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# The effects of elevated PCO<sub>2</sub> on the physiology of *Emiliana huxleyi*

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**THE EFFECTS OF ELEVATED PCO<sub>2</sub> ON THE  
PHYSIOLOGY OF *EMILIANA HUXLEYI***

by  
Tristen Wuori

Accepted in Partial Completion  
Of the Requirements for the Degree  
Master of Science

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## MASTER'S THESIS

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**THE EFFECTS OF ELEVATED PCO<sub>2</sub> ON THE  
PHYSIOLOGY OF *EMILIANA HUXLEYI***

A Thesis  
Presented to  
The Faculty of  
Western Washington University

In Partial Fulfillment  
Of the Requirements for the Degree  
Master of Science

by  
Tristen Wuori  
June 2012

## ABSTRACT

This study examined the effects of elevated CO<sub>2</sub> on the microalga *Emiliana huxleyi*. Two strains were compared, a calcifying (CCMP 2668) and a non-calcifying (CCMP 374) strain. The CO<sub>2</sub> levels used were 390 ppm, 760 ppm, and 1000 ppm. The effects of CO<sub>2</sub> on growth rate, cell size, calcification, particulate dimethylsulfoniopropionate (DMSPp), and chlorophyll were examined. Under elevated CO<sub>2</sub>, cell size in both strains and DMSPp in the calcifying strain increased. Calcification decreased under elevated CO<sub>2</sub>. DMSPp in the non-calcifying strain and chlorophyll content in the calcifying strain had non-linear responses when exposed to elevated CO<sub>2</sub>. Growth rate in both strains and chlorophyll in the non-calcifying strain were not affected by CO<sub>2</sub> level. The change in DMSPp may be increasing the volume of water inside the cells, therefore increasing the cells size. The change in DMSPp may be a stress response or a path for excess carbon waste. The change in chlorophyll in the calcifying strain may be a result of both an increase in light availability from decreased calcification and an increase in cell size. Both strains experienced physiological changes under elevated CO<sub>2</sub> that have implications for the food web and biogeochemical cycling. Increased cell size influences the types of predators that have access to *Emiliana huxleyi*, which may affect whether nutrients are transported through the food web or sink to the deep ocean and may lead to ecosystem shifts. Reduced calcification may cause a decrease in carbon exported to the deep ocean. It may also increase predation on *Emiliana huxleyi*. A change in DMSPp may alter the sulfur cycle and decrease grazing on *Emiliana huxleyi*.

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## INTRODUCTION

Atmospheric CO<sub>2</sub> has risen from pre-industrial concentrations of 280 ppm to current concentrations of 390 ppm (IPCC, 2007). Atmospheric CO<sub>2</sub> is expected to continue to rise in the next century to between 855 and 1130 ppm by 2100 (stabilization scenario VI, IPCC, 2007). During the last few decades, much research has been done to examine the effects of elevated atmospheric CO<sub>2</sub> on terrestrial systems (Bowes, 1993). Recently, oceanographers have been examining the effects of elevated atmospheric CO<sub>2</sub> on marine systems. The aim of this study was to examine how CO<sub>2</sub> effects the physiology of the microalgae, *Emiliana huxleyi*. Increasing atmospheric CO<sub>2</sub> decreases the pH of seawater (see below). This phenomenon is known as ocean acidification and is one of many consequences of elevated atmospheric CO<sub>2</sub>. It is predicted that ocean pH will decrease by 0.4 to 0.6 units by the end of the century, and 0.7 units by 2300 (IPCC IS92a scenario; Caldeira and Wickett, 2003). pH influences many chemical pools and processes in seawater that are critical to marine biota, including the carbonate system.

The carbonate system consists of multiple dissolved inorganic carbon (DIC) species. Gaseous CO<sub>2</sub> dissolves into the ocean and reacts with water to form carbonic acid (Archer, 2007). DIC may be present as CO<sub>2</sub>, carbonic acid (H<sub>2</sub>CO<sub>3</sub>), bicarbonate (HCO<sub>3</sub><sup>-</sup>), and carbonate (CO<sub>3</sub><sup>2-</sup>), depending on the pH of the solution, as indicated by the following chemical equation:



The proportions of each form (CO<sub>2</sub>, CO<sub>3</sub><sup>2-</sup>, and HCO<sub>3</sub><sup>-</sup>) depend on the pH of the solution. As pH decreases, the proportion of carbonate ions decreases, and the proportion of CO<sub>2</sub> increases. For example, the decrease in pH from 8.0 to 7.6 results in a 290% increase in CO<sub>2</sub>

concentration. Therefore, as CO<sub>2</sub> in seawater increases, carbonate decreases. At current pH levels, a small decrease in pH results in a relatively large decrease in carbonate concentration. Therefore, any decrease in pH could have huge impacts on the amount of available carbonate ions.

If there are fewer carbonate ions and more CO<sub>2</sub>, calcification is energetically less favorable. Calcification requires the precipitation of calcium carbonate from carbonate and calcium ions. The precipitation and stability of carbonate is dependent on the saturation state ( $\Omega$ ) of carbonate. The carbonate saturation state depends on the concentration of calcium and carbonate in seawater and the apparent saturation constant (Emerson & Hedges, 2008). A higher saturation state favors precipitation and stability of carbonate. A decrease of pH in seawater results in a decrease of the saturation state of carbonate. Therefore, at a lower pH, there are fewer carbonate ions, and calcification is less favored and requires more energy to complete. A decrease in pH may lead to a decrease in calcification and an increase in dissolution of marine calcium carbonate shells.

Coccolithophorids are a group of protist phytoplankton that form calcium carbonate plates called coccoliths. *Emiliana huxleyi* is a coccolithophorid species that is abundant globally and commonly forms blooms (Paasche, 2002); *E. huxleyi* can therefore have a large role in carbon cycling in the world oceans. Since coccolithophores such as *E. huxleyi* have calcium carbonate plates, they may be particularly vulnerable to an increase in atmospheric CO<sub>2</sub>. Several previous studies have looked at the calcification of *E. huxleyi* in response to an increase in CO<sub>2</sub>. Most studies have shown that calcification decreases with increased CO<sub>2</sub> (Riebesell *et al.*, 2000, Zondervan *et al.* 2001, Engel *et al.*, 2005). There is some variation in the response to

environmental stresses among strains of *E. huxleyi*. Strains are different isolates, collected from different locations, which vary genetically. There are several differences between the two strains used in this study. One difference is that one strain is in its calcifying stage, which means that it produces calcium carbonate coccoliths, and the other is not. Another difference is that the calcifying strain is diploid, while the non-calcifying strain is haploid. Yet another difference is that the non-calcifying strain has a flagellum, and the calcifying does not. There are other differences in physiology; for example, there is variability among strains with respect to growth rate and particulate organic and inorganic carbon under different CO<sub>2</sub> conditions (Langer *et al.*, 2009) and with respect to dimethylsulfoniopropionate (DMSP) lyase activity (Steinke *et al.*, 1998). In contrast to most research to date, one controversial study found that elevated CO<sub>2</sub> levels increased calcification in one calcifying strain of *E. huxleyi* (Iglesias-Rodriguez *et al.*, 2008); the difference between these results and others' may be due to the use of different *Emiliana huxleyi* strains. Alternatively, the experimental incubations used by Iglesias-Rodriguez *et al.* only lasted 1.5 to 3 d, which may not have allowed for enough generational turnover to accurately assess whether there was a treatment effect of CO<sub>2</sub> level (Riebesell *et al.*, 2008). In my study, I examined both calcifying and non-calcifying strains of *Emiliana huxleyi* in an attempt to isolate influences of elevated CO<sub>2</sub> on calcification and other aspects of cell biochemistry.

In addition to their potential for calcification, *Emiliana huxleyi* also produce dimethylsulfide (DMS) through enzymatic cleavage of DMSP (Steinke *et al.*, 1998). DMS is a climatic gas, which is oxidized in the atmosphere and acts as cloud condensation nuclei (Charlson *et al.*, 1987). Only one study so far has examined the effect of increased CO<sub>2</sub> on

production of DMS by phytoplankton (Wingenter *et al.*, 2007). That study, performed in mesocosms of natural planktonic communities at 760 ppm and 1150 ppm CO<sub>2</sub> concentrations, found that DMS concentrations increased in both elevated CO<sub>2</sub> treatments relative to the ambient CO<sub>2</sub> treatment. It is unknown how ocean acidification may affect DMS production by *E. huxleyi*.

DMSP is cleaved to DMS and acrylate by the action of DMSP lyase, a constitutive enzyme in some phytoplankton. Stress or damage to the cell is required for DMSP cleavage to occur, since DMSP and DMSP lyase are separated within the algal cell (Wolfe & Steinke, 1996). Stresses inducing DMSP cleavage include microzooplankton grazing on phytoplankton (Wolfe *et al.*, 1997). Ocean acidification may act as a stress, triggering this reaction. It has been demonstrated that increased DMSP lyase activity in *E. huxleyi* is associated with reduced grazing rates by microzooplankton (Strom *et al.*, 2003a). Dissolved DMSP can act as a chemical signal, which also reduces protist microzooplankton grazing (Strom *et al.*, 2003b). DMSP release may act as a signal to microzooplankton that DMSP cleavage may occur in response to grazing. Using DMSP as a signal may deter predators without requiring damage to the *Emiliania huxleyi* cell. In contrast, there is evidence that *Oxyrrhis marina* may be attracted to DMSP to increase encounter rates with prey (Brekels *et al.*, 2010), so there may be variability in the response of grazing to a change in cellular DMSP. If dissolved DMSP is altered by an increase in CO<sub>2</sub>, changes in grazing rates may follow, and changes in the quantity of dissolved DMSP may occur if changes in phytoplankton cellular DMSP content occur. The climatic significance, the potential to influence grazing and the large uncertainties make DMSP an important variable to examine for potential changes associated with elevated CO<sub>2</sub>. In addition to the influences of

decreased pH on cellular aspects, such as calcification and DMSP, algal cells may be directly influenced by CO<sub>2</sub> through carbon fertilization.

Elevated CO<sub>2</sub> may lead to carbon fertilization, in which *Emiliana huxleyi* cells increase their biomass either by increasing their cell size or by increasing their cellular division rate and, therefore, cell density. This may happen because the increase in CO<sub>2</sub> may relieve energy demands within the cell and that energy may be used for growth. RUBISCO, a CO<sub>2</sub>-fixing enzyme found in algae, is less than half saturated under present day CO<sub>2</sub> levels (Giordano *et al.* 2005). Energy is required to concentrate CO<sub>2</sub> for RUBISCO within the cell through carbon concentrating mechanisms (CCMs) (Giordano *et al.* 2005). If the concentration of CO<sub>2</sub> is higher in the environment, and therefore higher within the cell, less energy will be required for CCMs. This energy can then be used for growth. The influences of both pH and CO<sub>2</sub> on cell physiology lead to several experimental questions.

### **Experimental questions**

The broad question that this study aims to answer is: How is the physiology of *Emiliana huxleyi* affected by an increase in CO<sub>2</sub>? To examine this question, semi-continuous culture laboratory experiments were conducted to examine the effect of increased CO<sub>2</sub> on *E. huxleyi* growth rate, cell size, calcification, concentration of intracellular DMSP, and chlorophyll content. In addition to revealing the physiological responses of *Emiliana huxleyi* to elevated CO<sub>2</sub>, these findings have the potential to give insight into changes in putative defenses, including calcification and DMSP production.

Hypothesis 1: Growth rate will increase in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>.

Hypothesis 2: Cell size will increase in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>.

Hypothesis 3: Calcification will decrease in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>.

Hypothesis 4: The concentration of intracellular DMSP will increase in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>.

Hypothesis 5: Chlorophyll content will not change in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>.

## **METHODS**

### **Species used**

*Emiliana huxleyi* is a coccolithophorid, which is a photosynthetic prymnesiophyte. Two strains were used in this study, both obtained from the National Center for Marine Algae and Microbiota: CCMP 374, a non-calcifying phenotype, isolated from the Gulf of Maine in 1990; and CCMP 2668, a calcifying phenotype, isolated from the Gulf of Maine in 2002.

### **Culture maintenance conditions**

*Emiliana huxleyi* (non-axenic) cultures were maintained in batch culture, in f/50 medium without added silicate. They were kept in an incubator at 15°C and a 12:12 light:dark cycle. All media was prepared from seawater collected from Shannon Point Marine Center (salinity ~ 30 psu), filtered with a 0.2 µm pore size cartridge filter, then autoclaved for 60 min.

