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Whitings as a Potential Mechanism for Controlling Atmospheric Carbon Dioxide Concentrations – Final Project Report

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ABSTRACT

Species of cyanobacteria in the genera *Synechococcus* and *Synechocystis* are known to be the catalysts of a phenomenon called "whitings", which is the formation and precipitation of fine-grained $CaCO_3$ particles. Whitings occur when the cyanobacteria fix atmospheric CO_2 through the formation of $CaCO_3$ on their cell surfaces which leads to precipitation to the ocean floor and subsequent entombment in mud. Whitings represent one potential mechanism for CO_2 sequestration.

Research was performed to determine the ability of various strains of *Synechocystis* and *Synechococcus* to calcify when grown in microcosms amended with 2.5 mM HCO_3^- and 3.4 mM Ca^{2+}. Results indicated that while all strains tested have the ability to calcify, only two, *Synechococcus* species, strains PCC 8806 and PCC 8807, were able to calcify to the extent that $CaCO_3$ was precipitated. Enumeration of the cyanobacterial cultures during testing indicated that cell density did not appear to have an effect on calcification. Factors that had the greatest effect on calcification were CO_2 removal and subsequent generation of alkaline pH. As CO_2 was removed, growth medium pH increased and soluble Ca^{2+} was removed from solution. The largest increases in growth medium pH occurred when CO_2 levels dropped below 400 ppmv.

Precipitation of $CaCO_3$ catalyzed by the growth and physiology of cyanobacteria in the Genus *Synechococcus* represents a potential mechanism for sequestration of atmospheric CO_2 produced during the burning of coal for power generation. *Synechococcus* sp. strain PCC 8806 and *Synechococcus* sp. strain PCC 8807 were tested in microcosm experiments for their ability to calcify when exposed to a fixed calcium concentration of 3.4 mM and dissolved inorganic carbon concentrations of 0.5, 1.25 and 2.5 mM. *Synechococcus* sp. strain PCC 8806 removed calcium continuously over the duration of the experiment producing approximately 18.6 mg of solid-phase calcium. Calcium removal occurred over a two-day time period when *Synechococcus* sp. strain PCC 8807 was tested and only 8.9 mg of solid phase calcium was produced. The ability of the cyanobacteria to create an alkaline growth environment appeared to be the primary factor responsible for $CaCO_3$ precipitation in these experiments.

These research results demonstrate the potential of using cyanobacterial catalyzed "whitings" as a method to sequester CO_2 from the atmosphere.

SUMMARY

During the research that was performed; the following specific conclusions were drawn regarding the precipitation of calcium carbonate by cyanobacteria:

- All species of *Synechococcus* and *Synechocystis* tested were able to calcify which was evident by the formation of calcium carbonate.

- Only two species of *Synechococcus* (PCC 8806 and PCC 8807) were able to form calcium carbonate to the extent of what could be considered "whitings"; the precipitation of fine grained calcium carbonate crystals.

- Of the factors measured during microcosm experiments, cell density appeared to have the least effect on formation of calcium carbonate.

- Factors that appeared to have the greatest effect on calcium carbonate formation was carbon dioxide removal and generation of alkaline pH in the growth medium.

- Removal of calcium ions in the microcosm appeared to be catalyzed by both microbiological processes as well as chemically mediated processes such as increased growth medium pH.

- Fixation of carbon dioxide into biomass appeared to be a minor mechanism for removal during microcosm experiments.

- Molecular tools developed to monitor expression of genes associated with inorganic carbon transport in cyanobacterial cells appeared to have limited utility as a tool to estimate when calcium carbonate precipitation would occur.

- During large laboratory scale experiments (35 gallon), under growth conditions that would be expected in the environment; calcium carbonated formation was not initiated.

Calcification by *Synechococcus* sp. strain PCC 8806 appears to be a feasible alternative for sequestration of CO_2 through precipitation as $CaCO_3$. Calculated estimates scaling up calcification in microcosm studies to the area of whitings events that have been demonstrated on the Great Bahama Bank showed that *Synechococcus* sp. strain PCC 8806 produced enough $CaCO_3$ to account for over half of the CO_2 that would be produced during the operation of a 500 MW coal-fired power plant for one year.

ACKNOWLEDGMENTS

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1. BACKGROUND

Ice cores from the Antarctic have indicated that atmospheric CO_2 concentrations have been steadily increasing for the past 250 years (2003). Atmospheric CO_2 concentrations measured at the Mauna Loa Observatory in Hawaii indicate an increase from approximately 316 ppmv in March of 1958 to a high of 374 ppmv in December of 2004 (Keeling and Whorf 2004) Anthropogenic CO_2 emissions have increased by an average of 1.2% per year during the past 12 years with estimated emissions for 2002 reaching 5.8×10^9 metric tons of CO_2 (2003). Economic growth as well as increased demand indicates that this trend may continue. While the effects of elevated CO_2 levels on global climate are uncertain, there is consensus among scientists that a doubling of atmospheric CO_2 concentrations in the foreseeable future could have a variety of adverse environmental consequences.

The U.S. Department of Energy published a report in 1999 titled, "Carbon Sequestration Research and Development," (Reichle et al. 1999) that details research needs related to CO_2 capture and sequestration. Part of this report focuses on developing a roadmap for carbon capture and sequestration research and development, which includes the development of enhanced ocean processes and advanced biological adsorption technologies. For this reason, new and innovative carbon capture and control technologies must be developed to control projected increases in CO_2 emission over the next several decades. Microbial-based technologies, specifically those utilizing photoautotrophs, represent a promising solution once methods of carbon uptake and disposition by the cell are determined.

In considering the earth's carbon distribution, it is relatively easy to ascertain that much of the carbon that is represented in the global carbon cycle is sequestered (for the most part permanently) primarily as calcium and calcium-magnesium carbonates (Ehrlich 1996). In many cases, the carbonates are of biogenic origin, some precipitated by bacteria, cyanobacteria, and fungi. Calcium or calcium-magnesium carbonates are precipitated by numerous mechanisms, one of which is photo- and chemosynthetic autotrophy in the presence of Ca and Mg counterions.

Factors important in $CaCO_3$ precipitation are: (1) calcium concentration; (2) dissolved inorganic carbon (DIC) concentration; (3) the pH of the growth environment; and (4) the availability of nucleation sites for the formation of $CaCO_3$ (Hammes and Verstraete 2002). Many bacterial species have been implicated in the precipitation of $CaCO_3$ (Castanier et al. 1999; Milliman et al. 1999; Fujita et al. 2000; Hammes and Verstraete 2002; Cacchio et al. 2003; Hammes et al. 2003); a phenomenon that occurs in diverse environments (e.g., travertine deposits in psycrophilic (Sanchez-Moral et al. 2003)as well as thermophilic environments (Pentecost 2003)). Microbes facilitate these processes by: increasing surface area for nucleation (Dittrich et al. 2004); creating alkaline environments near the cell surface (de Vrind-de Jong and de Vrind 1997); and increasing concentrations of DIC through their physiological activity (Dittrich et al. 2003).

Precipitation of carbonaceous sediments by oxygenic photosynthetic bacteria classified as cyanobacteria is of particular interest because cyanobacteria represent a diverse group of photosynthetic prokaryotes that exhibit versatile physiology and wide ecological tolerance that has allowed them competitive success in a broad spectrum of environments. They are found in numerous terrestrial environments; but, more importantly, they are common in freshwater bodies, such as the Great Lakes, and the cyanobacterium, *Synechococcus*, contributes up to 50% of chlorophyll a biomass in oligotrophic

oceans (Waterbury 1999). In addition, marine cyanobacteria are responsible for an estimated 20–40% of carbon fixation in oceans (Partensky et al. 1999).

2. MATERIALS AND METHODS

2.1 Species Screening

2.1.1 Cultures and Growth Conditions

Various *Synechocystis* and *Synechococcus* strains (Table 1) were grown on Modified ASN-III medium: 427mM NaCl, 9.8mM $MgCl_2 \cdot 6H_2O$, 6.7mM KCl, 8.8mM $NaNO_3$, 14.2mM $MgSO_4 \cdot 7H_2O$, 3.4mM $CaCl_2 \cdot 2H_2O$, 0.19mM Na_2CO_3, 0.09mM K_2HPO_4, 0.015mM citric acid, 0.015mM ferric ammonium citrate, 0.0015mM disodium magnesium EDTA, 1 ml Trace Metal Mix A5+Co (see below), 20 µg/L Vitamin B_{12}. (Trace metal mix A5+Co ($g \cdot l^{-1}$): H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.222, $Na_2MoO_4 \cdot 2H_2O$ 0.390, $CuSO_4 \cdot 5H_2O$ 0.079, $Co(NO_3)_2 \cdot 6H_2O$ 0.049). All chemicals were from Fisher Scientific (Fair Lawn, NJ).

Cultures were maintained in 50 ml of ASN-III medium in a 125-ml Erlenmeyer culture flask. The cultures were incubated at a temperature of 25°C, with a 12-hour light/dark cycle, 28µmol $s^{-1}m^{-2}$ of photon irradiance, and shaking at 120 rpm in a Psycrotherm Environment Incubator/Shaker Model G27 w/illumination programmer (New Brunswick Scientific, Edison, NJ). Five percent of the culture was transferred to fresh medium every 2 to 3 weeks.

2.1.2 Experimental Set-up

The cultures listed above were tested for microbe-initiated calcification. The experiment took place in clear glass serum bottles (500 ml) containing 200 ml Modified 617 medium (see below) and sealed with a rubber septum and aluminum crimp ring. Modified 617 medium consisted of the following: 17.65 mM $NaNO_3$, 0.30 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 171mM NaCl, 0.18mM K_2HPO_4, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM disodium magnesium EDTA, 1 ml Trace metal mix A5 + Co (see above under "Cultures and Growth Conditions"), 20 µg Vitamin B_{12}. To eliminate residual CO_2 the medium and bottle headspace were purged with nitrogen, then oxygen was reintroduced to a final concentration of 21% (v/v). Sodium bicarbonate was added to each bottle to a final concentration of 152 mg bicarbonate/L (2.5 mM). The bottles were inoculated with each of the above mentioned cultures to a target concentration of 1×10^5 cells ml^{-1}. Cell numbers were determined by direct count on a Nikon Eclipse E600 epifluorescence microscope with an Opti Quip Model 1600 power supply equipped with a blue/violet filter (ca. 420 nm). The pH was adjusted to pH = 8.0 using 1M NaOH. Each

Table 1. Cyanobacterial used in this experiment.

Genus	Strain	Environment of Isolates
Synechocystis	PCC 7338	Tidal pool, Bodega Head, CA, USA
	PCC 7339	Tidal pool, Bodega Head, CA, USA
	ATCC 29152	Bank above high water, Bodega, CA, USA
	ATCC 29108	Brackish water, Oregon, USA
	ATCC 27266	Hypersaline lake, Salton Sea, CA, USA
Synechococcus	PCC 8806	Water sample, lagoon, Port Gentil, Gabon
	PCC 8807	Water sample, lagoon, Port Gentil, Gabon
	PCC 7336	Sea water tank, Berkeley, CA, USA
	ATCC 27265	Sand sample at edge of clam bed, Greenwich, CT, USA

Note: ATCC—American Type Culture Collection (Rockville, MD, USA)
PCC—Pasteur Culture Collection (Paris, France)

culture was set up in triplicate. During the calcification studies, the bottles were incubated at 25°C, at a photon irradiance of 28 μmol $s^{-1}m^{-2}$, using a 12 hour light/dark cycle, and were shaken at 60 rpm in a Psycrotherm Environment Incubator/Shaker. Analyses were performed on samples taken periodically over a two-week period.

