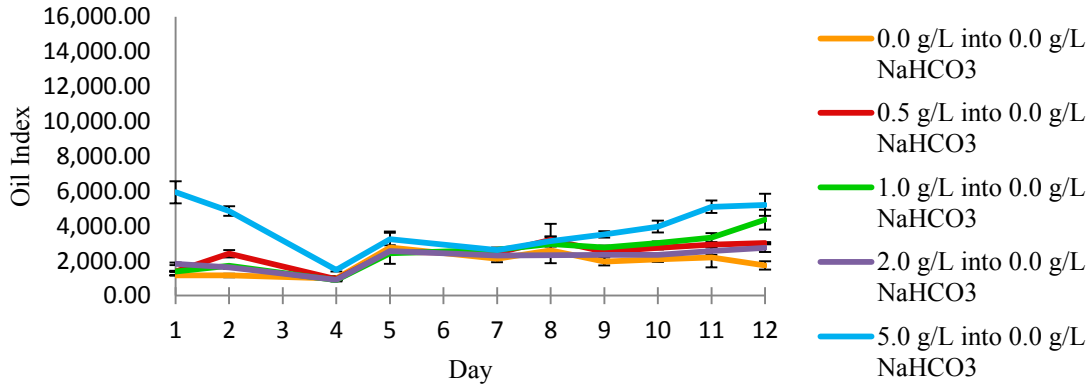


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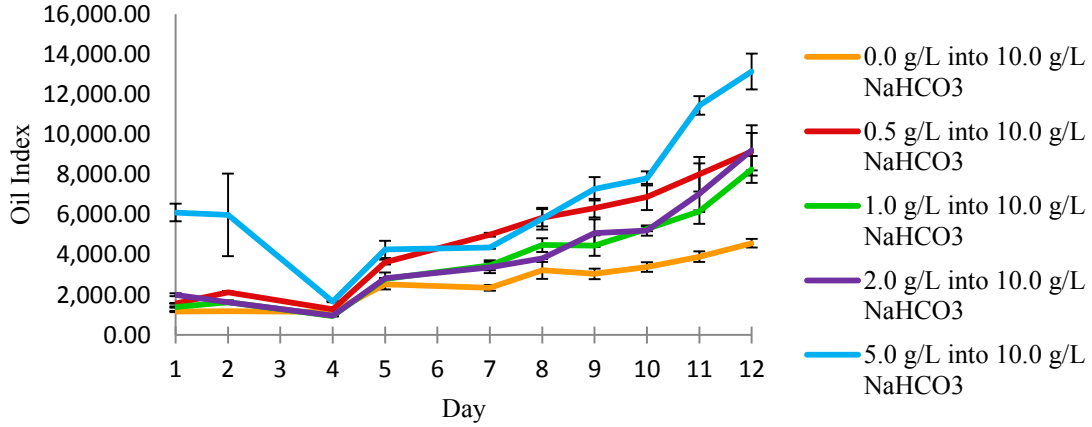


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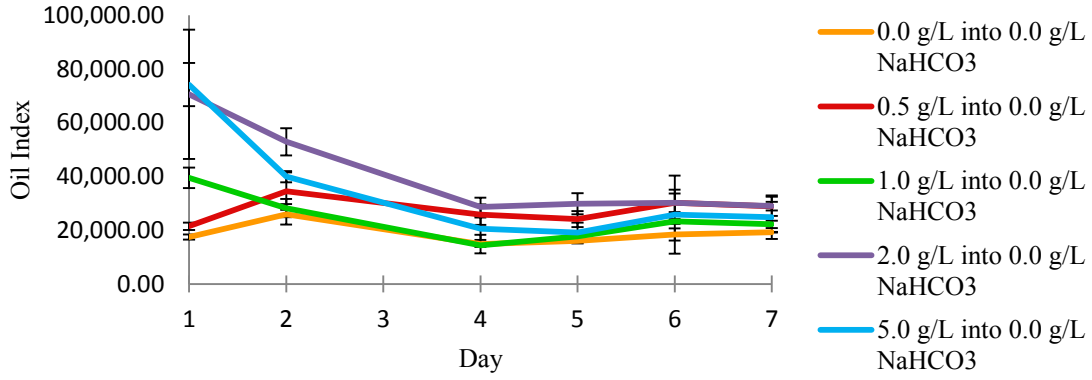