### **Experiment culture conditions**

During experiments, non-axenic cultures of *Emiliana huxleyi* were maintained with a semi-continuous culturing method. Semi-continuous culturing was performed by diluting cultures daily with f/50 medium, without added silicate, by a calculated amount based on the growth rate of the algal cells. The atmospheric CO<sub>2</sub> levels were 400 ppm, 760 ppm, and 1000 ppm. Medium, for both initial cultures and dilutions, was directly bubbled with air at the respective CO<sub>2</sub> treatment level for at least 24 hr before inoculation with algal cells. Our goal was to maintain an *Emiliana huxleyi* cell concentration of approximately 100,000 cell ml<sup>-1</sup>. This concentration was established during a preliminary batch culture experiment, which determined



the cell density at which photosynthesis consumed enough CO<sub>2</sub> to significantly alter the pH of the water. Semi-continuous cultures were started by maintaining batch cultures for 2 to 4 d in exponential growth before starting daily dilutions.

Cultures were kept in gas-tight, temperature-controlled chambers (which have the dimensions of 57.15 cm x 39.37 cm x 31.75 cm) at 15°C with a 14:10 light:dark cycle. The light level depended on the experiment. The atmosphere of each chamber was continuously supplied with a gas mixture to achieve target treatment CO<sub>2</sub> levels. Gas mixtures were created by adding compressed CO<sub>2</sub> to air from outside the laboratory building. These mixtures were controlled by Sierra Smart-Trak 2 mass flow controllers, and pCO<sub>2</sub> in air entering and leaving the chambers was monitored by a LiCor LI820 CO<sub>2</sub> sensor.

### **Algal cell counts**

Cell density was determined daily using the BD FACSCalibur flow cytometer. One hundred µl of a 2.0 µm fluorescent bead solution, made with Flow Check intermediate intensity level 1 fluorescent beads and ultrapure water, was added to each subsample of 1.9 ml. Beads were used to measure the exact volume analyzed by the flow cytometer. Bead concentration in the bead solution was determined by counting the number of beads filtered from a 300 µl volume of bead solution (using a 0.6 µm polycarbonate filter with a 0.65 µm polycarbonate backing filter) on an epifluorescent microscope using UV light and a counting grid within the eyepiece. Samples were held in glass tubes. Beads were added and the mixture vortexed in the glass tube. However, since the flow cytometer only holds specific plastic tubes, and the fluorescent beads stick to plastic, the sample was poured into the plastic tube immediately before being run on the

flow cytometer. Samples were run on the flow cytometer for 60 s on low velocity. Beads in the culture subsample were counted using the green fluorescence (FL1) parameter, and *Emiliana huxleyi* cells were counted using side scatter and red fluorescence (FL3) parameters. Events with very low red fluorescence and very low side scatter were excluded from cell counts. A ratio between beads and cells was used to calculate the cell concentration. Subsamples were also fixed with acid Lugol's solution for manual cell counts using a Sedgewick-Rafter counting chamber.

### **CO<sub>2</sub> chemistry measurements**

pH was measured daily using a Metrohm 888 Titrande titrator with a Metrohm Ecotrode combined electrode calibrated with TRIS and AMP buffers on the total H ion pH scale (Dickson et. al., 2007). Subsamples, each 20 ml, were brought to temperature in a water bath at 20°C for approximately an hour, then pH was measured. Alkalinity was measured using a Metrohm 888 Titrande titrator. Subsamples were taken every other day. One hundred ml subsamples were gravity filtered with a 0.7 µm effective pore size 25 mm glass fiber filter, then placed in a 6°C incubator. Alkalinity subsamples were titrated within two weeks of sampling with gran titration using approximately 0.1 N HCl and NaCl titrant. Prior to analysis, subsamples were incubated in a water bath at 20°C. The titrator was calibrated before each use for pH or alkalinity using seawater buffers. Buffers were prepared by combining prepared sea salts and HCl with 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) and 2-aminopyridine. pCO<sub>2</sub> was calculated by inputting pH and alkalinity values into CO2SYS using the Millero et. al. constants (Lewis & Wallace, 1998).

### **Cell size measurements**

Cell size was measured on live cells using an Olympus CH30 compound microscope. ImagePro Plus 5.0 software and a Photometrics CoolSNAP cf microscope camera were used to capture the images. The first fifteen to twenty cells encountered on the slide were imaged per replicate per analysis day. ImageJ software was used to measure width of the spherical cells. Cell volume was then calculated using the measured diameter, assuming spherical cell shape.

### **Chlorophyll *a* measurements**

Chlorophyll *a* was extracted from subsamples and measured using a Turner Designs 10-AU fluorometer. Ten ml subsamples were gravity filtered with a 0.7  $\mu\text{m}$  effective pore size 25 mm glass fiber filter. Glass fiber filters were stored at  $-70^{\circ}\text{C}$  for less than one month. The chlorophyll *a* was extracted from the glass fiber filters in 6 ml of 90% acetone in glass tubes for 24 hr in the dark at  $\sim -16^{\circ}\text{C}$ . The tubes were then vortexed, the filters removed, and the tubes centrifuged at 3600 rpm for 5 min. Fluorescence was measured before and after the addition of 2 drops of 1 N HCl. Both readings were used to calculate chlorophyll *a* content.

### **Carbon and nitrogen measurements**

Carbon and nitrogen content of the algal cells was measured using a CE Elantech Flash EA 1112 elemental analyzer. Duplicate subsamples of 60 ml were taken from each culture and filtered using glass fiber filters, type A/E 13 mm, that had been pre-combusted at  $450^{\circ}\text{C}$  for 4 hr in a muffle furnace. One subsample filter was placed in a small tin foil boat. The other

subsample filter was placed in a similar silver foil boat, since the tin boats cannot be placed in an acidic environment. Both subsample filters were dried at 60°C for 24 hr. Then, the first subsample filter was tightly wrapped and stored in a desiccator. The second subsample filter was placed in an acid fume (a sealed desiccation chamber containing a beaker of HCl) for 24 hr to remove any particulate inorganic carbon (PIC). This second subsample filter was dried again at 60°C for 24 hr, then the filter and silver boat were placed in a tin foil boat, tightly wrapped, and stored in a desiccator.

Acetanilide standards (0.040 – 3.685 mg) were used to create a standard curve during carbon and nitrogen analysis. Blanks containing only tin foil boats were used to set a baseline for the analysis. Blanks consisting of a dry pre-combusted filter and tin foil boat, made during filtration and treated the same way as the samples, were used as a blank correction during calculation of sample carbon and nitrogen content. PIC was calculated by subtracting the amount of organic carbon (the sample treated with the acid fume) from the amount of total carbon (the sample not treated with the acid fume).

It was determined that only approximately 2% of total coccoliths were passing through the 0.7 µm effective pore size, 25 mm diameter, glass fiber filters and the pre-combusted type A/E 13 mm glass fiber filters, in both control and high CO<sub>2</sub> treatments. This was done by analyzing the filtrate on the BD FACSCalibur flow cytometer.

### **Dimethylsulfoniopropionate (DMSPp) measurements**

Intracellular (particulate) dimethylsulfoniopropionate (DMSPp) was measured using a Shimadzu GC-14A gas chromatograph equipped with a flame photometric detector. Glass fiber

filters, 0.7  $\mu\text{m}$  effective pore size, 25 mm diameter, and glass vials were pre-combusted at 450°C for 4 hr in a muffle furnace. Subsamples of 25 ml were gravity filtered. Filters with algal cells were placed in 3 ml of 10 N NaOH in the muffled vials, then sealed with butyl septa and metal seals, and vortexed. Standards were made at the time of sampling. Standards were made using DMSP Cl dissolved in ultrapure water to 250  $\mu\text{M}$  DMSP, which was then added to the vials containing NaOH in different amounts in order to obtain a range of DMSP concentrations. Samples were stored in the dark until gas chromatography analysis according to the method of Wolfe et al. (2002).

### **Preliminary chamber effect experiment**

A preliminary experiment examined any possible effect of the gas-tight chamber, by comparing *Emiliana huxleyi* growth rates across all chambers. Batch cultures of strain CCMP 2668 were grown for 8 d. In batch cultures, cultures were not diluted after the inoculation. Culture volume was 200 ml in a 500 ml square polycarbonate bottle. Temperature was monitored constantly inside the chambers using a HOBO temperature logger. Cultures were grown at an average of 17 °C and an average light intensity of 230  $\mu\text{mol photons sec}^{-1}\text{m}^{-2}$ . All chambers were at ambient CO<sub>2</sub> levels. Triplicate cultures were incubated in each of the 3 different gas-tight chambers. In vivo fluorescence was measured daily as a proxy for cell concentration. In vivo fluorescence was measured on 6 ml subsamples using a Turner Designs 10-AU fluorometer. Growth rate was estimated from the slope of the linear portion of the relationship between the natural log of the in vivo fluorescence and time (i.e. the period of

exponential growth). The primary conclusion of this preliminary experiment was that the chambers did not affect growth rate (see appendix).

### **Preliminary volume experiment**

A preliminary experiment established the volume of culture that would not inhibit air-water gas exchange, allowing maintenance of the desired pH, while also providing enough volume for daily measurements. Batch cultures of strain CCMP 2668 were grown for 7 d at 15°C with an average light intensity of 133  $\mu\text{mol photons sec}^{-1}\text{m}^{-2}$ . Triplicate cultures of 500 ml, 700 ml, and 900 ml culture volume in 1000 ml square polycarbonate bottles were incubated in the gas-tight chambers at the high CO<sub>2</sub> level (1000 ppm). In vivo fluorescence, cell concentration by flow cytometry, and pH were measured daily. Alkalinity was measured the first and last day. Light intensity was measured the first day. Temperature was constantly monitored by a HOBO. Growth rates were calculated based on changes in both cell concentration and in vivo fluorescence. Growth rate was estimated from the slope of the linear portion of the relationship between the natural log of the in vivo fluorescence and time (i.e. the period of exponential growth). The primary conclusion of this preliminary experiment was that 500 ml was the optimal volume, of the volumes tested, to use for future experiments (see appendix).

### **Preliminary batch culture experiment**

A preliminary experiment compared algal cell density to pH over time in batch culture. The goal of this experiment was to determine the maximum algal cell density at which semi-

continuous cultures could be maintained without allowing algal photosynthesis and calcification to influence the seawater pH. All cultures were inoculated in f/50 medium that was pre-equilibrated for at least 24 hr by bubbling directly with the respective CO<sub>2</sub> treatment level. CCMP 2668 cultures were inoculated at 2200 cells/ml, and CCMP 374 cultures at 2000 cells/ml. Separate batch cultures of strains CCMP 2668 and CCMP 374 were grown for 9 d. Quadruplicate cultures were incubated in the gas-tight chambers at ambient, moderate (760 ppm), and high (1000 ppm) CO<sub>2</sub> levels. Cultures were grown at 15°C with an average light intensity of 120 μmol photons sec<sup>-1</sup>m<sup>-2</sup>. In vivo fluorescence, pH, and temperature were measured daily. pH was also measured before and after inoculation on day 1. Alkalinity was measured on the first and last days. Cell concentration was measured every other day by flow cytometry. Samples for cell carbon and nitrogen content were taken on day 5 and the last day. Salinity and light intensity were measured the first day. Growth rates were calculated based on cell concentration and in vivo fluorescence. Growth rate was estimated from the slope of the linear portion of the relationship between the natural log of the in vivo fluorescence and time (i.e. the period of exponential growth). The primary conclusion of this experiment was that 100,000 cells ml<sup>-1</sup> was the best target cell density to use for semi-continuous culturing (see appendix).

### **Preliminary DMSPp experiment**

A preliminary experiment compared sampling methods for measuring DMSPp, because there is concern that DMSP may leak out of the cells into the dissolved pool if not carefully sampled (Kiene and Slezak, 2006). Strains CCMP 2668 and CCMP 374 were used, which were grown as described in “culture maintenance conditions” (above). Cell density in both cultures

was 100,000 cells ml<sup>-1</sup> at time of filtration. Gravity, hand pump-assisted, and low vacuum-assisted filtration methods were compared (see appendix). Filtration volumes of 10 ml, 25 ml, and 50 ml were compared. Two or three replicates were filtered for each method and volume. The primary conclusion of this preliminary experiment was that 25 ml gravity filtered was the best method, of the methods tested, to use for future DMSPP analyses (see appendix).