2.2 Species Comparison

2.2.1 Cyanobacterial Cultures

Agar slants of *Synechococcus* sp. Strain PCC 8806 and *Synechococcus* sp. Strain PCC 8807 were received from the Pasteur Culture Collection and cultures were maintained in ASN-III growth medium (Rippka et al. 1979). Cultures were maintained at 25°C, at a photon irradiance of 28 μmol $s^{-1}m^{-2}$, using a 12 hour light/dark cycle, and were shaken at 60 rpm in a Psycrotherm Environment Incubator/Shaker.

2.2.2 Experimental Set-up

Experiments were performed in clear glass serum bottles (500ml) containing 200 ml Modified 617 medium (see below) and sealed with a rubber septum secured by an aluminum crimp ring. Modified 617 medium consisted of the following: 17.65 mM $NaNO_3$, 0.30 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 171 mM NaCl, 0.18 mM K_2HPO_4, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM disodium magnesium EDTA, 1 ml Trace metal mix A5+Co (see above under "Cultures and Growth Conditions"), 20 μg Vitamin B_{12}. Modified 617 medium was used instead of ASN-III because the high NaCl concentration in ASN-III interfered with polarized light microscopy (See below).

To eliminate residual CO_2 the medium and bottle headspace were purged with nitrogen, then oxygen was reintroduced to a final concentration of 21% (v/v). Sodium bicarbonate was used as the DIC source and was added to the growth medium to generate concentrations of 0.5, 1.25, and 2.5 mM. A control containing cells and no bicarbonate was also set up. The bottles were inoculated with each of the above mentioned cultures to a target concentration of 1×10^5 or 1×10^6 cells ml^{-1}. Cell numbers were determined by direct count on a Nikon Eclipse E600 epifluorescence microscope equipped with a blue/violet filter (ca. 420 nm). The pH was adjusted to 8.0 using 1M NaOH. Each bicarbonate concentration was set up in triplicate. During the calcification studies, the bottles were incubated at 25°C, at a photon irradiance of 28 μmol $s^{-1}m^{-2}$, using a 12 hour light/dark cycle, and were shaken at 60 rpm in a Psycrotherm Environment Incubator/Shaker. Analyses were performed on samples taken periodically over a two-week period.

During analyses, triplicate microcosms were sampled for each bicarbonate concentration tested and the values were averaged. The mean value from each triplicate set of microcosms was graphed; including error bars showing the standard deviation from each set.

Bicarbonate-free controls for the microcosm experiments were set up similar to the active microcosms. Briefly, headspace gases were removed by purging the medium with nitrogen. Oxygen was added to the headspace at a concentration of 21% and then 3.4 mM calcium was added to the liquid medium.

2.3 Large Laboratory Scale

2.3.1 Culture Conditions

Cultures used to inoculate the large laboratory scale experiment were maintained at 25°C, at a photon irradiance of 28 μmol $s^{-1}m^{-2}$, using a 12 hour light/dark cycle, and were shaken at 60 rpm in a

Psycrotherm Environment Incubator/Shaker. Two 500 ml cultures were used to inoculate the large culture.

2.3.2 Experimental Conditions

A 40 gallon aquarium was used to run the large laboratory scale experiments. The growth medium in the tank was composed of 12 g/L Instant Ocean to better simulate nutrient conditions in the ocean. The initial calcium concentration in the tank was 200 mg/L. Nitrate and phosphate were added to the tank as macronutrients for growth of *Synechococcus* sp. strain PCC 8806 at the start of the experiment and was added on a weekly basis during the experiment. The tank was inoculated to a density of 2.13×10^4 cells per ml. Gentle mixing of the tank was performed using a variable speed batch mixer. The tank was sampled on a weekly basis for pH, calcium concentration, and cell density. Samples were also taken for analysis of gene expression.

2.4 Analyses

2.4.1 Calcium

Two ml of each sample was filtered through a 0.2 μm Gelman nylon acrodisc syringe filter to remove cells and calcium carbonate precipitate. The pH was measured and recorded then 0.1 ml of 1 N HCl was added. The samples were analyzed for calcium using a Thermo Jarrell Ash Iris II Inductively Coupled Plasma-Atomic Emission Spectrophotometer (ICP-AES). Before analyzing on the ICP-AES 1 gram of each sample was placed in a poly tube. Then 1 ml of Aqua Regia (which is made up of three parts HCl and one part HNO_3) was added to the sample and digested in a microwave oven for 20 minutes. After the samples cooled they were spiked with an internal standard (100 μl of 200 mg/L yttrium) and then analyzed on the ICP-AES.

2.4.2 Cell Counts

Cell growth was monitored by direct microscopic counts using epifluorescence. Cyanobacterial cells from 5 μl to 5 ml of growth medium were captured on a 25 mm diameter 0.2 μm black polycarbonate filters which were placed on a glass microscope slides. The cells were counted using a Nikon Eclipse E600 microscope with a blue/violet filter (exciter: 425/40, DM Mirror: 460, Barrier: 475). Cyanobacterial cells fluoresced bright red under these conditions; therefore, no staining was required.

2.4.3 Gas Chromatography

Carbon dioxide in the headspace of the vials was measured using gas chromatography. The gas chromatograph was a Hewlett-Packard 5890 equipped with a thermal conductivity detector and a Chrompack Poraplot Q column (0.32 μm dia, 27.5 m length). The carrier gas was helium at a flowrate of 2 ml min-1, with a split ratio of 1:10. The injection temperature was set at 125 °C, the detector temperature was set at 250 °C, and the oven temperature was maintained at 30 °C.

2.4.4 Alkalinity

The alkalinity was measured using a HACH phenolphthalein and total alkalinity kit (method 8203) with a digital titrator (HACH, Inc., Loveland, CO). The samples were filtered through a 0.2 μm Gelman nylon acrodisc syringe filter and diluted 1:20 with water. Samples were titrated to an endpoint pH of 5.1 using 0.16 N sulfuric acid.

2.4.5 Polarized Light Microscopy

Calcification was observed microscopically using a polarized light microscope (Nikon Eclipse E800). Samples were prepared by placing 10 µl of growth medium on a glass microscope slide and allowed to air dry. The cells were observed under 1000X magnification. $CaCO_3$ appeared as rainbow colored, amorphous crystals directly adjacent to or surrounding the cells.

2.4.6 Reverse Transcription Real Time PCR

2.4.6.1 RNA Extraction/Isolation. Cells were harvested via centrifugation or filtration depending on cell density at time of harvest. Cells were then stored in RNA Later at -20°C until RNA was extracted. Cells were again centrifuged to remove RNA Later and the following lysis protocol was followed; 300 µl 3 mg/ml lysozyme in TE buffer was added to the cell pellet and re-suspended pellet by vortexing. The cell slurry was then transferred to a MicroBead tube (MOBIO) and heated at 37°C for 10 minutes with brief vortexing every 3 minutes. The 300 µl of cells and lysozyme were divided into 3 100 µl aliquots in MicroBead tubes. Added 350 µl Buffer RLT (RNeasy Mini Kit from Qiagen for total RNA) with β-Mercaptoethanol to each and briefly vortexed. Beads were removed by centrifugation and then the supernatant was removed and placed in a clean 1.5 ml centrifuge tube. An RNeasy Mini Kit (Qiagen) protocol was used for bacterial RNA isolation. Following RNA extraction, the RNA samples were treated with DNase (DNA-free kit, Ambion) to remove any traces of DNA.

2.4.6.2 Synthesis of complementary DNA (cDNA). A SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize cDNA from RNA extracted from the cells. Synthesis of cDNA was attempted using both random hexamers and gene specific primers; the method for gene specific primers gave the best results. In addition, the primer concentration was increased 3X for optimum synthesis, and used only the reverse primers for cDNA synthesis. See the Table 2 below for the primers and housekeeping genes used for the reverse transcription and real-time PCR.

2.4.6.3 Real-Time Polymerase Chain Reaction. A Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) kit was used to amplify the cDNA prepared above as the template. The following thermocycler program was used for real-time amplification of the cDNA samples: the samples were held at 50°C for 5 mins to degrade any non-specific nucleic acid contamination. cDNA was then denatured by holding at 95°C for 5 mins, followed by 45 cycles of denaturation at 95°C for 20 secs, annealing 55°C for 30 secs, and extension at 72°C for 40 secs. A final extension was done at 72°C for 2 mins, and then a melt curve analysis was performed from 50°C to 99°C. The fluorescent signal was acquired on the FAM/Sybr channel following extension of the cDNA amplicon.

Table 2. Sequences used for synthesis of cDNA and for real-time PCR.

Primer	Sequence 5'→ 3'
7942 cmpA F	TCG CAA CTT CTT TAA CGT G
7942 cmpA R	TTG TAG GGA TAG GAG ACA CTG
cmpB F	TCG AAA AGC AAG TAC CTA ATG
cmpB R	AAC AGC ACC GAT ATA AAT CAC
cmpC F	AGG ATT GAT CAT GAG TTT ATT TG
cmpC R	CAA CTC TAG ACC AGC AAT CAG
Housekeeping gene	Sequence 5'→ 3'
ppc F	CCT CAA GCT CTC CTA TAT TC
ppc R	GAA TCA GCT TGA GAT CTT CG

3. RESULTS

3.1 Species Screening

Two separate sets of experiments were run to determine the ability of nine strains of cyanobacteria in the genera *Synechococcus* and *Synechocystis* to calcify when grown under identical conditions in regard to bicarbonate and calcium concentrations. Experiments were run under batch conditions, closed to the atmosphere with bicarbonate added as the sole source of dissolved inorganic carbon (DIC). The primary objective of the research was to compare the ability of different species of cyanobacteria to alter their growth environment to the extent that calcification and precipitation of $CaCO_3$ would occur. Each strain of cyanobacteria was tested in triplicate. During analyses, each microcosm was sampled and the values were averaged. The mean value from each triplicate set of microcosms was graphed; included error bars show the standard deviation from each set.

Utilization of inorganic carbon in the bottles was measured by monitoring CO_2 in the headspace above the growth medium. As DIC transport by the cyanobacteria proceeded, the pH of the growth medium increased leading to uptake of CO_2 from the headspace as bicarbonate became the dominant DIC species in accordance with carbonate equilibria. Prior to examining the data, it is important to note that fixation of CO_2 by cyanobacteria occurs via the Calvin reductive pentose phosphate pathway or Calvin cycle (14); consequently, some of the CO_2 removed during the experiment was fixed as biomass.

Removal of CO_2 from the first five strains tested can be seen in Figure 1A. Two *Synechococcus* species, PCC 8806 and PCC 8807 began removing CO_2 sooner in the experiment than the other three cyanobacterial species tested. *Synechococcus* PCC 8806 completely removed the CO_2 within five days and was the only species tested that was able to maintain the CO_2 concentration near zero. This was the case even when bicarbonate was added during the experiment on days 5, 8 and 12. *Synechococcus* PCC 8807 also began removing CO_2 relatively quickly, but could not remove the CO_2 to the extent seen by *Synechococcus* PCC 8806 during the first 12 days of the experiment. Complete removal of CO_2 by *Synechococcus* PCC 8807 was seen by day 12 of the experiment and at all sampling points thereafter. Strains ATCC 29152 and PCC 7339, both *Synechocystis* species, showed lower levels of CO_2 removal initially but by day 12 performed as well as *Synechococcus* PCC 8807. *Synechocystis* PCC 7338 demonstrated the slowest initiation of CO_2 removal and did not remove the CO_2 to the extent demonstrated by the other species tested in parallel. Significant removal of CO_2 by *Synechocystis* species PCC 7338, PCC 7339 and ATCC 29152 occurred between days five and seven of the experiment. Decreases in CO_2 concentration of nearly 400 ppmv occurred during this time. While no bicarbonate was added to the control, CO_2 levels in this bottle increased initially but then leveled out after ten days of incubation.