**Fig 13.** Lipid phase average oil indices for (a) non-boosted and (b) boosted *D. tertiolecta*, (c) non-boosted and (d) boosted *Mayamaea spp.*, and (e) non-boosted and (f) boosted *Synechococcus sp*

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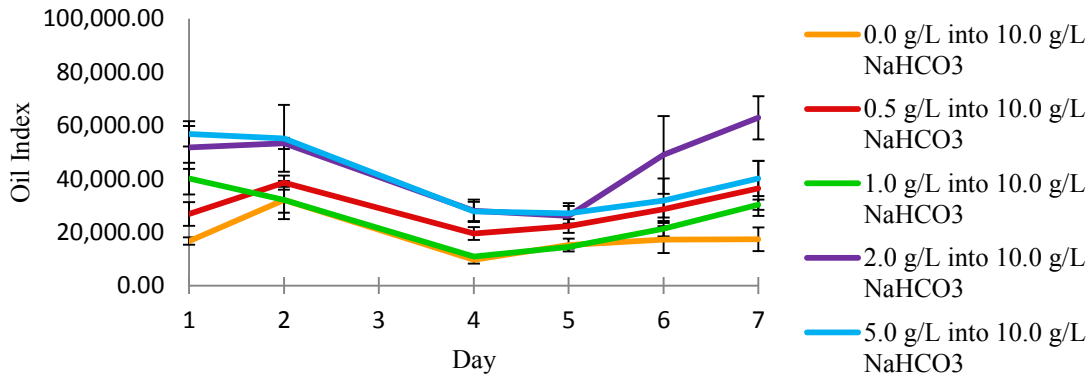


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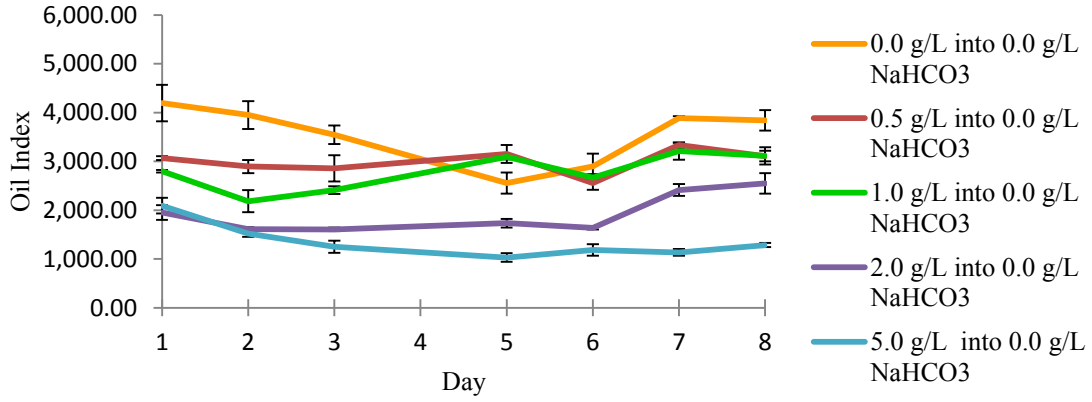