### **Preliminary semi-continuous experiment**

A preliminary experiment was conducted to determine whether to use in vivo fluorescence-based or flow cytometry-based growth rates to calculate dilution volumes for semi-continuous culturing methods. Separate semi-continuous cultures of strains CCMP 2668 and CCMP 374 were grown for 13 d. All cultures were started with f/50 medium which was pre-equilibrated with the respective CO<sub>2</sub> treatment level. Cultures were inoculated at 18,000 cells/ml. The first two days of growth were batch culture, the following 12 days were semi-continuous culture. Duplicate cultures were incubated at 15°C in the gas-tight chambers at ambient, moderate (760 ppm), and high (1000 ppm) CO<sub>2</sub> levels. Temperature, in vivo fluorescence, cell concentration by flow cytometry, and pH were measured daily. Samples for carbon and nitrogen content, chlorophyll *a*, cell concentration by microscopy, and DMSPP were taken on days 4, 9, and 13. Alkalinity was measured on the first and last day. Growth rates were calculated according to:

$$\text{Growth rate} = \ln (N_t / N_{t-1}) \quad \text{[Equation 1]}$$

where  $N_t$  is the cell concentration before dilution on the day of sampling, and  $N_{t-1}$  is the cell concentration after dilution on the previous day. Analogous growth rates were calculated from



in vivo fluorescence. The primary conclusion of this preliminary experiment was that this method of semi-continuous culturing, using Equation 1 and flow cytometry-based growth rates, was effective in maintaining cell densities at steady state (see appendix).

### **Non-calcifying experiment**

*Emiliana huxleyi* strain CCMP 374 was grown in semi-continuous cultures at three pCO<sub>2</sub> treatment levels: ambient (400.9 ± 69.7 ppm), moderate (665.2 ± 12.7 ppm), and high (871.9 ± 70.7 ppm) (Figure 1). Atmospheric CO<sub>2</sub> concentrations were 400 ppm (ambient), 760 ppm (moderate), and 1000 ppm (high). Moderate dissolved CO<sub>2</sub> levels were 12.5% below atmospheric CO<sub>2</sub> levels and high dissolved CO<sub>2</sub> levels were 12.8% below atmospheric CO<sub>2</sub> levels. Each treatment was replicated five times in separate culture bottles each with a culture volume of 500 ml in 1000 ml square polycarbonate bottles. Cultures were incubated for 14 d at 15°C with an average light intensity of 156 μmol photons sec<sup>-1</sup>m<sup>-2</sup>. Over the first 3 d, cultures were allowed to grow in batch culture mode to a cell density of 100,000 cells/ml; cultures were diluted daily thereafter. Average (± 1 SD) dilution volumes were 287 ± 71 ml. Subsamples were taken daily to measure pH and cell density using flow cytometry. Subsamples were taken on days 4, 6, 11, and 13 for carbon and nitrogen content analyses, DMSPP, chlorophyll *a*, and cell concentrations by microscopy. Subsamples were taken on days 5, 7, 12 and 14 for cell size and alkalinity analyses. Light intensity was measured on day 1 and day 7. Growth rates were calculated from flow cytometric estimates of cell densities, using equation [1] (above, see “preliminary semi-continuous experiment”). pCO<sub>2</sub> was calculated using CO<sub>2</sub>sys and pH and alkalinity data.

### **Calcifying experiment**

An experiment, nearly the same as the non-calcifying experiment, was conducted with *Emiliana huxleyi* strain CCMP 2668. The only differences, when compared to the non-calcifying experiment, were that light intensity was measured on day 5 and averaged  $134 \mu\text{mol photons sec}^{-1}\text{m}^{-2}$ , and average ( $\pm 1$  SD) dilution volumes were  $297 \pm 88$  ml. Actual  $\text{pCO}_2$  concentrations during the calcifying experiment (average  $\pm 1$  SD) were  $323.2 \pm 13.6$  ppm (ambient),  $664.5 \pm 31.7$  ppm (moderate), and  $798.4 \pm 23.4$  ppm (high) (Figure 1). Moderate dissolved  $\text{CO}_2$  levels were 12.6% below atmospheric  $\text{CO}_2$  levels and high dissolved  $\text{CO}_2$  levels were 20.2% below atmospheric  $\text{CO}_2$  levels.

### **Statistical methods**

Growth rates, carbon and nitrogen content, DMSPP, chlorophyll *a*, cell size, and chlorophyll content were compared among treatments with a repeated measures analysis of variance (ANOVAR). Sphericity was tested for each ANOVAR using a Mauchly's *W* test. If needed, a Tukey's post-hoc comparison was performed. Significant differences were defined as  $p < 0.05$  for all statistical analyses. An SPSS version 17.0 software package was used for all statistics. Data collected before the fifth day of the experiment were not included in the statistical analysis, because at the time that physiological measurements of the cells were taken (day 4) the cultures had not yet been diluted once and therefore could not be compared to semi-continuous cultures.

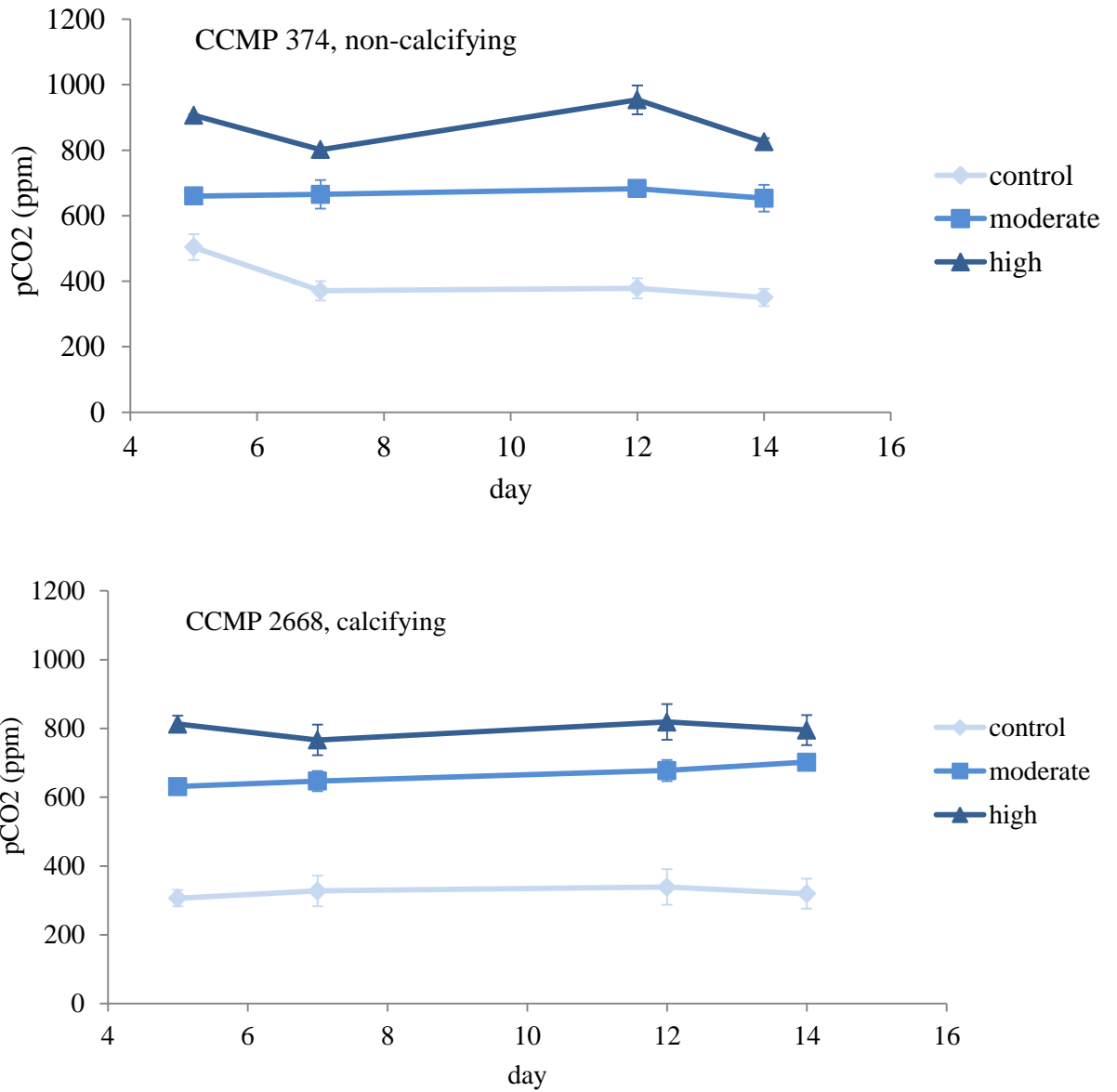


Figure 1. pCO<sub>2</sub> concentration of cultures during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5).

## RESULTS

Preliminary experiment results can be found in the Appendix.

### Growth rate

*Emiliana huxleyi* growth rate was not affected by elevated CO<sub>2</sub> (Figure 2, Table 1 & 2). Growth rate (average  $\pm$  1 SD) over time and all CO<sub>2</sub> treatments was  $0.91 \pm 0.09$  d<sup>-1</sup> for strain CCMP 374 and  $1.01 \pm 0.13$  d<sup>-1</sup> for strain CCMP 2668. Over time, growth rates of cultures for both strains were variable, which caused a significant time effect, but there was no directional trend (ANOVAR time effect p-value <0.001 and <0.001).

### Cell size

*Emiliana huxleyi* strain CCMP 374 had a significantly larger cell volume in high CO<sub>2</sub> than in ambient and moderate CO<sub>2</sub> (ANOVAR with Tukey's post-hoc comparison; p-value 0.004 and 0.011 respectively) (Figure 3, Table 1). Cell volume for strain CCMP 374 in high CO<sub>2</sub> was 17% larger than in ambient CO<sub>2</sub> and 15% larger than in moderate CO<sub>2</sub>. Similarly, strain CCMP 2668 had a larger cell volume in high CO<sub>2</sub> than in ambient and moderate CO<sub>2</sub>, and a larger cell volume in moderate than in ambient CO<sub>2</sub> (Figure 3, Table 2). Cell volume for strain CCMP 2668 in high CO<sub>2</sub> was 37% larger than in ambient CO<sub>2</sub> and 15% larger than in moderate CO<sub>2</sub>. Cell volume for strain CCMP 2668 in moderate CO<sub>2</sub> was 19% larger than in ambient CO<sub>2</sub>. For strain CCMP 374, there was an initial increase in cell volume, followed by a decrease under all CO<sub>2</sub> conditions (ANOVAR time effect p-value <0.001). For strain CCMP 2668, there was an

Table 1. Average  $\pm$  standard deviation of physiological parameters measured during the non-calcifying experiment (strain CCMP 374). Bold font indicates significant effect of CO<sub>2</sub> treatment. P-values presented are regarding CO<sub>2</sub> treatment effects (ANOVAR,  $\alpha=0.05$ ).