Three additional species of cyanobacteria were tested in a second set of experiments to monitor the ability of different species of cyanobacteria to calcify. Figure 1B shows CO_2 removal demonstrated by these cultures during the experiment. The three species, *Synechocystis* ATCC 29108, *Synechococcus* ATCC 27265 and *Synechocystis* ATCC 27266 demonstrated comparable removal over the entire experiment. *Synechococcus* ATCC 27265 decreased the CO_2 in less time, but by day 12 of incubation, removal in all of the bottles was similar. Significant removal of CO_2 was again demonstrated by these strains four days into the experiment. Between days four and six, all three strains removed high amounts of CO_2 from the serum bottle headspace.

The CO_2 concentration in the control remained stable for most of the experiment with the exception of a slight increase in CO_2 concentration prior to day six of the experiment.

Cell growth of the cyanobacterial species used for testing is shown in Figures 2A and 2B. Experiments were initiated at a target, uniform cell density of 1×10^5 cells ml^{-1}. Since bicarbonate was added to the microcosm bottles over the course of the experiment, most of the cultures used for testing did not reach a stationary phase of growth during the experiment, with the exception of the species *Synechocystis* 29152 and *Synechococcus* PCC 8807 (Figure 2A). The cyanobacterial cell density of *Synechocystis* 29152 reached an apparent stationary phase of growth eight days into the experiment. *Synechococcus* PCC 8807 cell numbers dropped significantly after reaching a maximum cell density on day eight of the experiment. While the apparent growth rate of all the species was similar during the first few days of the experiment, by day ten of the experiment, *Synechococcus* PCC 8807 had grown to a cell density significantly higher than the other species tested in the same experiment (Figure 2A), as well as the species tested in the second set (Figure 2B). The most significant data was the decrease in cell density shown in the bottle containing *Synechococcus* PCC 8807 after day nine of growth. From data generated, this decrease in cell density was associated with precipitation of cells due to encrustation with $CaCO_3$.

Like decreasing CO_2 concentrations in the headspace, increasing pH is another indicator of active DIC uptake by cyanobacteria, and is also advantageous for calcification due to the decreased solubility of $CaCO_3$ at elevated pH. Increasing pH may be caused by one of two mechanisms; first, hydroxide ions produced during the conversion of the bicarbonate ion by the carbonic anhydrase enzyme within the cell to provide CO_2 for photosynthesis is transferred to the exterior of the cell leading to increased pH (de Vrind-de Jong and de Vrind 1997). A second potential mechanism is an increase in pH due to the import of H^+ when Ca^{2+} is transported from the cell (Yates and Robbins 2001; Hammes and Verstraite 2002). By this mechanism acidic as well as alkaline regions are formed on the surface of the cell. In one location on the cell surface, as Ca^{2+} is transported into the cell, two molecules of H^+ are transferred outside the cell and acidic regions are formed. In alternate locations of the cell, as Ca^{2+} is actively transported out of the cell, H^+ is sequestered as charge is balanced and alkaline regions are formed. Typically under environmental conditions, the increase in pH occurs near the surface of the cell within the hydrodynamic boundary, while in the batch conditions used for testing the effect of the pH increase at the cell surface is eventually reflected in the bulk growth medium. Figure 3A shows the increase in pH associated with strains PCC 8807, PCC 8806, PCC 7339, PCC 7338 and ATCC 29152. The pH of the control increased from 8.0 at the beginning of the experiment and stabilized near 8.5 and remained at this level for the rest of the experiment. *Synechocystis* ATCC 29152 showed the least change in pH over the test period, generating a maximum pH of approximately 9.0 by day 12. *Synechococcus* PCC 8807 produced the highest pH during the test, with a maximum of 10.7. Growth medium pH for this strain increased sharply between days three and six, followed by a more gradual increase between days six and thirteen. Strain PCC 8806 demonstrated the next highest increase in pH, but the rise in pH was more gradual compared to PCC 8807. Both *Synechocystis* strains PCC 7338 and 7339 gradually increased the pH to 9.5 by day 12 of the experiment. While *Synechococcus* strains PCC 8806 and PCC 8807 began to increase pH from the onset of the experiment, *Synechocystis* strains PCC 7339, PCC 7338 and ATCC 29152 all required a 5-day lag period before producing an increase in pH.

Synechocystis strains ATCC 29108 and ATCC 27266 and *Synechococcus* ATCC 27265 raised the pH of the growth medium to above 10, but as described for the strains tested during the first leg of the experiment there was a 4-day lag prior to this increase (Figure 3B). The pH level in the control for this leg of the experiment remained near 8.0 for the entire experiment.

As the pH of the growth medium increases, carbonate will be the dominant inorganic carbon species; thereby, favoring calcification by the incorporation of carbonate ion into a growing $CaCO_3$ crystal. Alkalinity of the bulk growth medium was used as an indicator of inorganic carbon speciation during the experiment. Figure 4A shows alkalinity data for the first leg of the experiment in which *Synechococcus* strains PCC 8806 and PCC 8807 and *Synechocystis* strains PCC 7338, 7339 and ATCC 29152 were tested. Alkalinity for these vials was tested at the beginning of the experiment ($t = 0$)

and at the conclusion of the experiment (t = 12 days). At time zero, alkalinity from bicarbonate was less than 25 mg/L in all of the test bottles and was near 50 mg/L in the control bottle. By day 12 of the experiment, alkalinity had shifted entirely toward CO_3^{2-} and OH^- for *Synechococcus* strains PCC 8806 and 8807, and was by far the highest in the growth medium containing PCC 8807. For *Synechocystis* strains PCC 7338 and PCC 7339, alkalinity was nearly evenly split between HCO_3^- and CO_3^{2-} but was not represented by OH^-. Alkalinity in the bottle containing *Synechocystis* ATCC 29152 was predominantly HCO_3^- reaching a level of nearly 200 mg/L, with slight alkalinity from CO_3^{2-}. Alkalinity in the control was entirely from HCO_3^- but the level remained below 100 mg/L.

Alkalinity values for *Synechocystis* strains ATCC 29108 and ATCC 27266 and *Synechococcus* strain 27265 were uniform over the duration of the second experiment (Figure 4B). Alkalinity levels were checked at times 0, 7 and 18 days. The effect on alkalinity from HCO_3^- was near 140 mg/L at times 0 and 7, but dropped to near zero on day 18. By day 18, CO_3^{2-} alkalinity had increased to between 60 and 80 mg/L while alkalinity from OH^- increased to near 30 mg/L. In the control vial, alkalinity levels hovered near 120 mg/L as HCO_3^- for the entire experiment.

Concentrations of calcium in the first leg of the experiment can be seen in Figure 5A. The largest amount of calcium was removed by the microcosm containing *Synechococcus* PCC 8806. In addition to removing the most calcium by any of the strains tested, calcium removal occurred earlier in the experiment; beginning shortly after day five of the experiment. While *Synechococcus* PCC 8807 began removing calcium earlier in the experiment than the other strains tested, the overall amount removed was similar. The other strains tested (PCC 7339 and PCC 7338) all began removing calcium after day twelve of the experiment. These results were generated after pH values in the growth medium had reached a maximum (See Figure 3A). No calcium removal occurred in the microcosm containing ATCC 29152. Interestingly, the pH in the growth medium of this microcosm only reached a level of approximately nine, while pH in the growth medium of the other strains tested was above 9.5. This is no table because the aqueous carbonate equivalence point in which carbonate becomes the dominant form of inorganic carbon is reached at a pH of approximately 9.5 (Stumm and Morgan 1996). Therefore, precipitation of $CaCO_3$ would be dominant in microcosms in which the pH was above 9.5 due to incorporation of carbonate and calcium into the forming solid.

Cyanobacterial strains ATCC 29108, ATCC 27265 and ATCC 27266 all began removing calcium after day eleven of the experiment (Figure 5B). In a manner similar to the strains tested in the first leg of the experiment, calcium removal began after pH levels had reached maximum levels above pH 10 in the growth medium, as shown in Figure 3B.

Calcification was verified in the microcosms microscopically using plane-polarized light birefringence. Samples (10 µl) were taken at the end of the experiment, dried and then viewed using a Nikon Eclipse E800 polarized light microscope. This analysis was used as a qualitative measure of calcite formation in the microcosms. No differences were noted between the microcosms at the end of the experiment, cells from all of the microcosms demonstrated birefringent ring on the periphery of the cells upon analysis. While quantification of the $CaCO_3$ was not attempted, the presence of calcite on the cell surface indicates that all of these strains were able to calcify.

The amount of carbon fixed as biomass versus how much was removed by incorporation into $CaCO_3$ was determined for strains PCC 8807 and PCC 8806. Carbon fixed as biomass was estimated using average carbon to cell volume ratios for *Synechococcus* of 0.47 pg C μm^{-3} established by Verity et al (1992). Polarized light microscopy indicated cell diameters for PCC 8807 and PCC 8806 of 1.5 and 2.0 µm, respectively. Carbon removed via incorporation into the $CaCO_3$ crystal was estimated by assuming a 1:1 molar ratio of carbon removed per calcium removed from solution. Since PCC 8807 generated a higher cell density during the experiment, more carbon was removed as biomass (2.21 mg C)

compared to 1.32 mg C removed as biomass by PCC 8806. Conversely, more carbon was removed via formation of $CaCO_3$ by PCC 8806 (4.8 mg C), while PCC 8807 removed only 2.16 mg C as CaCO3. More $CaCO_3$ formed on the surface of the bottles used when testing PCC 8806 than 8807 which may explain this difference in carbon removed as $CaCO_3$. This would indicate that more $CaCO_3$ may have been removed by precipitation away from the cell than cell associated as was seen with PCC 8807.

3.2 Species Comparison

Microcosms experiments were performed to determine the effect of bicarbonate concentration on $CaCO_3$ precipitation by *Synechococcus* sp. strain PCC 8806 and *Synechococcus* sp. strain PCC 8807. Both of these strains demonstrated $CaCO_3$ precipitation levels greater than those shown by other species of *Synechococcus* and *Synechocystis* (Lee et al. 2004). Indirect properties monitored to follow calcification included; cell density, growth medium pH and soluble calcium. In addition, direct evidence of calcification was determined using polarized light microscopy to monitor the presence or absence of $CaCO_3$ on the cell surface or in the vicinity of cells by the presence of a zone of birefringence around the cells. Finally the study was completed by determining partitioning of the calcium into different phases in the microcosm.

Changes in cell density for *Synechococcus* PCC 8806 can be seen in Figure 6. Results showed that growth occurred at HCO_3^- concentrations of 1.25 and 2.5 mM but not at 0.5 mM. While cyanobacterial growth would be expected at an HCO_3^- concentration of 0.5 mM (Badger and Price 2003) it is not known why the cell density did not increase in this microcosm. As might be expected, the maximum cell density increased as HCO_3^- concentration increased. A slight decrease in *Synechococcus* PCC 8806 cell density was noted in the microcosms containing 1.25 and 2.5 mM HCO_3^- after day 13. Cell density remained stable during the experiment in the 0 mM HCO_3^- control.

Synechococcus sp. strain PCC 8807 showed similar growth characteristics in response to varying HCO_3^- concentrations, except this strain grew when exposed to 0.5 mM HCO_3^- (Figure 7). During the first three days of the experiment, cell density in the control increased along with cell density in the test microcosms. This growth could have been caused by residual CO_2 in the vial or within the cell upon inoculation. Patterns of cell growth were similar when the culture was grown at 0.5 and 1.25 mM HCO_3^-. Cell density in both microcosms increased until day six of the experiment; followed by slight decreases in cell density when sampled on days 9 and 14 of the experiment. The most noteworthy data from the comparison came from the microcosm in which cells were grown at 2.5 mM HCO_3^-. After day three of sampling, cell density increased at a greater rate than cells grown at 0.5 and 1.25 mM HCO_3^- until day nine of sampling. Following sampling on day 9, the cell density decreased in the culture growing at 2.5 mM HCO_3^-.