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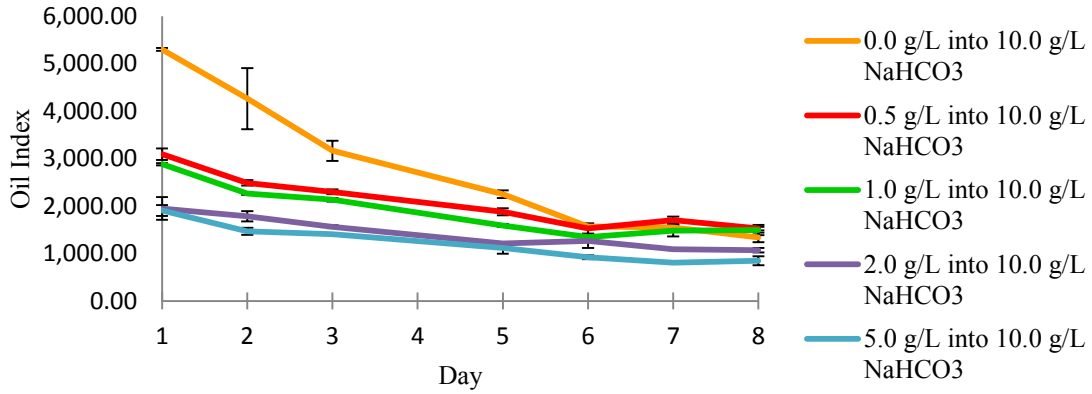


**Fig 13.** Continued

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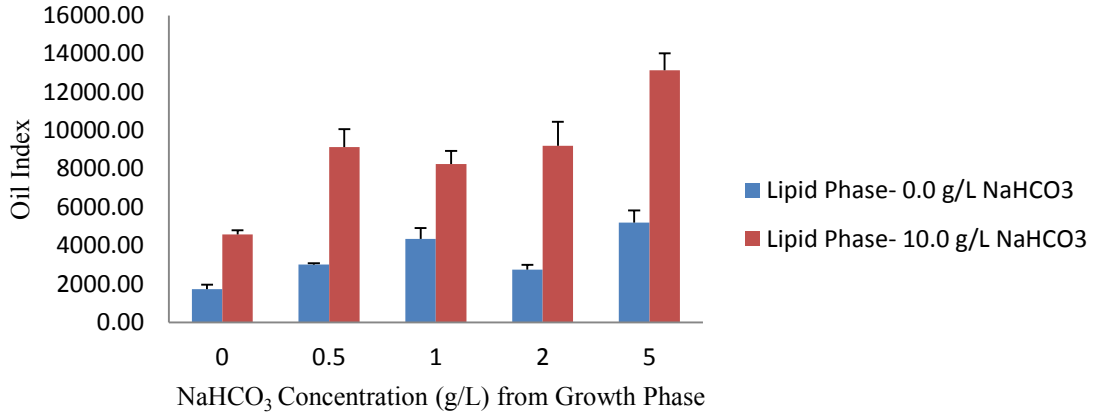


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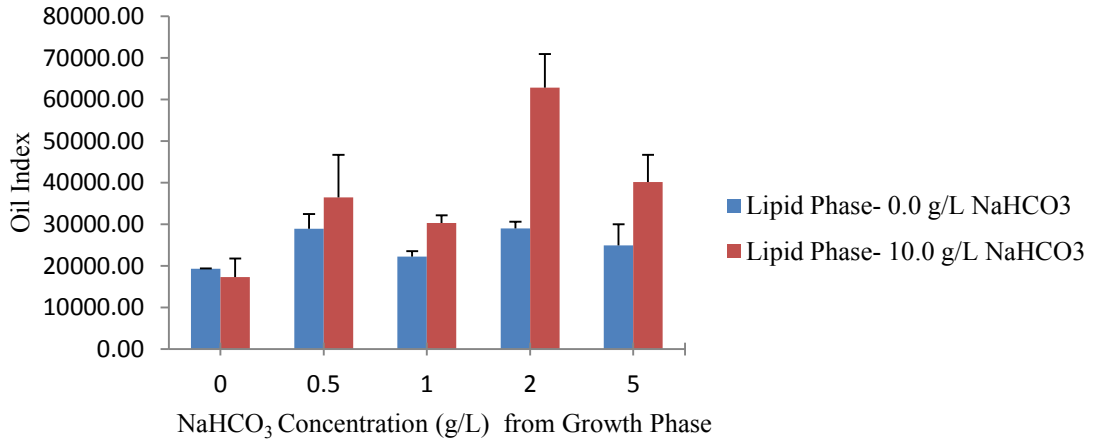