Parameter	ambient	moderate	high	p-value	time effect?
growth rate (d <sup>-1</sup> )	0.89 $\pm$ 0.10	0.91 $\pm$ 0.11	0.94 $\pm$ 0.07	0.205	Yes
<b>cell volume (<math>\mu\text{m}^3</math>)</b>	<b>71.5 <math>\pm</math> 7.7</b>	<b>73.2 <math>\pm</math> 7.2</b>	<b>83.9 <math>\pm</math> 6.5</b>	<b>0.003</b>	<b>yes</b>
POC/cell (pg/cell)	12.2 $\pm$ 0.6	12.7 $\pm$ 0.3	12.3 $\pm$ 0.7	0.673	no
POC/ $\mu\text{m}^3$ (pg/ $\mu\text{m}^3$ )	0.17 $\pm$ 0.03	0.17 $\pm$ 0.02	0.15 $\pm$ 0.02	0.387	no
POC:PON	12.1 $\pm$ 6.1	11.0 $\pm$ 4.5	10.2 $\pm$ 4.5	0.060	yes
<b>DMSPp/cell (pg/cell)</b>	<b>1.61 <math>\pm</math> 0.22</b>	<b>1.41 <math>\pm</math> 0.25</b>	<b>1.80 <math>\pm</math> 0.18</b>	<b>0.012</b>	<b>yes</b>
<b>DMSPp/POC (pg DMSP/pg POC)</b>	<b>0.13 <math>\pm</math> 0.01</b>	<b>0.11 <math>\pm</math> 0.02</b>	<b>0.15 <math>\pm</math> 0.02</b>	<b>0.003</b>	<b>yes</b>
DMSPp/ $\mu\text{m}^3$ (fg/ $\mu\text{m}^3$ )	22.6 $\pm$ 5.5	19.6 $\pm$ 6.0	22.1 $\pm$ 4.5	0.302	yes
chlorophyll/cell (pg/cell)	0.103 $\pm$ 0.003	0.102 $\pm$ 0.005	0.103 $\pm$ 0.007	0.739	no
chlorophyll/POC (pg chlorophyll/pg POC)	0.0085 $\pm$ 0.0007	0.0081 $\pm$ 0.0003	0.0085 $\pm$ 0.0003	0.491	no
chlorophyll/ $\mu\text{m}^3$ (pg/ $\mu\text{m}^3$ )	1.43 $\pm$ 0.13	1.44 $\pm$ 0.23	1.25 $\pm$ 0.12	0.109	yes

Table 2. Average  $\pm$  standard deviation of physiological parameters measured during the calcifying experiment (strain CCMP 2668). Bold font indicates significant effect of CO<sub>2</sub> treatment, and italic font indicates a significant interaction between time and CO<sub>2</sub> treatment. P-values presented are regarding CO<sub>2</sub> treatment effects, p-values not given for interactions. \*Significance and means presented are excluding the outlier data point (ANOVAR,  $\alpha=0.05$ ).

Parameter	ambient	moderate	high	p-value	time effect?
growth rate (d <sup>-1</sup> )	1.01 $\pm$ 0.15	1.00 $\pm$ 0.16	1.02 $\pm$ 0.08	0.932	yes
<i>cell volume (<math>\mu\text{m}^3</math>)</i>	<i>192.5 <math>\pm</math> 12.0</i>	<i>229.0 <math>\pm</math> 25.9</i>	<i>264.4 <math>\pm</math> 43.7</i>		
POC/cell (pg/cell)	21.2 $\pm$ 0.9	21.9 $\pm$ 1.5	24.0 $\pm$ 2.1	0.125	yes
POC:PON	6.4 $\pm$ 0.6	6.8 $\pm$ 0.9	6.3 $\pm$ 0.6	0.193	yes
PIC/cell (pg/cell)	16.9 $\pm$ 0.9	15.0 $\pm$ 0.6	16.5 $\pm$ 2.4	0.542	no
<b>PIC:POC*</b>	<b>0.84 <math>\pm</math> 0.08</b>	<b>0.70 <math>\pm</math> 0.02</b>	<b>0.69 <math>\pm</math> 0.04</b>	<b>0.043</b>	<b>no</b>
<b>DMSPP/cell (pg/cell)</b>	<b>2.30 <math>\pm</math> 0.19</b>	<b>2.37 <math>\pm</math> 0.11</b>	<b>2.88 <math>\pm</math> 0.10</b>	<b>&lt;0.001</b>	<b>yes</b>
<i>DMSPP/POC (pg DMSP/pg POC)</i>	<i>0.11 <math>\pm</math> 0.01</i>	<i>0.11 <math>\pm</math> 0.01</i>	<i>0.12 <math>\pm</math> 0.01</i>		
<b>chlorophyll/cell (pg/cell)</b>	<b>0.182 <math>\pm</math> 0.021</b>	<b>0.172 <math>\pm</math> 0.016</b>	<b>0.198 <math>\pm</math> 0.008</b>	<b>0.043</b>	<b>yes</b>
chlorophyll/POC (pg chlorophyll/pg POC)	0.0086 $\pm$ 0.0010	0.0079 $\pm$ 0.0004	0.0081 $\pm$ 0.0005	0.265	yes

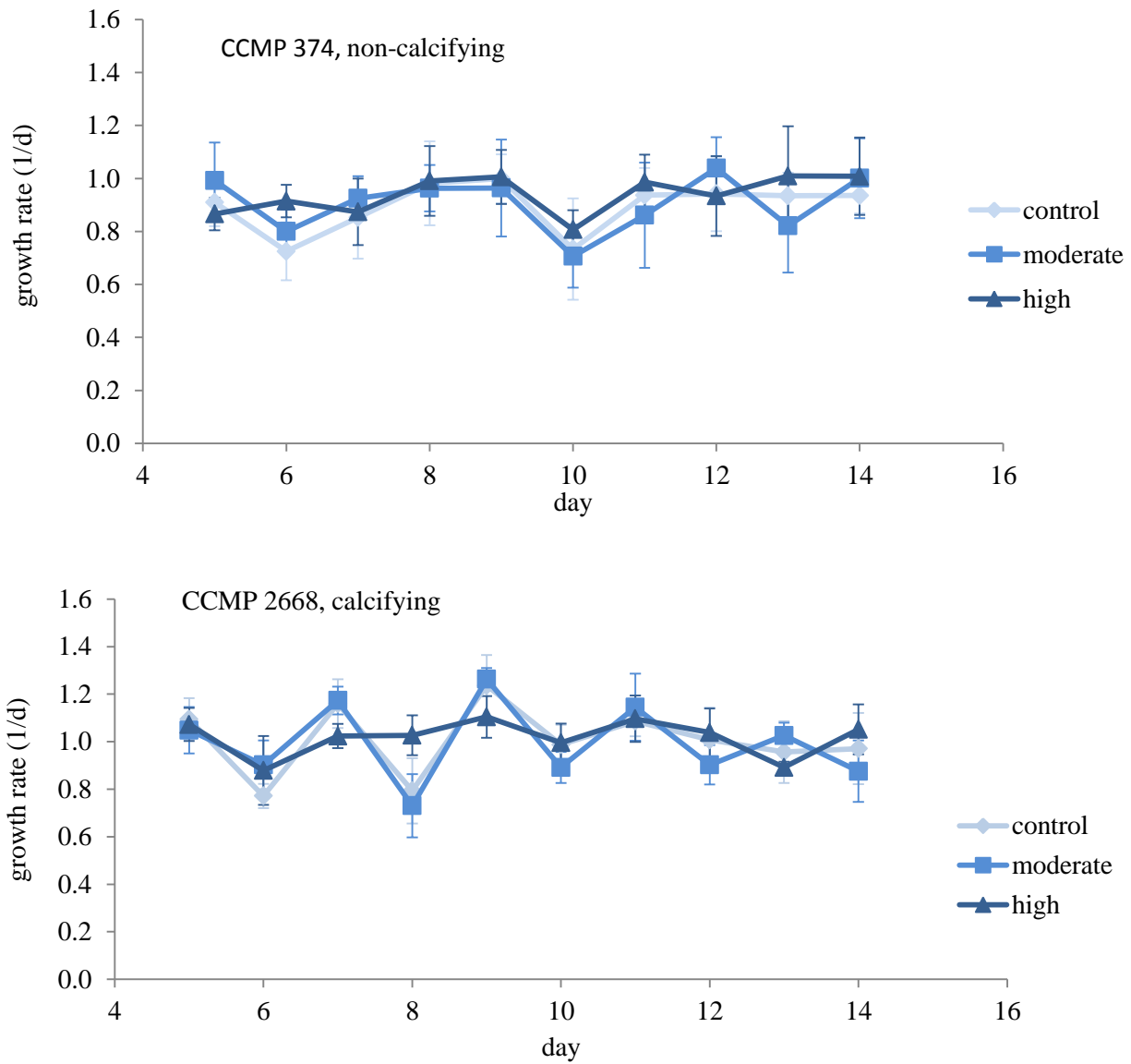


Figure 2. Growth rates of cultures during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5).

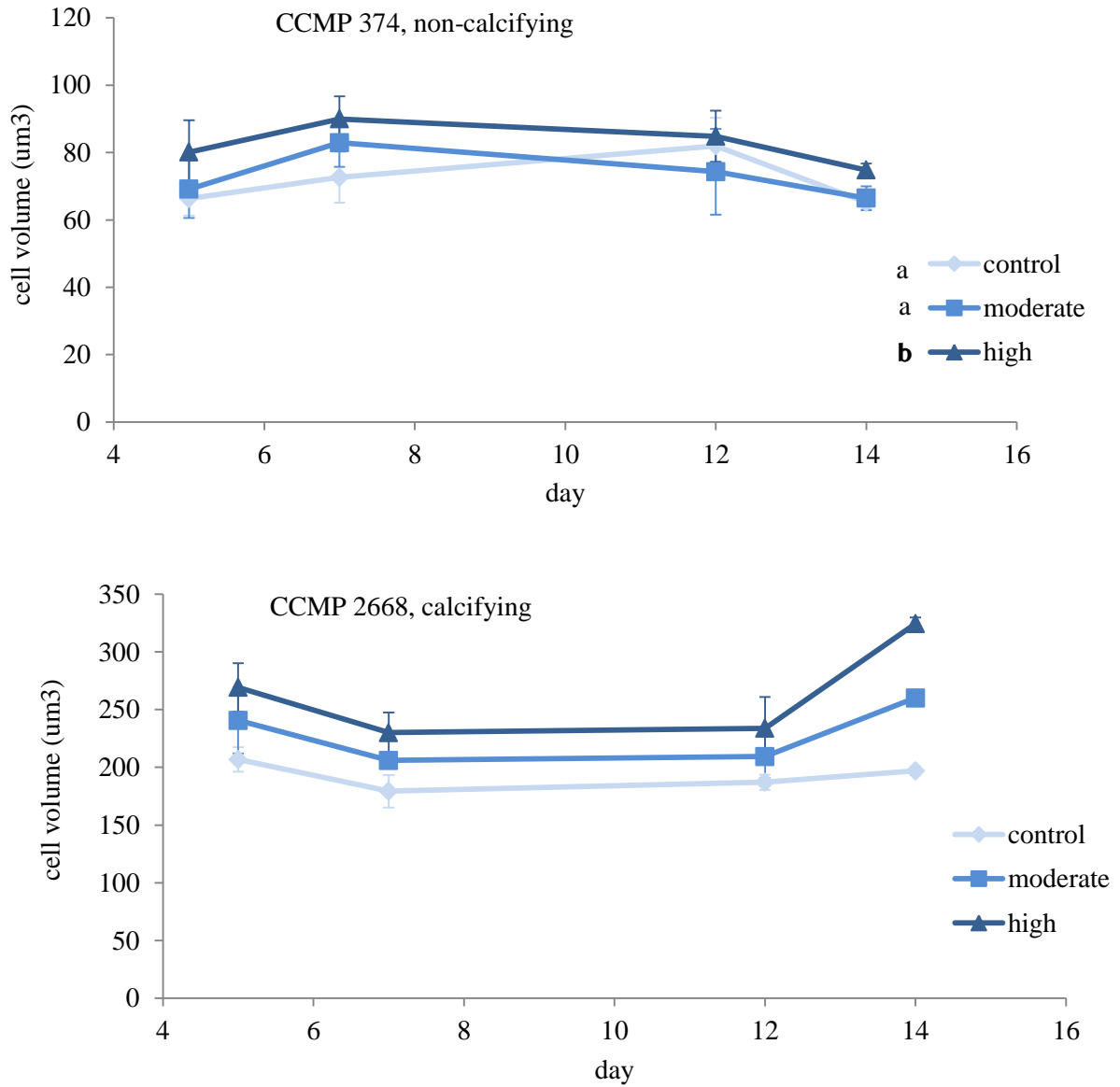


Figure 3. Cell volume of cells in culture during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Note the difference in the y-axis scales. Treatments with shared letters (a,b) are not statistically different.



initial decrease in cell size, followed by an increase in the elevated CO<sub>2</sub> treatments. For strain CCMP 2668, cell volume did not increase over time in ambient CO<sub>2</sub>, but did increase in moderate and high CO<sub>2</sub> (ANOVAR interaction p-value 0.003).

### **Carbon and nitrogen**

*Emiliana huxleyi* particulate organic carbon (POC) per cell was not affected by elevated CO<sub>2</sub> (Figure 4, Table 1 & 2). Although not statistically significant, there was a tendency for CCMP 2668 cultures grown in high CO<sub>2</sub> to have higher POC cell<sup>-1</sup> than those grown in ambient and moderate CO<sub>2</sub>. Over time, POC cell<sup>-1</sup> significantly increased for strain CCMP 2668 under all CO<sub>2</sub> conditions (ANOVAR time effect p-value 0.006).