A second experiment was performed to monitor factors important to calcification using *Synechococcus* PCC 8807 grown at 2.5 mM HCO_3^-. More time points were taken over the duration of the experiment to help understand what was happening in the culture (See Figure 7; □). While the initial cell density in the culture was higher than the previous experiment, the results were very similar. Cell density increased during the first seven days of the experiment, with a maximum of approximately 1×10^7 cells/ml being achieved on day seven. Following day seven, cell density gradually decreased at each successive sampling point. Finally, cell density decreased to below the initial level.

Increase in pH over time demonstrated for *Synechococcus* PCC 8806 is shown in Figure 8. By day eight of the experiment the cells grown at an HCO_3^- concentration of 0.5 mM had increased the pH of the growth environment to an average pH of 10.8 for the three replicate microcosms. The pH of the growth medium in the cells grown at 1.25 and 2.5 mM HCO_3^- increased to near 10 by day eight of the experiment. By the conclusion of the experiment on day 15 the pH was similar for all three HCO_3^-

concentrations tested. The pH of the growth medium in controls containing no HCO_3^- remained between 7 and 8 for the duration of the experiment.

Increases in the growth medium pH for *Synechococcus* sp. strain PCC 8807 at all of the HCO_3^- concentrations tested were identical over the duration of the test (Figure 9). Growth medium pH remained fairly stable during the first three days of testing; increasing slightly (i.e., ~0.2 pH units). By day six of the experiment, the pH had increased to an average pH of 9.9. With the exception of cells grown at 2.5 mM HCO_3^- the pH was stable for the remainder of the experiment. Cells grown at 2.5 mM HCO_3^- demonstrated an increase in pH to just above 10 by the conclusion of the experiment. The pH in a microcosm containing cells but no HCO_3^- increased slightly at the beginning of the experiment but remained at a pH of just below 8 for the entire experiment.

Soluble calcium is important to monitor as an indicator of $CaCO_3$ formation by cyanobacteria. Removal of calcium from solution by *Synechococcus* sp. strain PCC 8806 can be seen in Figure 10. Calcium levels decreased at all HCO_3^- concentrations tested; whereas, no calcium removal was demonstrated in a control receiving no HCO_3^-. More calcium was removed as HCO_3^- concentration increased; the lowest calcium removal was demonstrated at 0.5 mM HCO_3^- while the maximum was demonstrated at 2.5 mM HCO_3^-. Interestingly, calcium removal was continuous over the duration of the experiment in microcosms receiving 0.5 and 2.5 mM HCO_3^-, while at 1.25 mM HCO_3^- calcium removal lagged until day four of the experiment followed by a sharp decrease during days four through eight. Interestingly, cell density (Figure 6) increased on day four which coincided with initiation of calcium removal in the 1.25 mM HCO_3^- microcosm.

Calcium removal results for the microcosm experiments run for *Synechococcus* sp. strain PCC 8807 are shown in Figure 11. In contrast to results demonstrated for PCC 8806, calcium removal was not seen at all HCO_3^- concentrations tested. Microcosm experiments in which PCC 8807 cells were exposed to 2.5 mM HCO_3^- showed the only calcium removal; and calcium removal did not begin until after day six of the experiment. In addition, less calcium was removed overall in these experiments when compared to PCC 8806. Removal of calcium by PCC 8807 tested at 0.5 and 1.25 mM HCO_3^- did not differ significantly from the control containing no HCO_3^-.

Analyses were performed at the conclusion of the experiment to determine calcium partitioning in the experiment. Calcium concentrations in the microcosms receiving 2.5 mM HCO_3^- for both species were compared. Fractions measured included; calcium in solution, calcium in the cell pellet and calcium bound to the microcosm vessel. Results from these analyses can be seen in Figure 12. Calcium added was the mass measured for each microcosm at the beginning of the experiment, while total calcium was that recovered in all fractions at the conclusion of the experiment.

Data for the HCO_3^--free controls indicated that irregardless of test conditions, approximately 1% of the calcium added was bound to the glass during the experiment. Roughly 4% of the calcium added at the beginning of the experiment adsorbed to the cells and was removed when the cells were separated from the growth medium. Some of this calcium could have been carried over in residual growth medium that remained on the cells following centrifugation. With the exception of the small amount bound to the glass, nearly all of the calcium in the microcosm with no cells remained in solution. Total recovery of calcium in the PCC 8806 microcosm without HCO_3^- was 102%, while recovery of calcium in the HCO_3^--free control for PCC 8807 was 99.7%.

More calcium was removed by *Synechococcus* sp. strain PCC 8806 than *Synechococcus* sp. strain PCC 8807 in the microcosms containing 2.5 mM HCO_3^-. Calcium removal through precipitation of $CaCO_3$ by PCC 8807 in the presence of HCO_3^- was only slightly higher than cells with no HCO_3^-. PCC 8806 was able to remove nearly 2.64 mg more of cell-associated calcium than PCC 8807 by the end

of the experiment. From the data generated it appears that the primary mechanism for removal of calcium was by adsorption onto the glass vessel used for experimentation, but only when cells were present, growing and presumably increasing the pH in the growth environment allowing nucleation of $CaCO_3$ on the surface of the glass. No determination was made as to whether the $CaCO_3$ crystals were nucleating in scratches on the glass surface or whether cells or cell material had absorbed to the glass and $CaCO_3$ was nucleating on the cyanobacterial cells. Maintenance cultures of these cyanobacteria show that cells do grow on the surface of the glass in experiments where lower levels of calcium are added to the growth medium. Average recovery of calcium in the microcosms for PCC 8806 and PCC 8807 was 96% and 92.7%, respectively. Calcium lost from this mass balance is assumed to be a product of inefficient extraction of calcium from the glass surfaces of the bottles. The relatively low value for PCC 8807 may have also been a factor of calcium lost with cells during harvest. The amount of calcium recovered with the cells seems lower than would be expected.

Assuming a 1:1 molar ratio between calcium and $CaCO_3$ and the duration of the experiment, calculations indicate a relatively large amount of $CaCO_3$ was precipitated per day of activity. Production rates for each strain tested at 2.5 mM bicarbonate were calculated by dividing the total amount of calcium measured at the end of the experiment by the number of days after calcium removal was initiated in either microcosm. While different rates of calcium removal are expected from day to day an average value was used to simplify calculations. Calcification by *Synechococcus* sp. strain PCC 8806 accounted for approximately 3.1 mg of $CaCO_3$ per day; while *Synechococcus* sp. strain PCC 8807 removed roughly 1.7 mg of $CaCO_3$ over a similar time period. Rate calculations for PCC 8806 were more straightforward since calcium removal was for the most part continuous over the duration of the experiment (Figure 10). Calcium removal was not continuous for PCC 8807; most calcium was removed between days six and eight of the experiment.

Figure 13 represents a series of polarized light micrographs showing $CaCO_3$ formation on the surface of cyanobacterial cells. Preparations of the cells were viewed as both wet and dry mounts and results for both were similar. The only exception was that there was no formation of other crystals, such as NaCl, on the wet mounts. Dry mounts were used for testing because the samples lasted for longer periods of time. Calcium carbonate formation was qualitatively verified by the presence of a zone of birefringence around the periphery of the cell. In the gray scale images used, birefringence is seen as bright white areas in the micrographs. Under conditions where there was no HCO_3^- added there was no birefringence noted for either *Synechococcus* sp. strain PCC 8806 or *Synechococcus* sp. strain PCC 8807 (Figures 13A and 13B, respectively). The larger crystals generated by the drying of other minerals in the growth medium can also be seen in the micrographs; birefringence was noted in relationship to these crystals, but was presumed to be $CaCO_3$ crystals forming on the surface of the NaCl crystals. In addition, uncharacterized areas of birefringence can be seen that are not associated with the cells (Figure 13B).

Figure 8B shows *Synechococcus* sp. strain PCC 8807 cells (indicated by arrows) among other culture debris that was dried during preparation of the sample. Large areas of birefringence can be seen in the micrograph that are presumed to be areas where $CaCO_3$ crystals have dried.

Results for *Synechococcus* sp. strain PCC 8806 grown at 2.5 mM HCO_3^- can be seen in Figure 13C. Arrows indicate cells demonstrating zones of birefringence; while the square shows cells that are presumed to have just divided that do not show any signs of the zone of birefringence. Birefringence was also noted at the edge of other crystals that formed due to the drying for viewing the sample. PCC 8806 cells were typically present as single cocci or as pairs. Rarely the cells were seen in groups and the groups were typically associated with large clusters of crystals presumed to be $CaCO_3$ due to the birefringence under the polarized light.

Figure 13D shows the most interesting results from the polarized light microscopy; chains of *Synechococcus* sp. strain PCC 8807 cells demonstrating the zone of birefringence. PCC 8807 cells appear to be grouped among a larger mass of $CaCO_3$. Pairs of cells were also noted that demonstrated birefringence that was not apparent in groups. Cells in this microcosm were typically in pairs or chains of the pairs. There did not appear to be cells that were not associated with $CaCO_3$ for this strain of *Synechococcus*.

On a practical basis; the findings of this research are substantial related to sequestration of CO_2 from the atmosphere. Using the $CaCO_3$ generation results for the experiments an estimate of total CO_2 removed during an extended whitings event can be calculated. Whitings events on the Great Bahama Bank can extend between 35 and 200 km^2 in area; with an average area of approximately 70 km^2 (Robbins et al. 1997). Table 1 shows the potential for CO_2 removal through the calcification process. Assumptions used during calculation were an average whitings area of 70 km^2 and an average depth of 5 m. While whitings are typically temporary occurrences; calculations were made assuming continuous calcification. Results indicate that *Synechococcus* sp. strain PCC 8806 could remove up to 2.5×10^{12} g of $CaCO_3$ in a single year; while *Synechococcus* sp. strain PCC 8807 demonstrated removal results of 1.4×10^{12} g of $CaCO_3$ in a single year. Comparing cell density and calcium removal during the microcosm experiments it appeared that calcification by PCC 8807 ended in loss of cells as well as cessation of calcium removal. Calcification by PCC 8806 appeared for the most part to be continuous. These results are comparable to yearly whiting mass numbers generated by Robbins *et al.* (Robbins et al. 1997) in a study of the lime mud budget of the Great Bahama Bank.

Using cellular carbon estimates from our previous research (Lee et al. 2004); and carbon to cell rations developed by Verity et al.; (Verity et al. 1992) it is estimated that an additional 8.1×10^5 g of CO_2 would be fixed as biomass during such an event. This finding indicates that the amount of CO_2 fixed as biomass by *Synechococcus* sp. strain PCC 8806 under the conditions tested is inconsequential compared to the amount fixed during calcification.

Using the net equation for the dissolution of CO_2 into the ocean and formation of $CaCO_3$ below:

$$Ca^{2+} + CO_2 + H_2O \leftrightarrow CaCO_3$$

We can see that for every mole of $CaCO_3$ formed there is one mole of CO_2 sequestered. Amounts of $CaCO_3$ generated by these species of cyanobacteria are substantial related to CO_2 production from coal fired power generation. A 500 MW power plant produces approximately 4×10^{12} g of CO_2 per year (Herzog and Golomb 2004). We see from the $CaCO_3$ precipitation results that approximately half of the CO_2 generated could be accounted for by $CaCO_3$ precipitation by *Synechococcus* sp. strain PCC 8806.

3.3 Large Laboratory Scale

Results for cell density and growth medium pH can be seen in Figure 14 below. The largest increase in pH appeared to be during an early stationary phase for the culture; at this point cell density increased minimally while growth medium pH increased greater than one unit, from approximately 7 to above 8. Logarithmic growth occurred in the culture during weeks one through four. During this time there were minimal changes in growth medium pH. After week four, cell density and pH decreased slightly.