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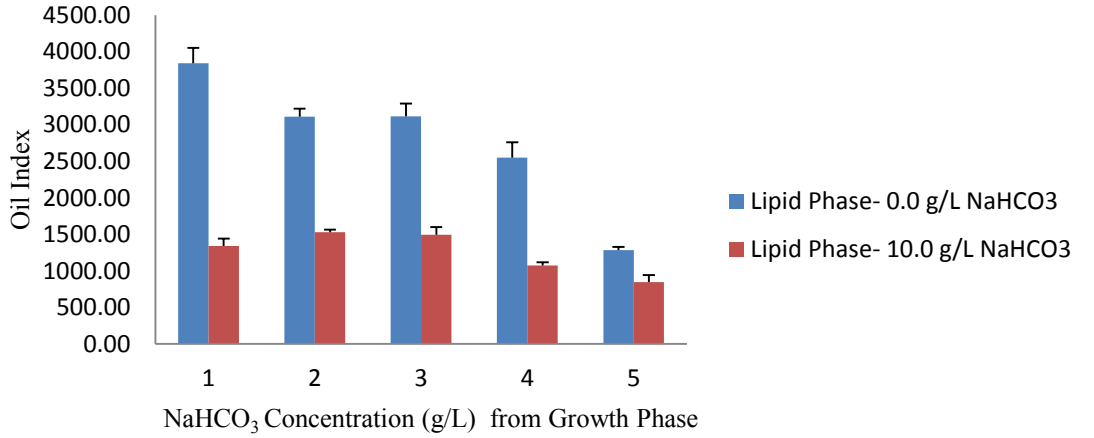
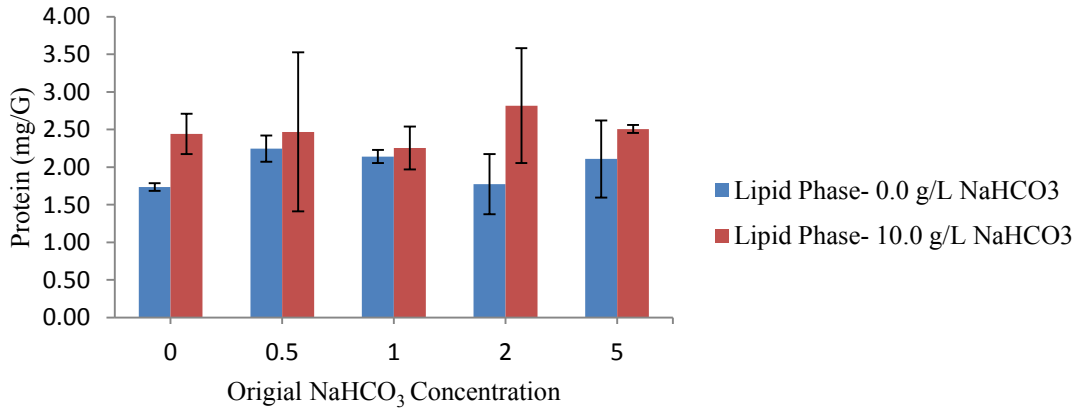



**Fig 14.** Comparison of final day oil averages between non-boosted and boosted treatments for (a) *D. tertiolecta*, (b) *Mayamaea spp.*, and (c) *Synechococcus sp*