*Emiliana huxleyi* strain CCMP 374 cell carbon density (POC μm<sup>-3</sup>) was not affected by elevated CO<sub>2</sub> (Figure 5, Table 1). Although it was not statistically significant, cells grown in moderate CO<sub>2</sub> tended to have higher carbon density than cells grown in high CO<sub>2</sub>. Time had no significant effect on carbon density. Organic carbon density is not reported for CCMP 2668, because in those cells, there is an organic carbon center and an inorganic carbon shell, so it is not accurate to represent carbon in a density measurement since organic carbon is not distributed uniformly throughout the cell. However, cells grown in high CO<sub>2</sub> were larger and tended to contain more POC.

*Emiliana huxleyi* POC:PON was not affected by elevated CO<sub>2</sub> (Figure 6, Table 1 & 2). Although not statistically significant, for strain CCMP 374 cultures grown in high CO<sub>2</sub> tended to have lower POC:PON than those grown in moderate and ambient CO<sub>2</sub>. For strain CCMP 374, POC:PON decreased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value <0.001).

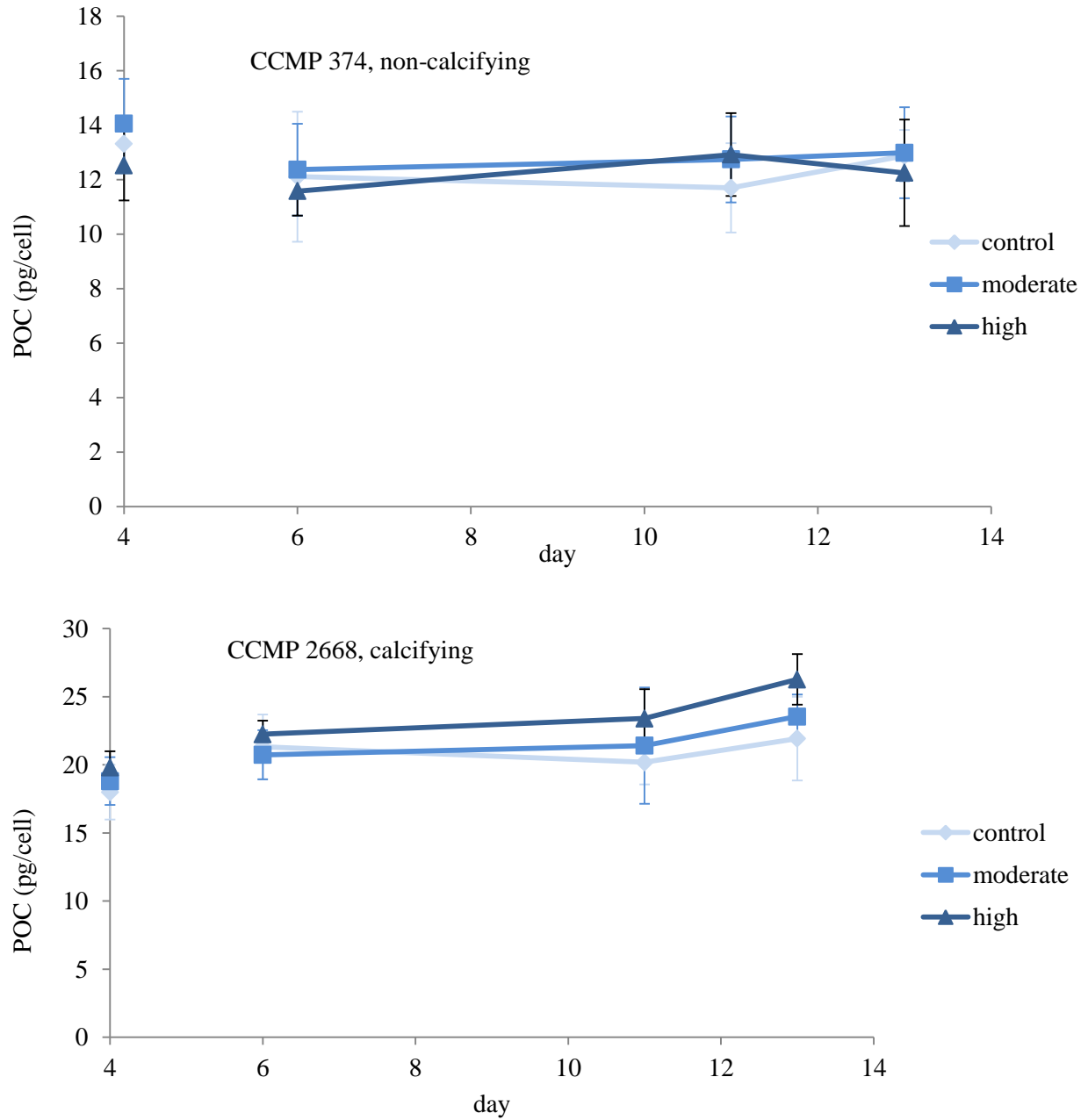


Figure 4. POC/cell of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Note the difference in the y-axis scales.

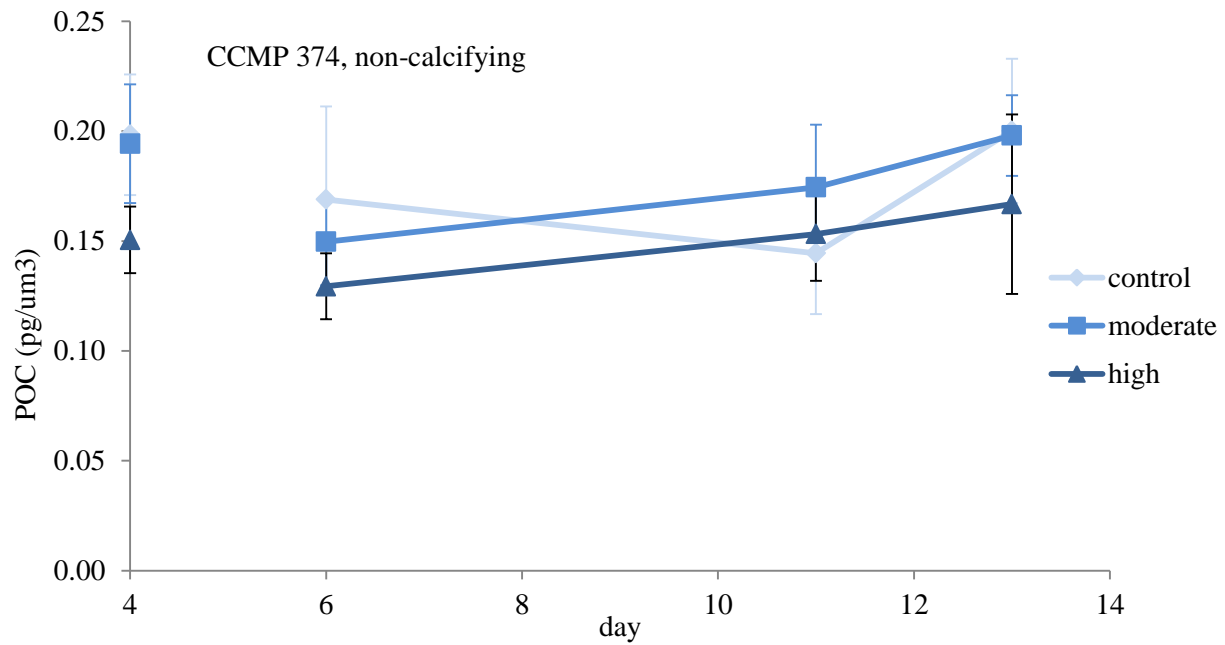


Figure 5. POC/ $\mu\text{m}^3$  *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions.

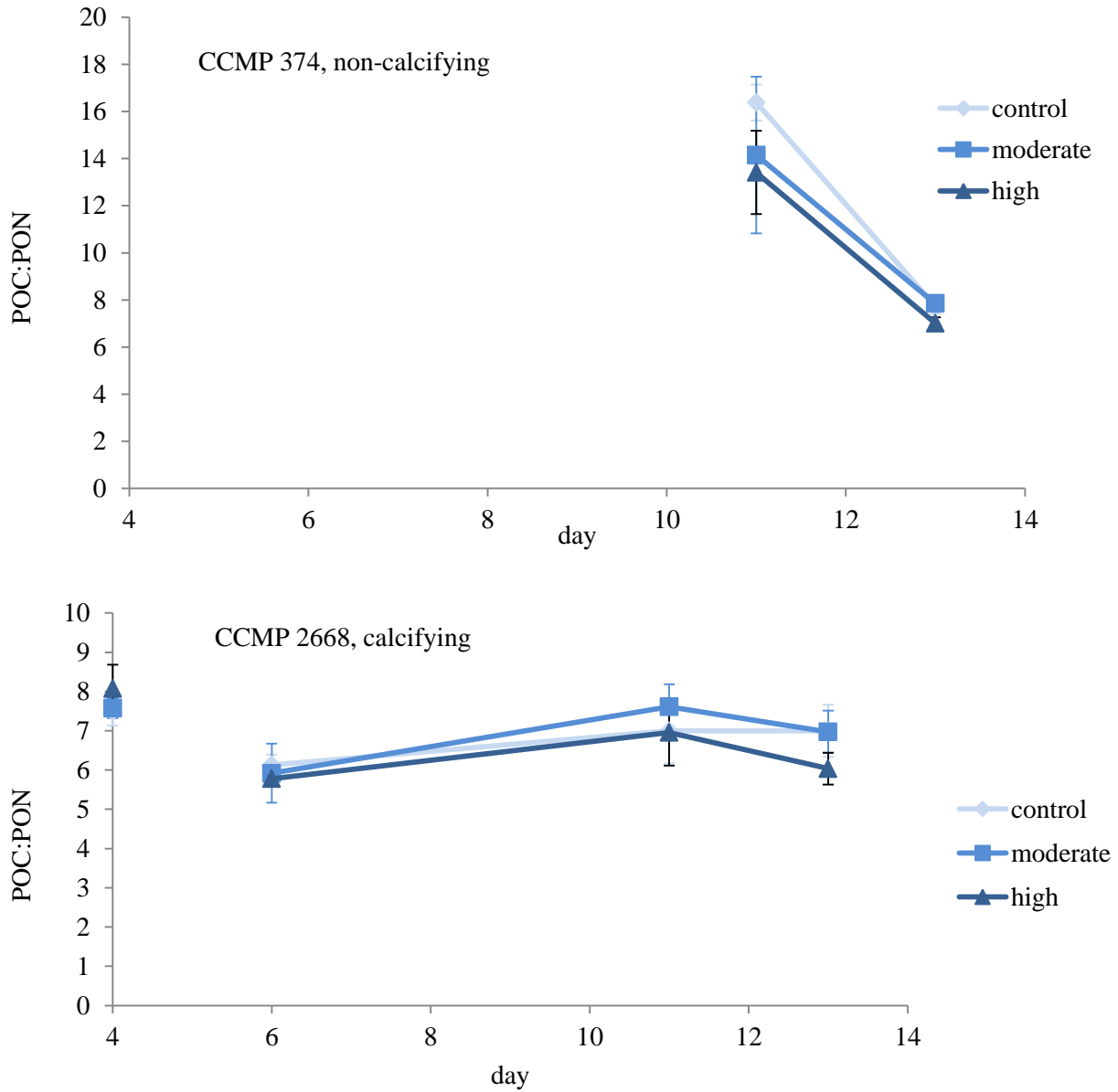


Figure 6. POC:PON of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Note the difference in the y-axis scales. Nitrogen data for days 4 and 6 were lost for strain CCMP 374.

For strain CCMP 2668, POC:PON initially increased, then decreased under all CO<sub>2</sub> conditions (ANOVAR time effect p-value <0.001).

*Emiliana huxleyi* strain CCMP 2668 particulate inorganic carbon (PIC) per cell was not affected by elevated CO<sub>2</sub> (Figure 7, Table 2). Time had no significant effect.