Macro- and microscopic analysis of cells in the tank revealed no indication of calcification. Samples are currently awaiting calcium analysis in another laboratory. The apparent lack of calcification may have been a result of conditions that were not conducive to nucleation of calcium carbonate crystals.

Higher cell density and pH seen during our previous batch microcosm studies appeared to be the catalyst for calcification.

Cyanobacterial cells were concentrated via filtration to generated biomass for DNA and mRNA extraction. Transport of inorganic carbon in the form of bicarbonate is controlled by a number of constitutive and inducible transport protein complexes. Genes for inducible bicarbonate transporters; represented by the *cmpABCD* operon, are expressed under low carbon dioxide concentrations. Expression of *cmpA* from cells harvested from the tank showed that these genes were expressed at higher levels during the first two weeks of the experiment and that as the experiment proceeded lower levels were noted. These results would indicate that as the *Synechococcus* PCC 8806 was growing exponentially that the carbon dioxide concentration that the cell was seeing was low causing expression of these genes. When cell density was at the maximum and the culture had reached an apparent stationary phase from approximately weeks three through four, expression returned to baseline levels.

4. CONCLUSIONS

Data from microcosm experiments used to determine the ability of various species of cyanobacteria to precipitate $CaCO_3$ demonstrated that all strains tested were able to calcify, but only two strains demonstrated calcification early in the experiment. *Synechococcus* species PCC 8806 and 8807 began removing CO_2 earlier in the experiment than the other strains tested, leading to alkaline pH levels conducive to $CaCO_3$ precipitation earlier in the experiment. This increase in pH led to the subsequent precipitation of $CaCO_3$ as indicated by the removal of Ca^{2+} from solution. Other strains tested demonstrated similar physiological characteristics, but these changes occurred later in the experiment.

Research presented indicates that calcification by various species of *Synechococcus* and *Synechocystis* may represent a feasible biological alternative for CO_2 sequestration. Specifically, *Synechococcus* sp. Strains PCC 8806 and PCC 8807 demonstrated significant levels of calcification to the extent of precipitating $CaCO_3$ from solution. Optimization of the $CaCO_3$ precipitation will require in depth analysis of mechanisms of inorganic carbon transport by these cyanobacteria. For instance, research to determine the effect of HCO_3^- transport on cell surface pH, which is the primary driver of $CaCO_3$ nucleation, counter ion incorporation and precipitation. Under conditions of high HCO_3^-, a limited number of constitutive transporters are more than likely functioning to provide CO_2 for carbon fixation by the cells. As conditions of low HCO_3^- are encountered, inducible, high-affinity transporters are expressed increasing the number of transporters in the cytoplasmic membrane. An increase in these transporters probably leads to more alkaline sites on the cell surface leading to nucleation of $CaCO_3$ crystals. Further research will be required to determine the exact link between inorganic carbon transport and $CaCO_3$ formation.

Results from microcosm experiments to determine the degree of calcification by *Synechococcus* sp. strain PCC 8806 and *Synechococcus* sp. strain PCC 8807 showed that a substantial amount of CO_2 could be sequestered as $CaCO_3$. Of the two strains tested, *Synechococcus* sp. strain PCC 8806 produced nearly twice as much $CaCO_3$ over the same time period. Rates of $CaCO_3$ production were also higher. $CaCO_3$ production by *Synechococcus* sp. strain PCC 8807 appeared to be a terminal event; at the conclusion of the experiment, calcium removal had ceased and cell density dropped to near initial cell numbers. Calcium removal was continuous over the duration of the experiment for *Synechococcus* sp. strain PCC 8806 and while growth slowed near the end of the experiment; there was not significant loss in cell density.

Increases in cell density appeared to coincide with the gradual decrease in calcium over time for *Synechococcus* sp. strain PCC 8806 as long as pH levels were high enough for CO_3^{2-} to be the major species of inorganic carbon present. Results for this species seem to indicate that cell growth continues while calcification is occurring. The opposite appeared to be true for *Synechococcus* sp. strain PCC 8807; calcium removal appeared to be a short term event and cell number decreased once calcification had been initiated.

Calcification by *Synechococcus* sp. strain PCC 8806 appears to be a feasible alternative for sequestration of CO_2 through precipitation as $CaCO_3$. Fixation of CO_2 into biomass appeared to be inconsequential compared to incorporation into the growing $CaCO_3$ crystal. Calculated estimates scaling up calcification in microcosm studies to the area of whitings events that have been demonstrated on the Great Bahama Bank showed that *Synechococcus* sp. strain PCC 8806 produced enough $CaCO_3$ to account for over half of the CO_2 that would be produced during the operation of a 500 MW coal-fired power plant for one year.

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6. FIGURES

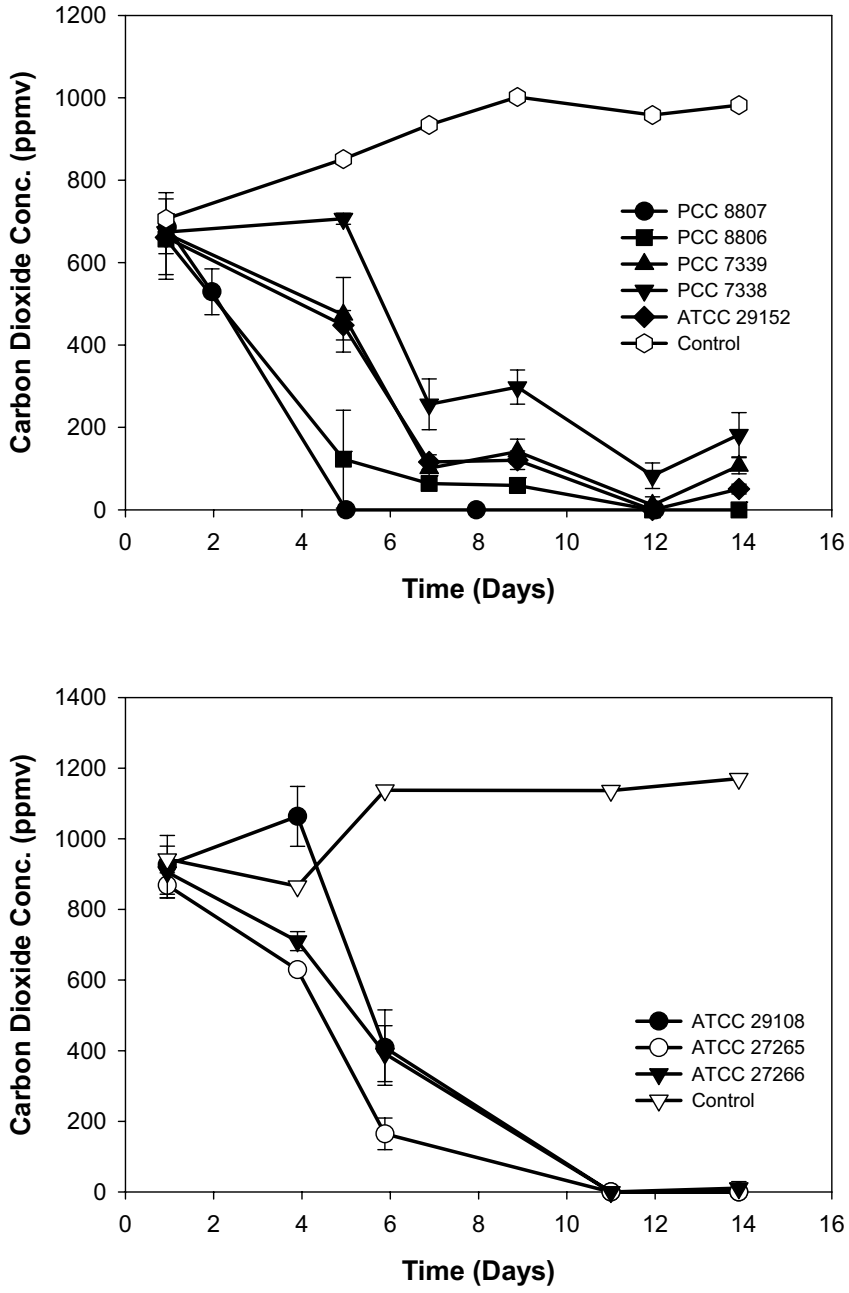

Figure 1. Removal of CO_2 from the headspace of microcosm experiments used to determine the ability of various strains of cyanobacteria to calcify. (A) First leg of the experiment in which *Synechococcus* sp. Strains PCC 8807 and 8806 and *Synechocystis* sp. Strains PCC 7338, PCC 7339 and ATCC 29152 were tested. (B) Second leg of the experiment in which *Synechococcus* sp. Strain ATCC 27265 and *Synechocystis* sp. Strains ATCC 27266 and ATCC 29108 were tested.

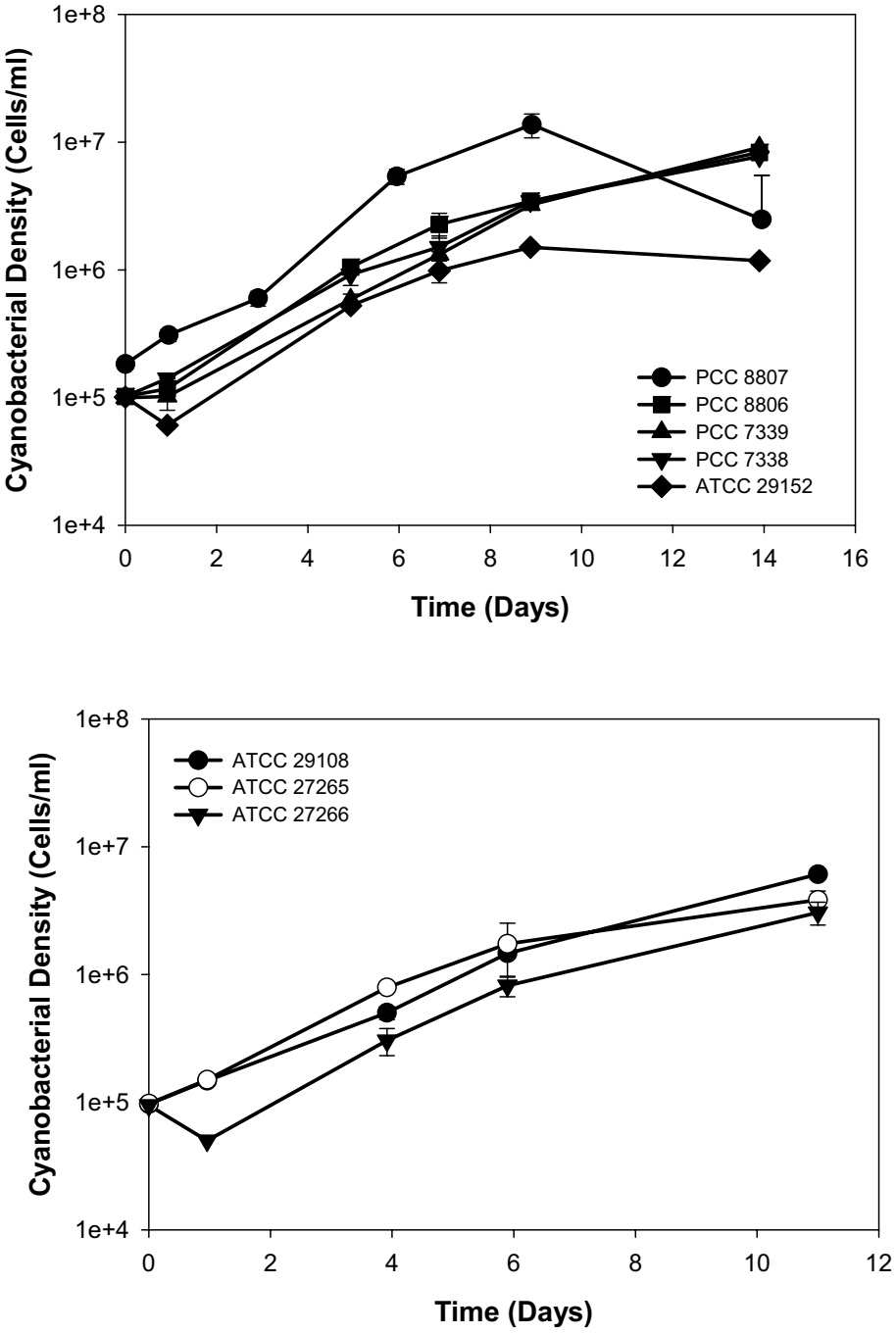

Figure 2. Growth of cyanobacterial cultures in microcosm experiments to determine the ability of various strains of cyanobacteria to calcify. (A) First leg of the experiment in which *Synechococcus* sp. Strains PCC 8807 and 8806 and *Synechocystis* sp. Strains PCC 7338, PCC 7339 and ATCC 29152 were tested. (B) Second leg of the experiment in which *Synechococcus* sp. Strain ATCC 27265 and *Synechocystis* sp. Strains ATCC 27266 and ATCC 29108 were tested.