Based on the average protein content in *D. tertiolecta* (Figure 15a), every treatment except for boosted 0.5 g/L and non-boosted 2.0 g/L and 5.0 g/L resulted in larger protein content than the control. Among all the ones that were higher than the control though, each fell within ±1 standard deviation of one another. While all of the boosted treatments did have higher averages than non-boosted, all the boosted treatments except 0.0 g/L fell within the standard deviations of the non-boosted treatments. For *Mayamaea spp*. the boosted treatments had higher averages against their non-boosted counterparts in all but the 5.0g/L concentration (Figure 15b). Similar to *D. tertiolecta* though, those treatments with higher protein did not show one clear ~~"b~~est" treatment for *Mayamaea spp*. since those highest treatments where all within ±1 standard deviation of each other. What is notably different however is when directly compared against the control, only the boosted 0.0g/L and 0.5 g/L had higher protein content that fell outside the standard deviation range of the control. For *Synechoccocus sp.* (Figure 15c) while all of the boosted treatments did have higher averages than their non-boosted counterparts, that difference was only larger than the standard deviation ranges for the 0.5 g/L and 2.0 g/L groups. All of the treatments except for the boosted 0.0 g/L and non-boosted 0.5 g/L and 0.2 g/L treatments had higher protein averages than the control group. Just as with the other two species, *Synechoccocus sp.* did not have a single treatment that was distinctly higher than the others when considering their standard deviations.

a

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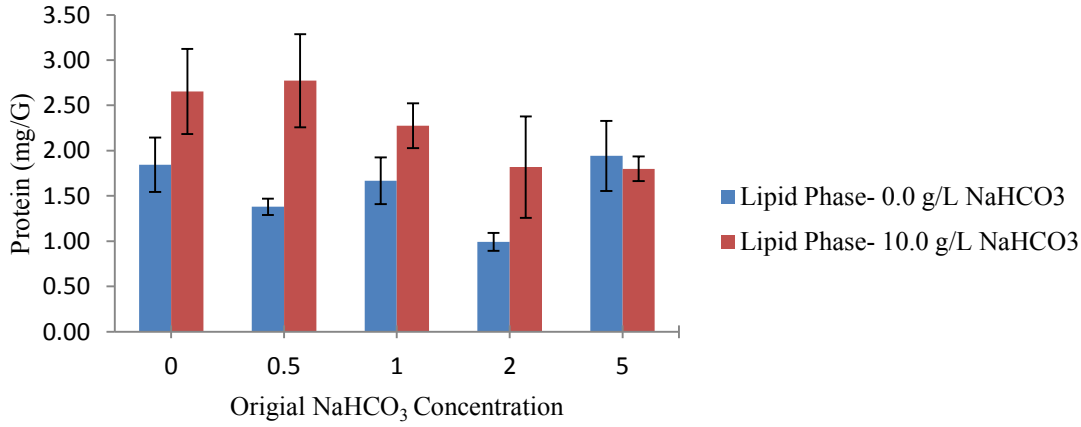


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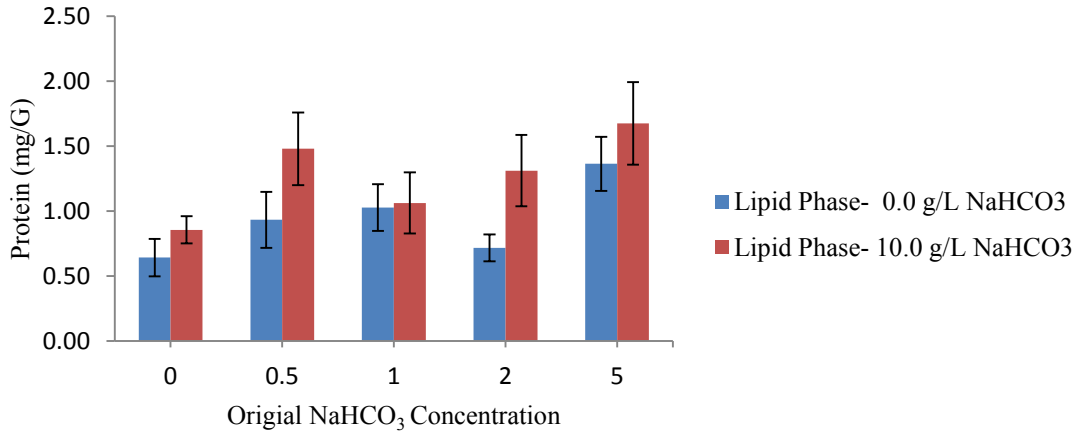