*Emiliana huxleyi* strain CCMP 2668 PIC:POC was not significantly affected by elevated CO<sub>2</sub> (Figure 8, Table 2). Although not statistically significant, for strain CCMP 2668 there was a tendency for cultures grown in ambient CO<sub>2</sub> to have higher PIC:POC than cultures grown in moderate and high CO<sub>2</sub>. Time had no significant effect. There was one data point which was an extreme outlier (a PIC:POC of 0.23 in ambient CO<sub>2</sub>, compared to the average of 0.79 in ambient CO<sub>2</sub>), which, when removed, yielded a significant ANOVAR result (ANOVAR p-value 0.043). PIC:POC in ambient CO<sub>2</sub> (average  $\pm$  1 SD) was  $0.79 \pm 0.004$  including the outlier, compared to  $0.84 \pm 0.08$  excluding the outlier. PIC:POC (average  $\pm$  1 SD) was  $0.69 \pm 0.04$  in high CO<sub>2</sub> and  $0.70 \pm 0.02$  in moderate CO<sub>2</sub>.

### **Particulate dimethylsulfoniopropionate (DMSPp)**

*Emiliana huxleyi* strain CCMP 374 had a significantly higher cellular DMSP content (DMSPp cell<sup>-1</sup>) in high CO<sub>2</sub> than in moderate CO<sub>2</sub> (ANOVAR with Tukey's post-hoc comparison; p-value 0.009) (Figure 9, Table 1). DMSPp cell<sup>-1</sup> for strain CCMP 374 in high CO<sub>2</sub> was 28% higher than in moderate CO<sub>2</sub>. Ambient DMSPp cell<sup>-1</sup> was not significantly different from that in high or moderate CO<sub>2</sub> conditions. Strain CCMP 2668 had significantly more DMSPp cell<sup>-1</sup> in high CO<sub>2</sub> than in ambient and moderate CO<sub>2</sub> (ANOVAR with Tukey's post-hoc comparison; p-value <0.001 and <0.001 respectively) (Figure 9, Table 2). DMSPp cell<sup>-1</sup> for

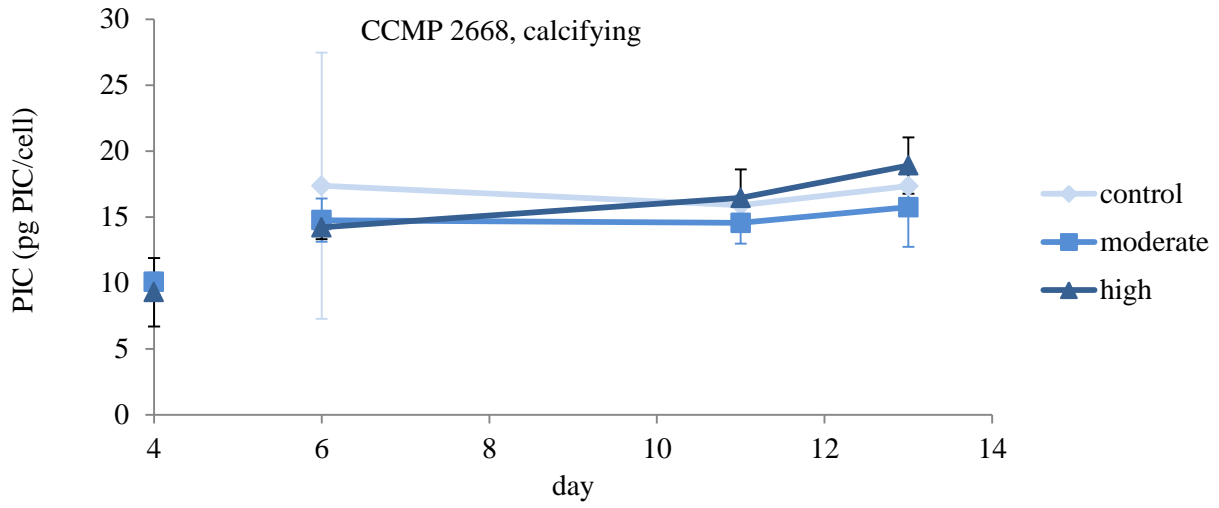


Figure 7. PIC/cell of *Emiliana huxleyi* during the calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions.

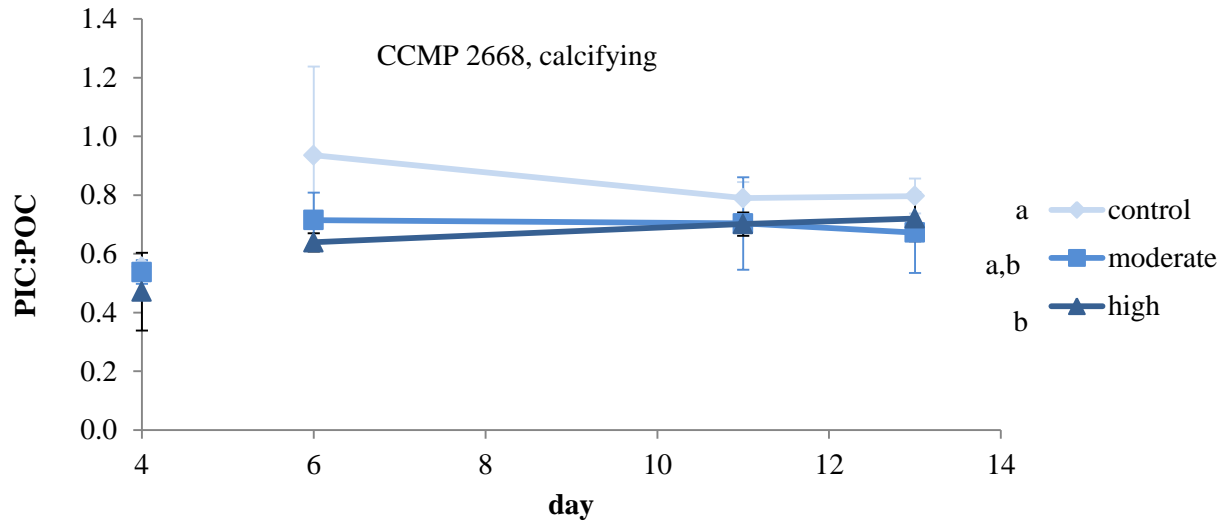


Figure 8. PIC:POC of *Emiliana huxleyi* during the calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Treatments with shared letters (a,b) are not statistically different.

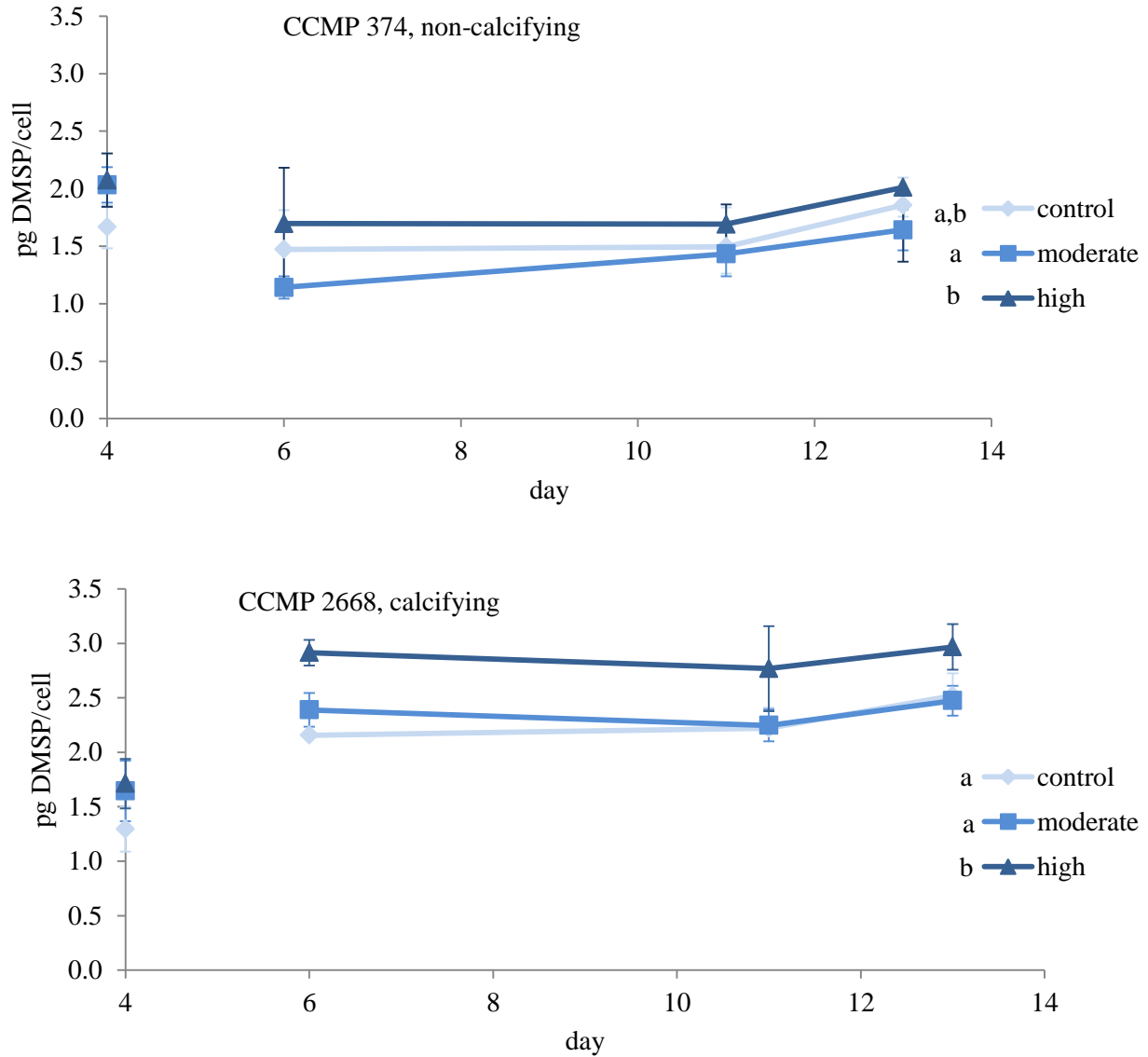


Figure 9. DMSP/cell of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Treatments with shared letters (a,b) are not statistically different.



strain CCMP 2668 in high CO<sub>2</sub> was 25% higher than ambient CO<sub>2</sub> and 22% higher than moderate CO<sub>2</sub>. For both strains, DMSPp cell<sup>-1</sup> slightly increased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value 0.006 and 0.010, CCMP 374 and CCMP 2668 respectively).

*Emiliana huxleyi* strain CCMP 374 had significantly more DMSPp POC<sup>-1</sup> in high CO<sub>2</sub> than in moderate CO<sub>2</sub> (ANOVAR with Tukey's post-hoc comparison; p-value 0.002) (Figure 10, Table 1). DMSPp POC<sup>-1</sup> for strain CCMP 374 in high CO<sub>2</sub> was 34% higher than in moderate CO<sub>2</sub>. Ambient DMSPp POC<sup>-1</sup> was not significantly different from either high or moderate CO<sub>2</sub>. Strain CCMP 2668 had more DMSPp POC<sup>-1</sup> in high CO<sub>2</sub> than in ambient and moderate CO<sub>2</sub> (Figure 10, Table 2). DMSPp POC<sup>-1</sup> for strain CCMP 2668 in high CO<sub>2</sub> was 10% higher than in both ambient and moderate CO<sub>2</sub>. For strain CCMP 374, DMSPp POC<sup>-1</sup> increased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value 0.015). For strain CCMP 2668, in moderate and high CO<sub>2</sub>, DMSPp POC<sup>-1</sup> decreased over time, and in ambient CO<sub>2</sub>, DMSPp POC<sup>-1</sup> increased over time (ANOVAR interaction p-value 0.026).

*Emiliana huxleyi* strain CCMP 374 volumetric DMSP content (DMSPp μm<sup>-3</sup>) was not affected by elevated CO<sub>2</sub> (Figure 11, Table 1). Over time, DMSPp μm<sup>-3</sup> increased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value <0.001).