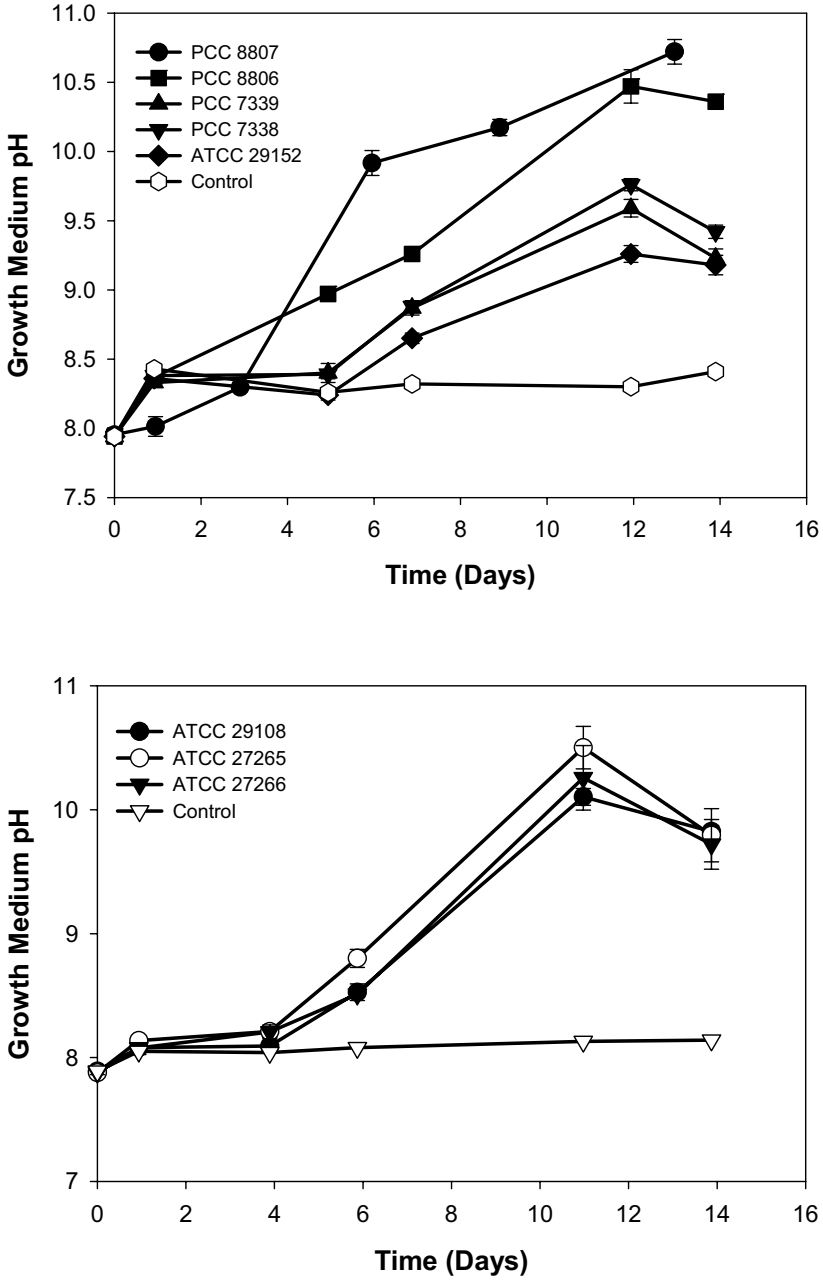

Figure 3. Change in pH over the duration of microcosm experiments to determine the ability of various strains of cyanobacteria to calcify. (A) First leg of the experiment in which *Synechococcus* sp. Strains PCC 8807 and 8806 and *Synechocystis* sp. Strains PCC 7338, PCC 7339 and ATCC 29152 were tested. (B) Second leg of the experiment in which *Synechococcus* sp. Strain ATCC 27265 and *Synechocystis* sp. Strains ATCC 27266 and ATCC 29108 were tested.

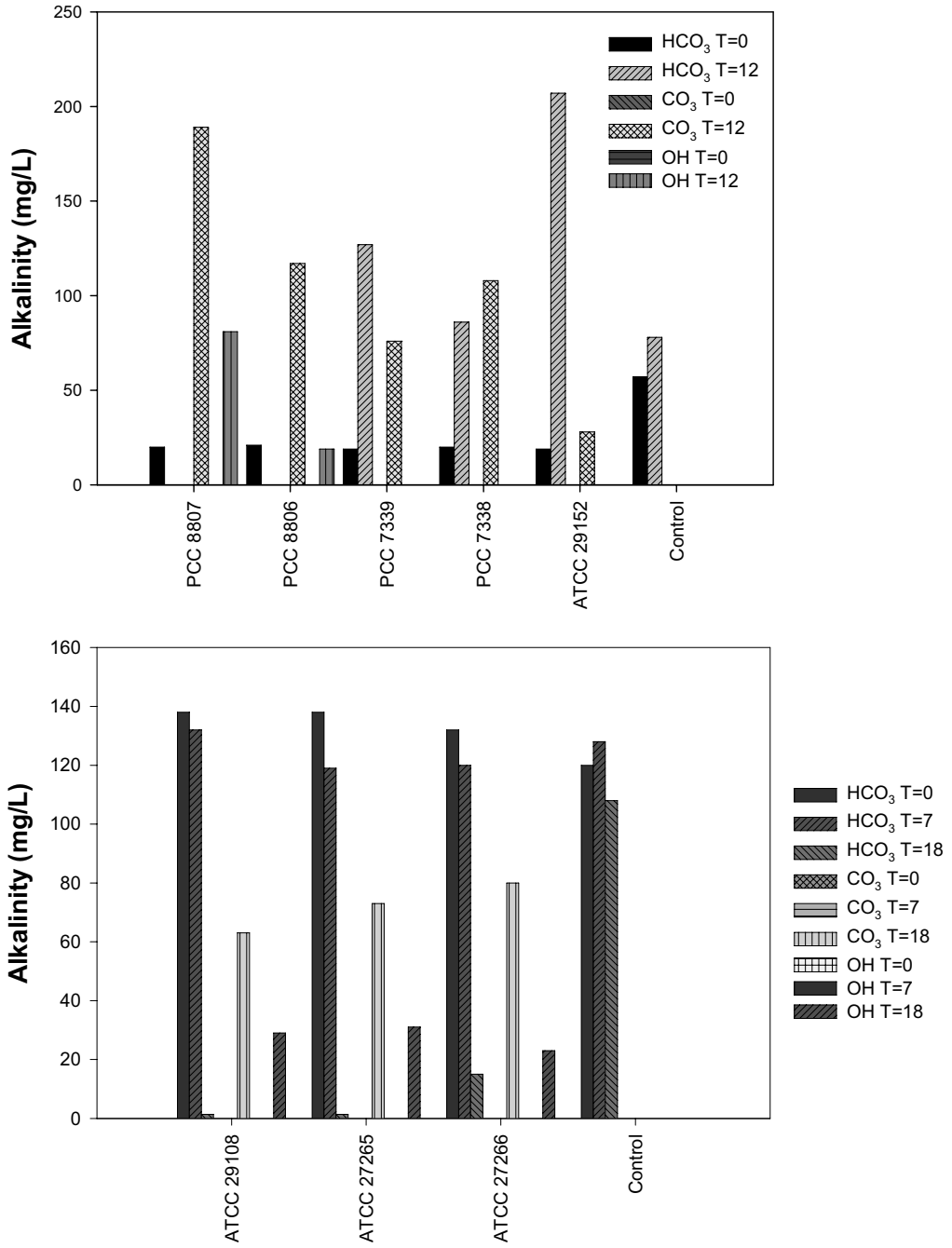

Figure 4. Changes in alkalinity over time in microcosms experiments to determine the ability of various strains of cyanobacteria to calcify. (A) First leg of the experiment in which *Synechococcus* sp. Strains PCC 8807 and 8806 and *Synechocystis* sp. Strains PCC 7338, PCC 7339 and ATCC 29152 were tested. (B) Second leg of the experiment in which *Synechococcus* sp. Strain ATCC 27265 and *Synechocystis* sp. Strains ATCC 27266 and ATCC 29108 were tested.

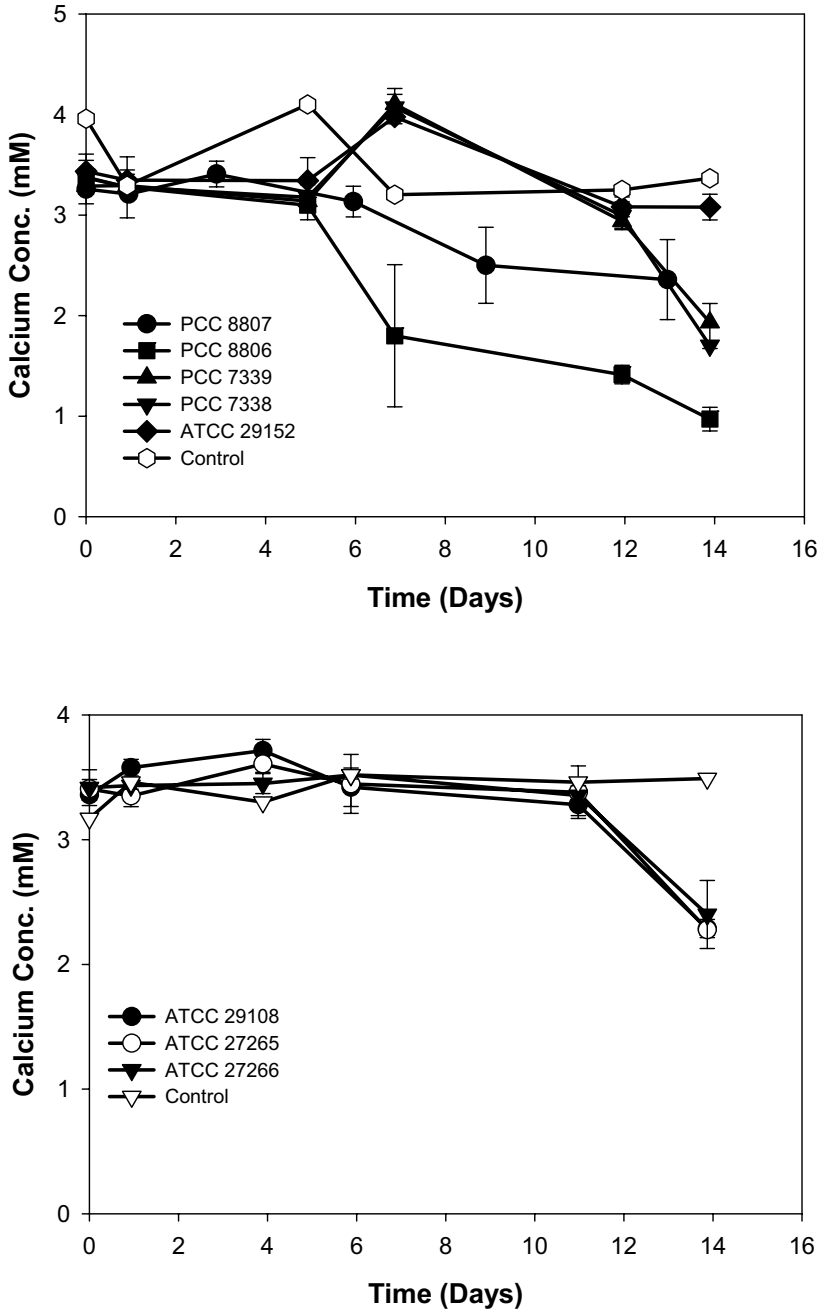

Figure 5. Removal of soluble calcium from growth medium obtained from microcosms to determine the ability of various strains of cyanobacteria to calcify. (A) First leg of the experiment in which *Synechococcus* sp. Strains PCC 8807 and 8806 and *Synechocystis* sp. Strains PCC 7338, PCC 7339 and ATCC 29152 were tested. (B) Second leg of the experiment in which *Synechococcus* sp. Strain ATCC 27265 and *Synechocystis* sp. Strains ATCC 27266 and ATCC 29108 were tested.