**Fig. 15** Final protein content (mg/G) of (a) *D. tertiolecta*, (b) *Mayamaea spp*., and (c) *Synechoccocus* sp. during lipid phase

# DISCUSSION

The species selected for this experiment all had one specific requirement, and that is to be a species able to survive in marine or brackish environments. The reason behind this was to encourage biofuels production that would not compete with freshwater resources. Additionally the three species come from three different families of phytoplankton, specifically a Chlorophyta (green), Bacillariphyta (diatom), and cyanobacteria species. These are representatives of each group and so the findings provide a result for how each particular group might respond to $NaHCO_3$. Also at the time of this research there was a lack of literature available for how cyanobacteria might respond to lipid accumulation with $NaHCO_3$ while several studies (Devgoswami et al., 2011; White et al., 2013) have examined the eukaryotic algae.

The first goal of this research was to determine which of the three species used would be able to successfully utilize $NaHCO_3$ as a photosynthetic carbon source. Based on the results of both the growth and lipid phase, all three species were able scale up from a small inoculation to a ~~h~~harvest" at the end of the lipid phase. While the cultures were able to get a small amount of atmospheric $CO_2$ available from the bottle headspace when they were shaken before sampling, that would not account for the difference in densities among each treatment. From the results there were some species-specific preferences, which were to be expected since the three species used were different types of algae (green, diatom, and cyanobacteria). In terms of which species appears to grow most efficiently by using $NaHCO_3$, *Synechoccocus sp*. had the highest overall growth rates across both the growth and lipid phase, followed by *Mayamaea spp*. then *D. tertiolecta*. This maybe a result of their differential abilities to use the various forms of carbon available in aquatic systems or may be because some species are known to employee a carbon concentrating or storage mechanisms to improve the efficiency of their photosynthetic activity (Chi et al., 2011).

For the growth phase, the best final density averages were measured in the 2.0 g/L for *D. tertiolecta* and *Mayamaea spp*., while *Synechoccocus* sp. was highest in 5.0

g/L $NaHCO_3$. The highest average growth rate was also found in the 5.0 g/L treatments.

This is inconsistent with findings that investigated different species and lower $NaHCO_3$ concentrations in a study by White et al., (2013). It showed 2.0 g/L treatments performed more poorly than, or at best case about equal to lower treatments of 1.0 g/L and 0.0 g/L $NaHCO_3$. In that study, the most efficient cell growth occurred in 1.0 g/L during the growth phase.

During the lipid phase, the two cultures with highest densities for each species occurred in treatments that had been boosted with 10.0 g/L $NaHCO_3$ compared to the control treatments. This was dependent on growth phase $NaHCO_3$ for all three species. These lipid phase density results were also inconsistent with White et al., (2013), since the boosted media had higher densities in the higher $NaHCO_3$ concentration groups. Other experiments worked with $NaHCO_3$ in concentrations less than 5 g/L (Devgoswami et al., 2011; Gardner et al., 2012; White et al., 2013), but this researched indicates that all three of the species used during these tests can acclimate and work in higher concentrations.

The Fv/Fm measurements throughout the growth and lipid phase also indicate that the overall photosynthetic efficiency of the cultures was enhanced by using $NaHCO_3$ as the primary carbon source. As mentioned in (Chi et al., 2011), many cyanobacteria and microalgae have the capacity to take up and utilize $HCO3^{-}$, so the presence of $NaHCO_3$ would likely help photosynthetic efficiency by providing a readily available carbon source and/or by buffering media pH. Throughout both the growth and lipid phase, *Mayamaea spp*. had an average Fv/Fm of 0.605 ±0.004 in all media types, while in *Synechoccocus* sp. the final average Fv/Fm was about 0.625 ±0.007 only in the all lipid phase media. Paradoxically, the growth phase Fv/Fm readings for *Synechoccocus* sp. were much lower, with readings below 0.3 in all growth phase $NaHCO_3$ concentrations. This does not indicate inefficient photosynthetic functions but rather the limitations of the instrument. *D. tertiolecta* also exhibited a somewhat similar result where the growth phase started peaked over days 3-5 all treatments showing Fv/Fm readings around 0.550 but then steadily dropping over the remainder of the