### **Chlorophyll**

*Emiliana huxleyi* strain CCMP 374 cellular chlorophyll content (chlorophyll cell<sup>-1</sup>) was not affected by CO<sub>2</sub> (Figure 12, Table 1). Strain CCMP 2668 had significantly more chlorophyll cell<sup>-1</sup> in high CO<sub>2</sub> than in moderate CO<sub>2</sub> (ANOVAR with Tukey's post-hoc comparison; p-value

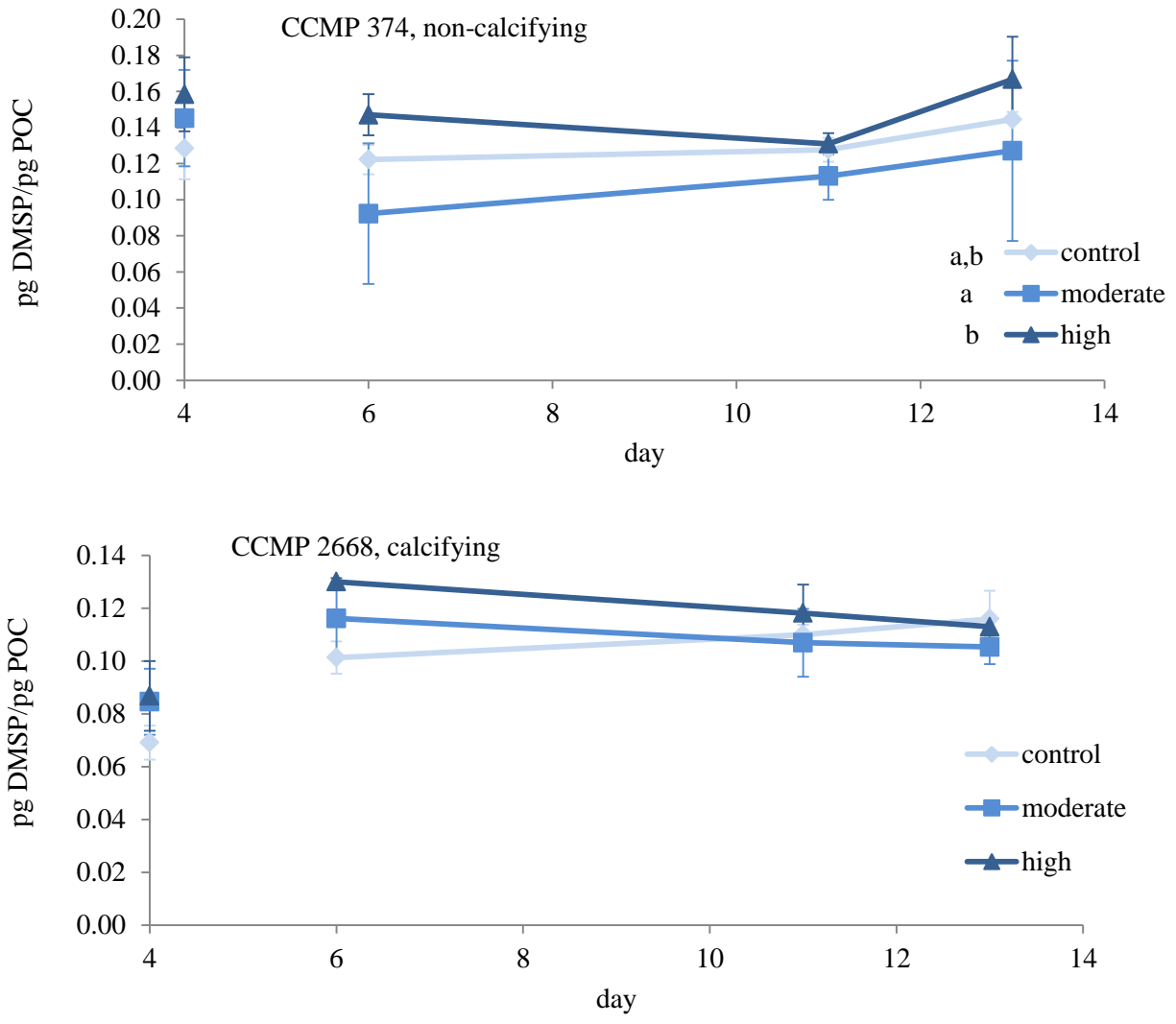


Figure 10. DMSP/POC of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Treatments with shared letters (a,b) are not statistically different.

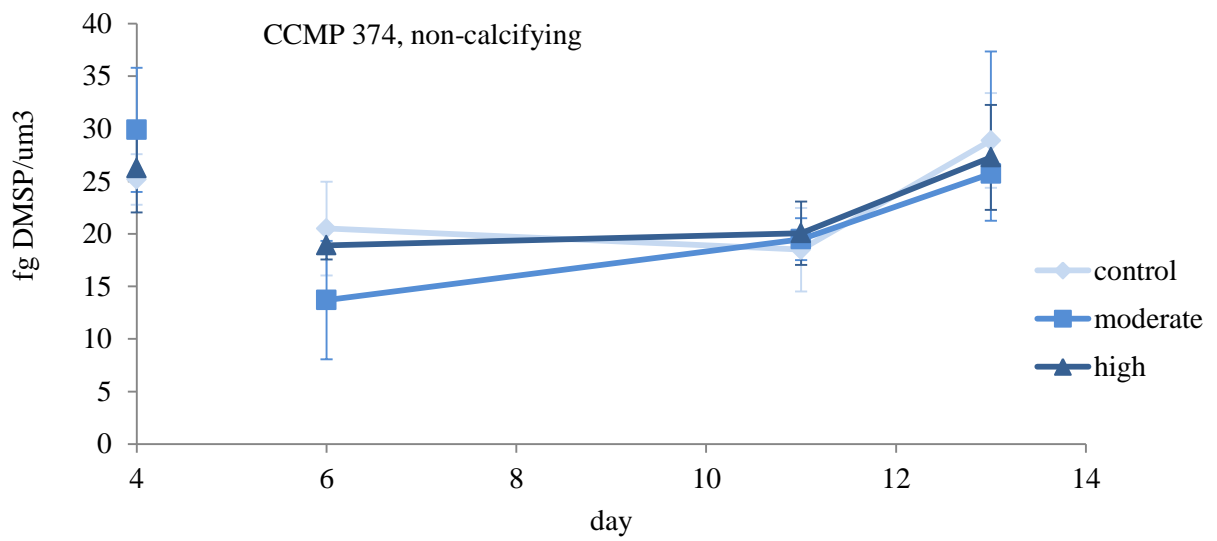


Figure 11. DMSP/ $\mu\text{m}^3$  of *Emiliania huxleyi* during the non-calcifying experiment (CCMP 374). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions.

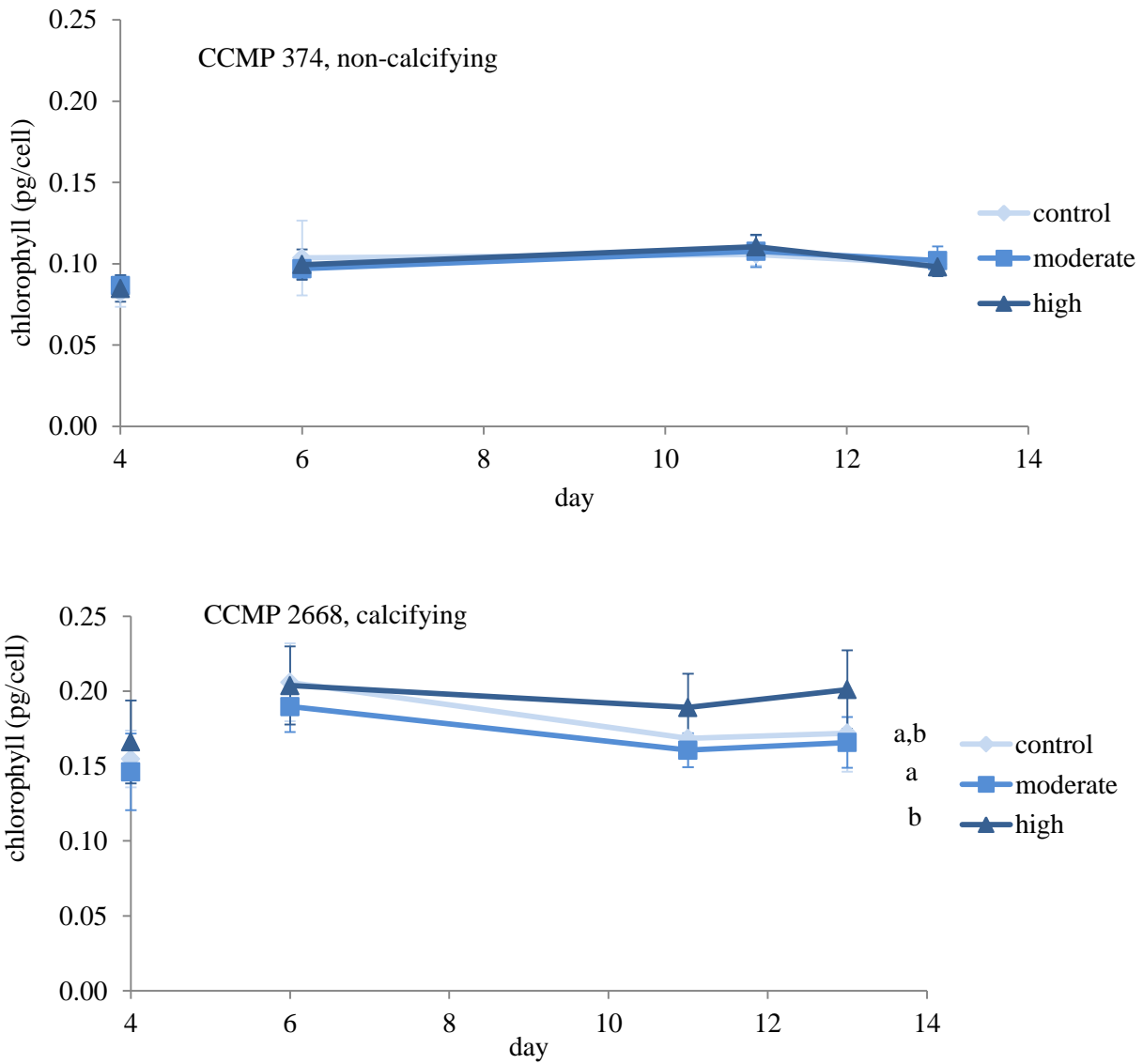


Figure 12. Chlorophyll/cell of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions. Treatments with shared letters (a,b) are not statistically different.

0.039) (Figure 12, Table 2). Chlorophyll cell<sup>-1</sup> for strain CCMP 2668 in high CO<sub>2</sub> was 15% higher than in moderate CO<sub>2</sub>. Ambient chlorophyll cell<sup>-1</sup> was not significantly different from high or moderate CO<sub>2</sub>. For strain CCMP 2668, there was an initial decrease in chlorophyll cell<sup>-1</sup>, followed by a slight increase under all CO<sub>2</sub> conditions (ANOVAR time effect p-value 0.003).

*Emiliana huxleyi* chlorophyll POC<sup>-1</sup> was not affected by elevated CO<sub>2</sub> (Figure 13, Table 1 & 2). For strain CCMP 2668, chlorophyll POC<sup>-1</sup> decreased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value <0.001).

*Emiliana huxleyi* strain CCMP 374 cell chlorophyll density (chlorophyll μm<sup>-3</sup>) (Figure 14, Table 1) was not affected by elevated CO<sub>2</sub>. Although not significant, there was a trend toward lower chlorophyll μm<sup>-3</sup> in cultures grown in high CO<sub>2</sub> relative to cultures grown in ambient and moderate CO<sub>2</sub>. Chlorophyll μm<sup>-3</sup> generally increased over time under all CO<sub>2</sub> conditions (ANOVAR time effect p-value 0.028).