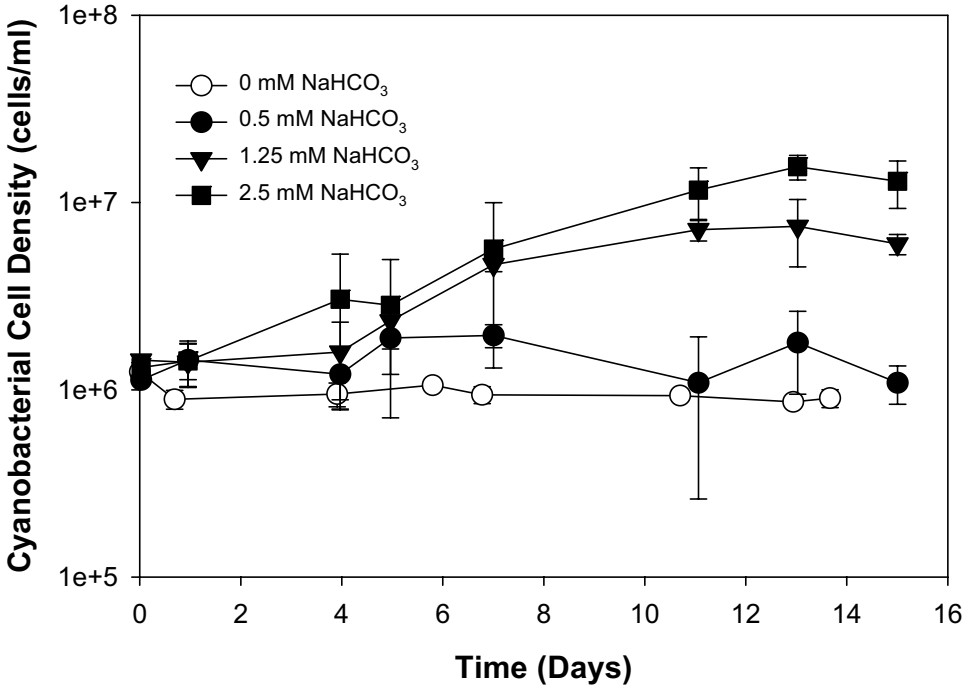

Figure 6. Cell density of *Synechococcus* sp. strain PCC 8806 when exposed to bicarbonate concentrations of 0.5, 1.25 and 2.5 mM. Experiments were used to determine the effect of bicarbonate concentration on growth.

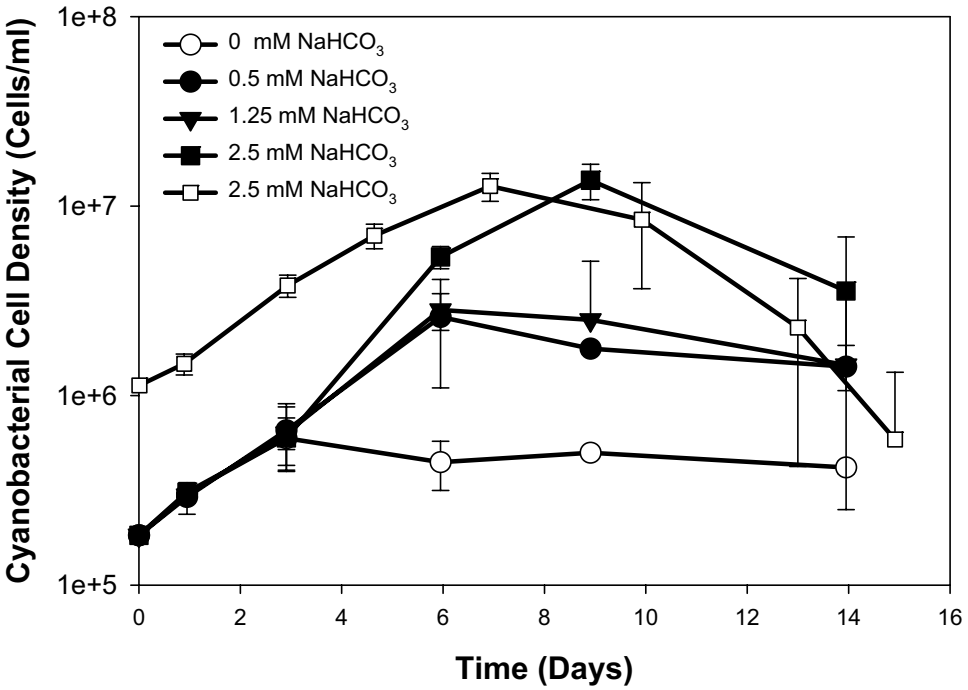

Figure 7. Changes in cell density of *Synechococcus* sp. strain PCC 8807 associated with exposure to bicarbonate concentrations of 0.5, 1.25 and 2.5 mM. Experiments were used to determine the effect of bicarbonate concentration on growth.

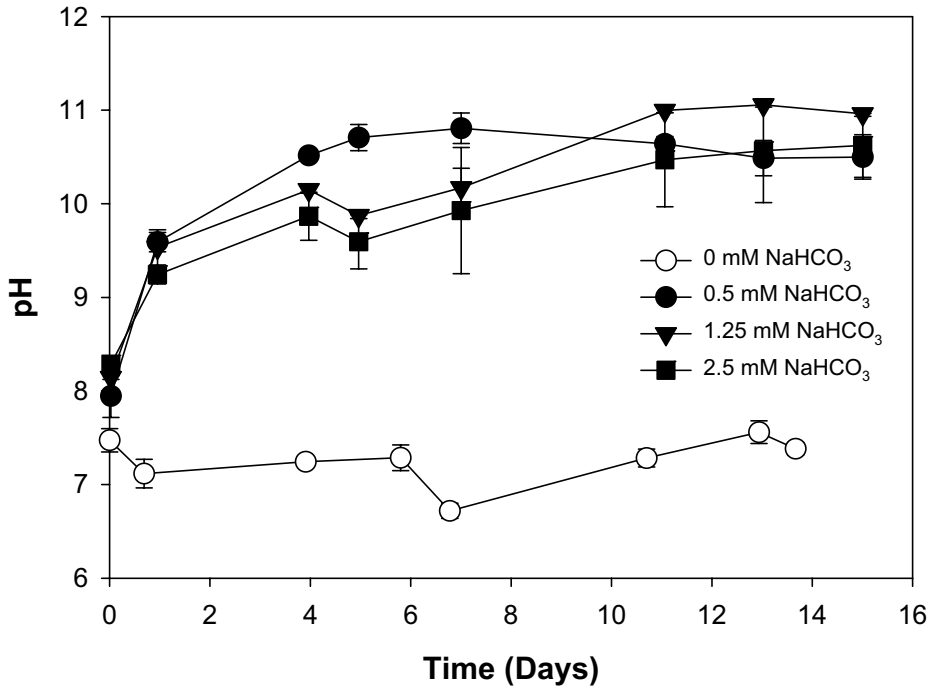

Figure 8. Graph demonstrating changes in pH over time when *Synechococcus* sp. strain PCC 8806 is exposed to 0.5, 1.25 and 2.5 mM bicarbonate.

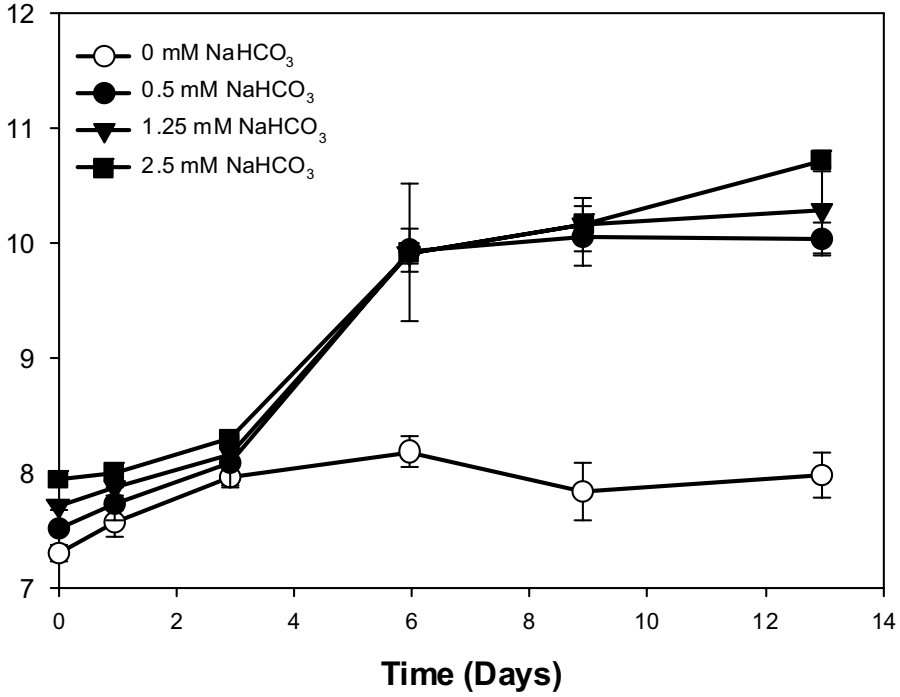

Figure 9. Effect of bicarbonate concentration on growth of *Synechococcus* sp. strain PCC 8807. Changes in pH were determined for the growth medium when cells were exposed to 0.5, 1.25 and 2.5 mM bicarbonate.