growth phase with the final average among all treatments at 0.408 ±0.017. Previous studies have shown that different species of phytoplankton respond differently to media. Also, a perusal at the websites that provide algae commercially (e.g., the National Center for Marine Algae and Microbiota - https://ncma.bigelow.org/) recommend specific media for specific species or groups of algae.

The second major goal of this was to examine the effects of $NaHCO_3$ had on lipid accumulation, especially using the mechanism of suddenly adding a significantly higher concentration of $NaHCO_3$. Based on the literature, this could cause or increase lipid production (Devgoswami et al., 2011; Gardner et al., 2012; White et al., 2013). The results obtained in this research were in agreement with their research showing $NaHCO_3$ can cause additional lipid accumulation.

For both *Mayamaea spp.* and *D. tertiolecta* the highest oil indices were measured in the 10.0 g/L ~~b~~boosted" $NaHCO_3$ concentrations during lipid phase from cultures which previously had been grown in the 2.0 g/L and 5.0 g/L concentrations respectively during growth phase. As an example of how the species react differently, *Mayamaea spp.* which had the highest overall oil index of all species for this experiment, had an oil index almost 4.8 times higher than the highest final average oil index in *D. tertiolecta*. This result was expected though with diatoms having been shown to typically have higher lipid (fatty acid) content (Volkman et al., 1989).

*Synechoccocus* sp. behaved in a completely different manner though. Not only the highest oil index measured in lipid media that had no $NaHCO_3$ added to it, but the treatments with the top four final average oil indices for this species were in non-boosted lipid media. This could be explained by looking at the growth data during the lipid phase, which showed that the culture densities of the boosted media were typically much higher i.e. undergoing more photosynthesis. This extra growth could indicate that *Synechoccocus* sp. was not being stressed in the boosted lipid media and was instead shifting back into a period of high growth, while the lipid media without additional $NaHCO_3$ was more stressful from a lack of fresh carbon input. The oil index data also supports this idea because in the boosted media, the oil indices dropped then stayed

consistently lower over time so there was not a need for the cells to focus on lipid production.

Beyond the effects on growth and lipid formation, using $NaHCO_3$ in production can assist in maintaining a stable pH by acting as a buffer. While this capacity did vary among each of the three species, $NaHCO_3$ use tended to result in either less acidic conditions or reduced variation in the pH during the time course of the growth and lipid phase experiments. It is especially noticeable in the lipid phase, particularly for *Mayamaea* spp. and *Synechoccocus* sp., that the boosted lipid media all resulted in both lower and more consistent pH values. For examples, the pH was 8.93 ±0.000 and 8.73 ±0.056 for boosted 5.0 g/L *Mayamaea* spp. and *Synechoccocus* sp. respectively, compared to the pH of 9.48 ±0.053 and 9.1 ±0.062 in their respective non-boosted 5.0g/L treatments.

While the *Synechoccocus* sp. grown in the 5.0 g/L growth phase treatment and then transferred to the control or non-boosted lipid phase treatment did initially show a close resemblance to the pH of the boosted concentrations, it showed a larger change from start to end date particularly in the final three days. Where the boosted media only increased in pH about 0.25 over the final three days, that non-boosted concentration increased by 0.5 over the same time.

It is already established that many species can utilize $NaHCO_3$ as a carbon source (Larsson & Axelsson, 1999), and the three species from this experiment can clearly do so even though they belong to different groups of phytoplankton. At the very least providing more $NaHCO_3$ results in more dissolved inorganic carbon available, some of which converts to $CO_2$. Adding $NaHCO_3$ to the media could therefore provide the possibility for a species to benefit from $NaHCO_3$ without actually using it. It would appear that based on the growth data (OD 750, growth rate, and Fv/Fm) that all of them could potentially be scaled up into a larger setting such as a raceway pond using $NaHCO_3$ instead of or in conjunction with $CO_2$, especially if cultures are given an adequate acclimation period. This would hopefully overcome the lowering Fv/Fm values found only in the growth period for *D. tertiolecta* and *Synechoccocus* sp.