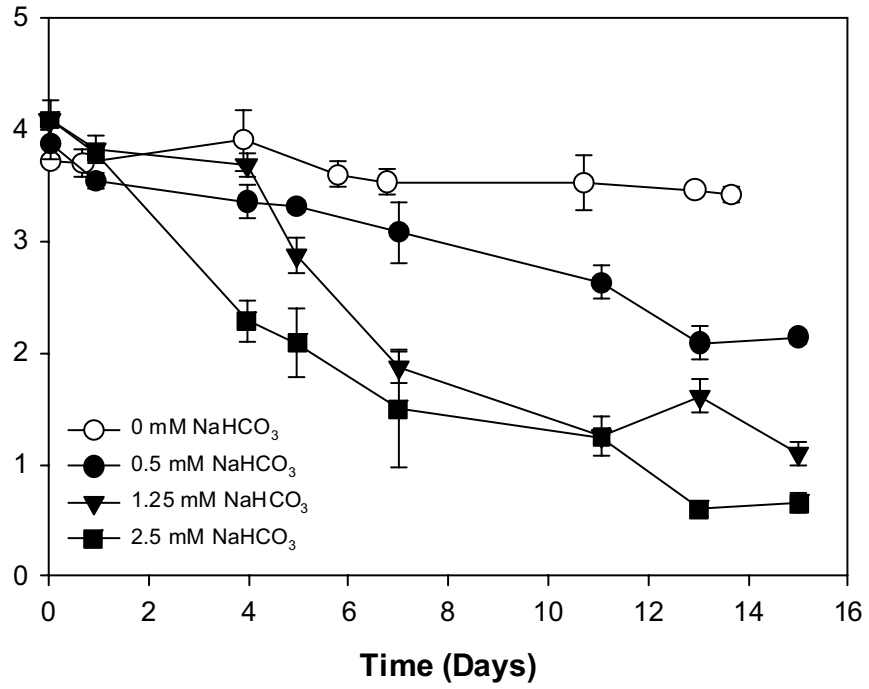

Figure 10. Calcium removal over time when *Synechococcus* sp. strain PCC 8806 was exposed to 0.5, 1.25 and 2.5 mM bicarbonate.

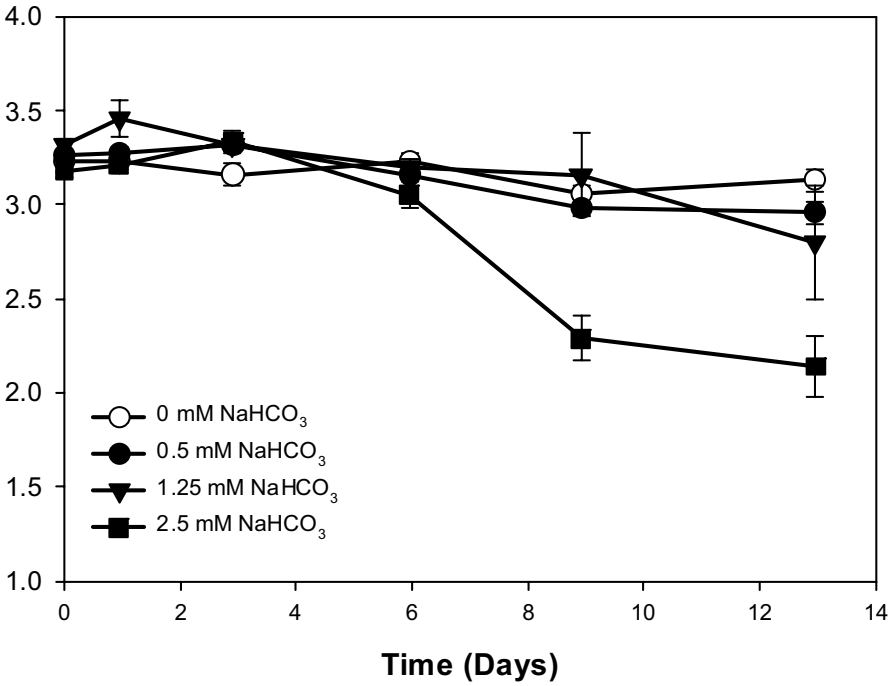

Figure 11. Removal of calcium over time when *Synechococcus* sp. strain PCC 8807 was exposed to 0.5, 1.25 and 2.5 mM bicarbonate.

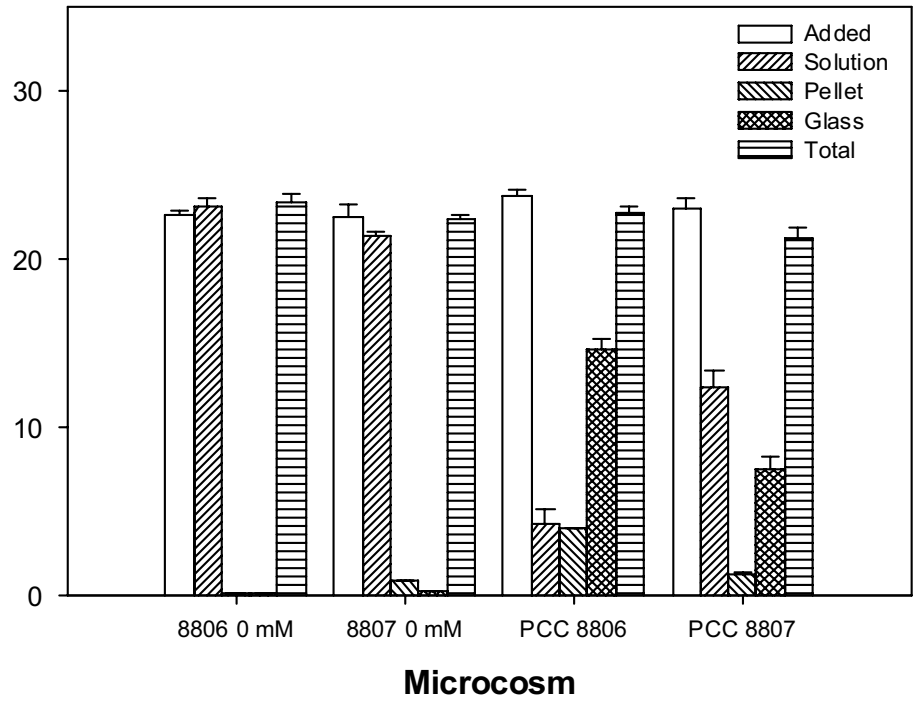

Figure 12. Mass of calcium in solution, precipitated with the cell pellet and adsorbed on the microcosm vessel compared to calcium added and total calcium recovered from the experiment. Results were generated from microcosms experiments when *Synechococcus* sp. strain PCC 8806 and *Synechococcus* sp. strain PCC 8807 was exposed to 2.5 mM bicarbonate and from controls containing cells and no bicarbonate or bicarbonate and no cells.

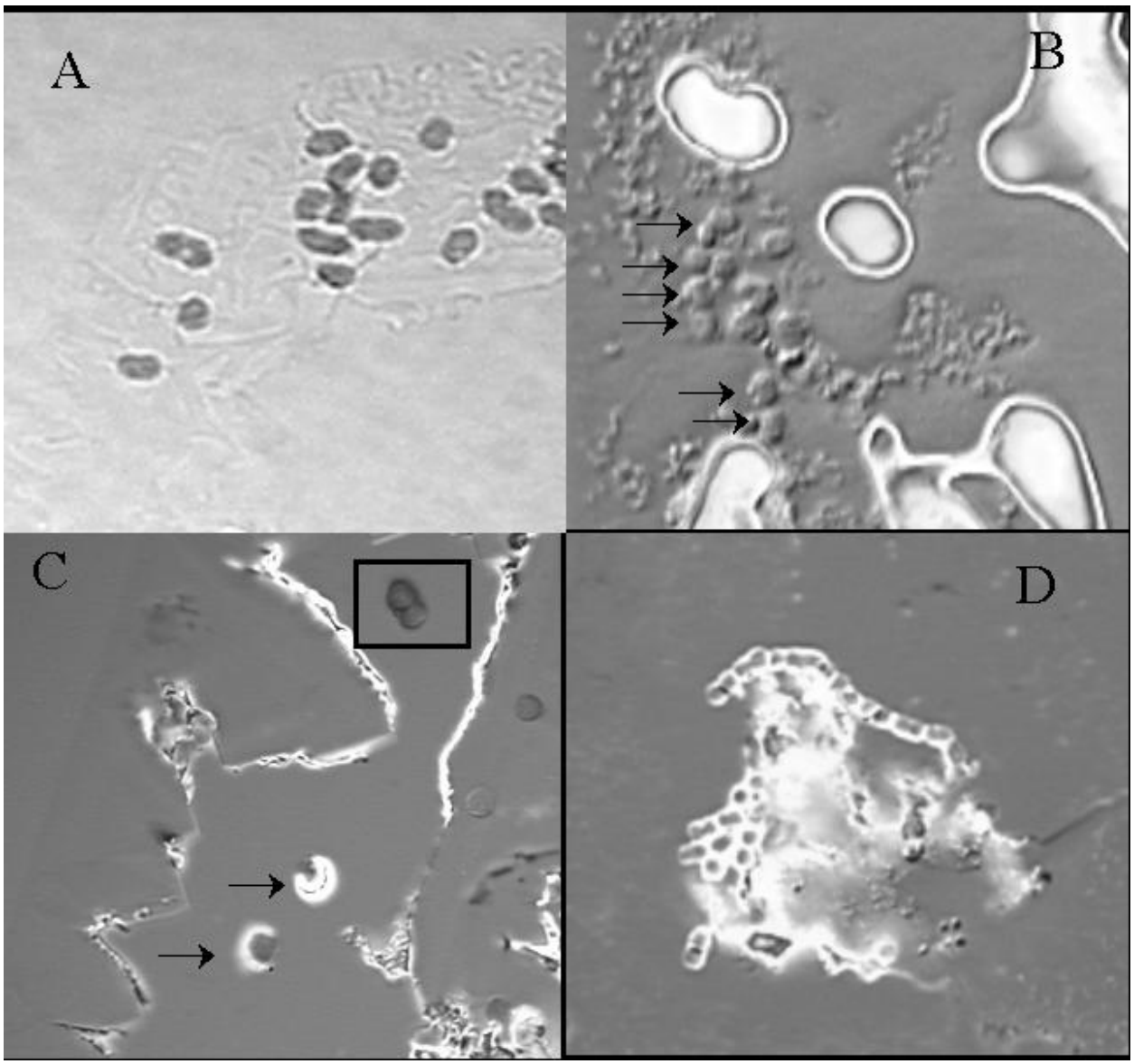

Figure 13. Polarized light micrographs of *Synechococcus* sp. strain PCC 8806 and *Synechococcus* sp. strain PCC 8807 when exposed to no bicarbonate and 2.5 mM bicarbonate. (A) PCC 8806 with no exogenous bicarbonate added; (B) PCC 8807 with no external bicarbonate added; (C) PCC 8806 at the conclusion of the experiment when exposed to 2.5 mM bicarbonate; and (D) PCC 8807 at end of experiment after growth at a bicarbonate concentration of 2.5 mM.

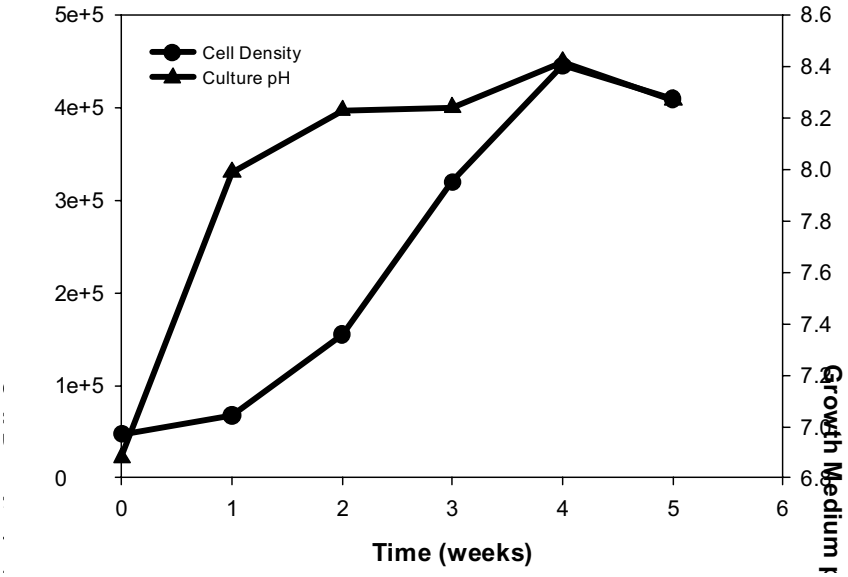

Figure 14. Cell density and growth medium pH for *Synechococcus* PCC 8806 growing at a large laboratory scale.