Of the cultures tested, only *D. tertiolecta* and *Mayamaea* spp. showed signs of increased oil production during the lipid phase. This is a positive outcome in terms of biofuel production, because it indicates that $NaHCO_3$ can induce higher oil content. This is comparable with Gardner et al. (2012) which also found that $NaHCO_3$ can trigger lipid accumulation (triacylglycerols in their study). Gardner et al. (2012) also indicated that there could be some dependence on nitrogen depletion for $NaHCO_3$ to act as a trigger, but while $NaHCO_3$ and nitrogen might have an effect on each other, this research also showed that nitrogen depletion is not a requirement for lipid accumulation to occur. The next significant direction for biofuel research would be to continue looking at the interaction of $NaHCO_3$ and nitrogen depletion on lipid accumulation in algae. Would using just one method result in higher lipid content or to they work synergistically together to give a higher amount?

Beyond the lipid production, using $NaHCO_3$ does provide additional benefits larger scale operations. Particularly in the lipid phase the boosted treatments all showed signs that $NaHCO_3$ was acting in some kind of buffering capacity, either by keeping the final pH lower or keeping its variation over the entire phase lower than the non-boosted counterparts. Particularly in the large scale open raceway ponds, $NaHCO_3$ can be used as an alternative to bubbling $CO_2$ multiple times each day. Most of the $CO_2$ used in this method is simply wasted since raceway ponds are not deep to allow for photosynthesis, so there is only a small interface where the gas can go into solution. The overall loss to the atmosphere can constitute about 90% of the total $CO_2$ that is being added into the system (Becker, 1993). Personal communications with Dr. Ronald Lacey have included a biofuels production model that atmospheric loss increases the mass ratio of $CO_2$ input to biomass to be approximately 15:1 when using 1.5 g $CO_2$ for every 1.0 g of biomass as the stochiometric balance. This ratio suggests it takes approximately 15.0 kg $CO_2$ for 1.0 kg biomass. Those personal communications with Dr. Lacey also assumed that 1,000 kg of $CO_2$ would cost $19, so that would mean 1.0 kg of biomass would require $0.285 in $CO_2$ expense. That cost would vary among production sites and their specific needs, also factoring in things such as proximity and access to a $CO_2$ sources,

transportation, and storage needs. That escaped $CO_2$ is not only part of the expense (Norsker et al., 2011), but is putting a greenhouse gas directly back into the atmosphere, decreasing the carbon offsetting potential. Using $NaHCO_3$ would provide a readily available, dissolved inorganic carbon source for photosynthesis.

# CONCLUSION

Algae biofuels still require continued research to make them a more viable fuel source, but the potential for them to become viable is there. This research was to find a suitable alternative to using gaseous $CO_2$ as the inorganic carbon source not only for growth, but to potentially help with lipid production as well. The end result is that $NaHCO_3$ can be a suitable replacement for $CO_2$ in both growth and lipid accumulation, but two additional areas to take this research need to be addressed in the future. Specifically the next step should investigate how does $NaHCO_3$ compare to using nitrogen depletion (competitively and at the same time). While the cultures were using nitrogen, the lipid phase media provided a fresh source and it is unlikely for the *D. tertiolecta* and *Mayamaea* spp. cultures to have depleted it enough to cause their highest oil indices to increase as fast as they did. The other thing to investigate in what situations could a large scale production using $NaHCO_3$ (exclusively or supplementary to $CO_2$) result in any cost savings. Continuing to make the growth and lipid accumulation stages of algal biofuel production more efficient will help increase the chances for algae biofuels to become a successful industry.

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