### **Strain and parameter comparison**

Cell volume was the parameter most influenced by CO<sub>2</sub> in both strains (Figure 15). The second most affected parameter by CO<sub>2</sub> was calcification (PIC:POC) in the calcifying strain (Figure 16). DMSPp content and chlorophyll content were also affected by CO<sub>2</sub> (Figures 17, 18, and 19). For strain CCMP 2668 cell volume increase was more dramatic than for strain CCMP 374 under elevated CO<sub>2</sub> (Figure 15). POC cell<sup>-1</sup>, DMSP cell<sup>-1</sup>, and chlorophyll cell<sup>-1</sup>, all increased, to various degrees, with cell size in strain CCMP 2668 (Figure 20, 17 and 19). Therefore, cell parameters measured, with the exception of calcification, scaled up with cell size

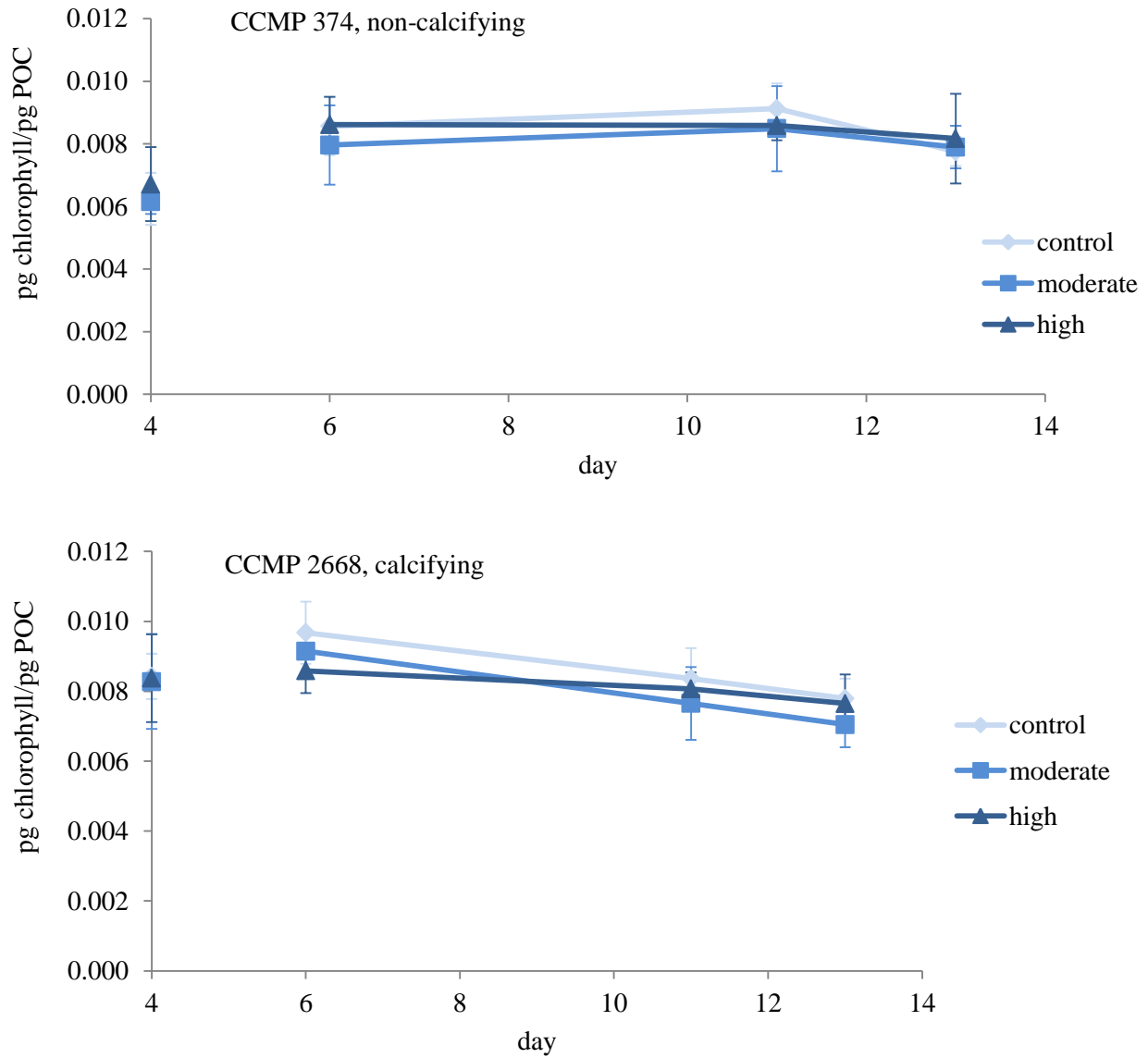


Figure 13. Chlorophyll/POC of *Emiliana huxleyi* during the non-calcifying experiment (CCMP 374) and calcifying experiment (CCMP 2668). Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions.

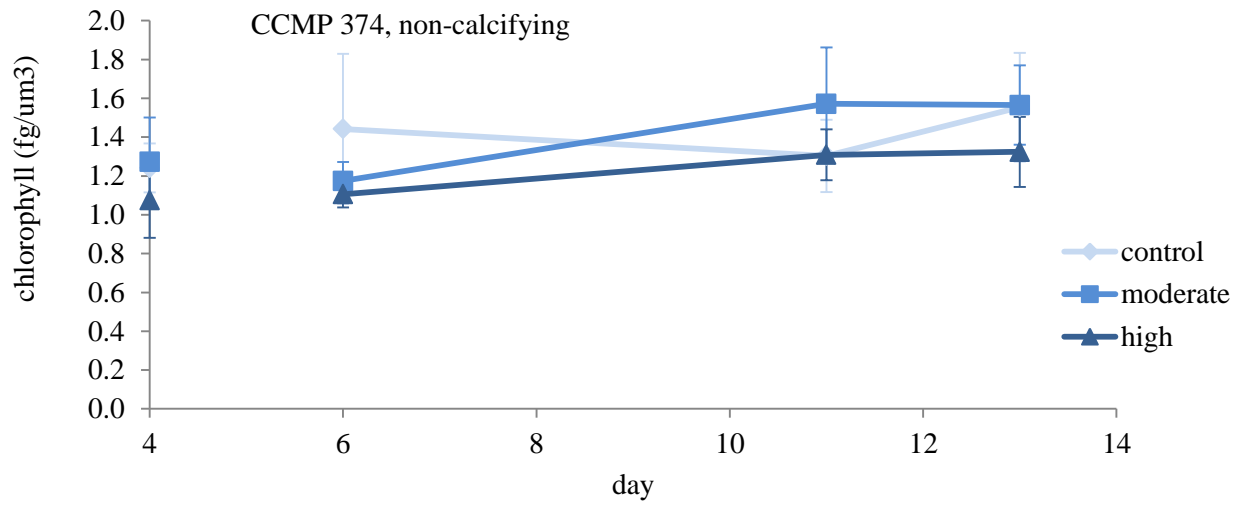


Figure 14. Chlorophyll/ $\mu\text{m}^3$  of *Emiliana huxleyi* during the non-calcifying experiment. Error bars represent  $\pm 1$  SD (n=5). Data on day 4 represent batch cultures sampled just prior to the first dilution; all other data represent semi-continuous culture conditions.

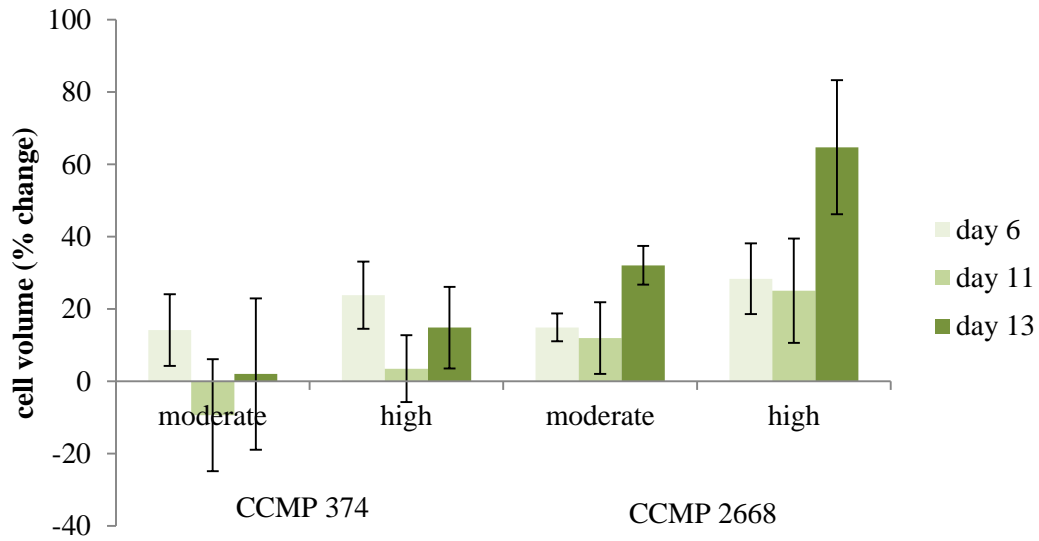


Figure 15. Percent change in cell volume relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).



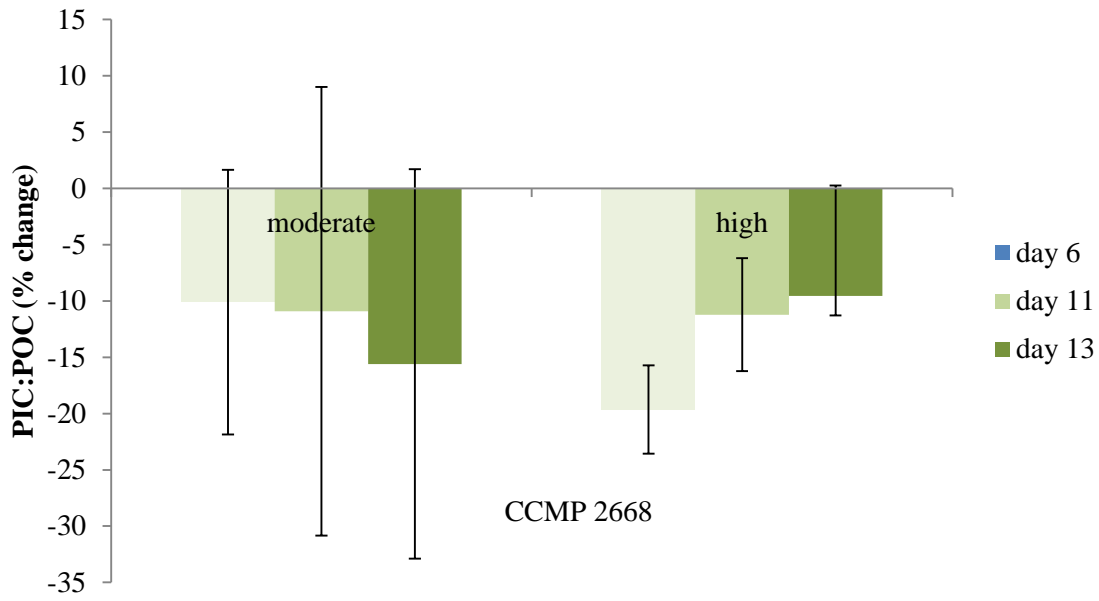


Figure 16. Percent change in PIC:POC relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent  $\pm 1$  SD (n=5).

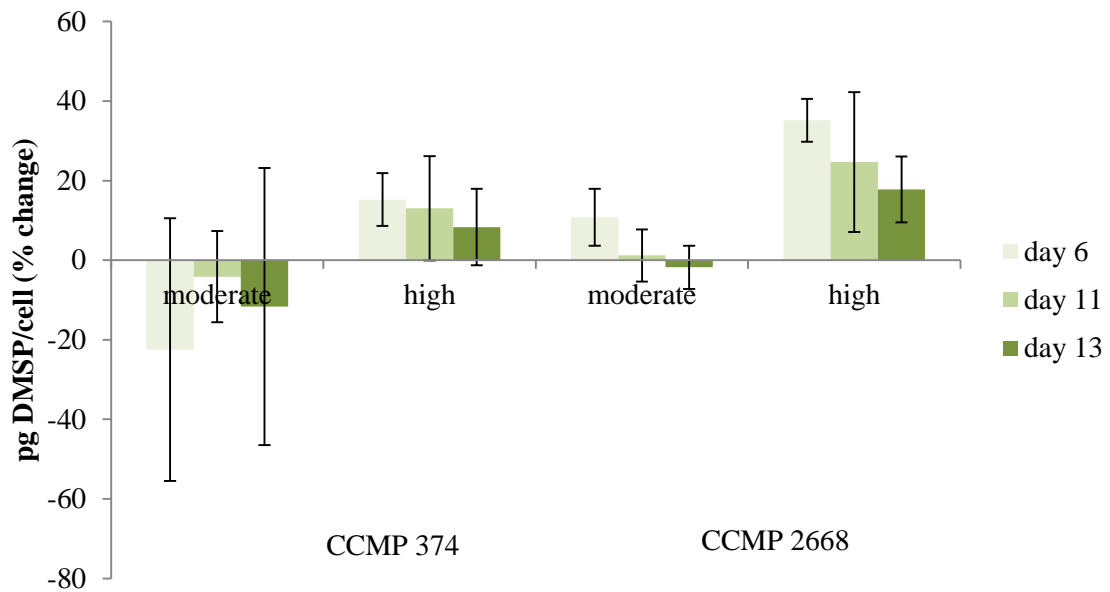


Figure 17. Percent change in DMSP/cell relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

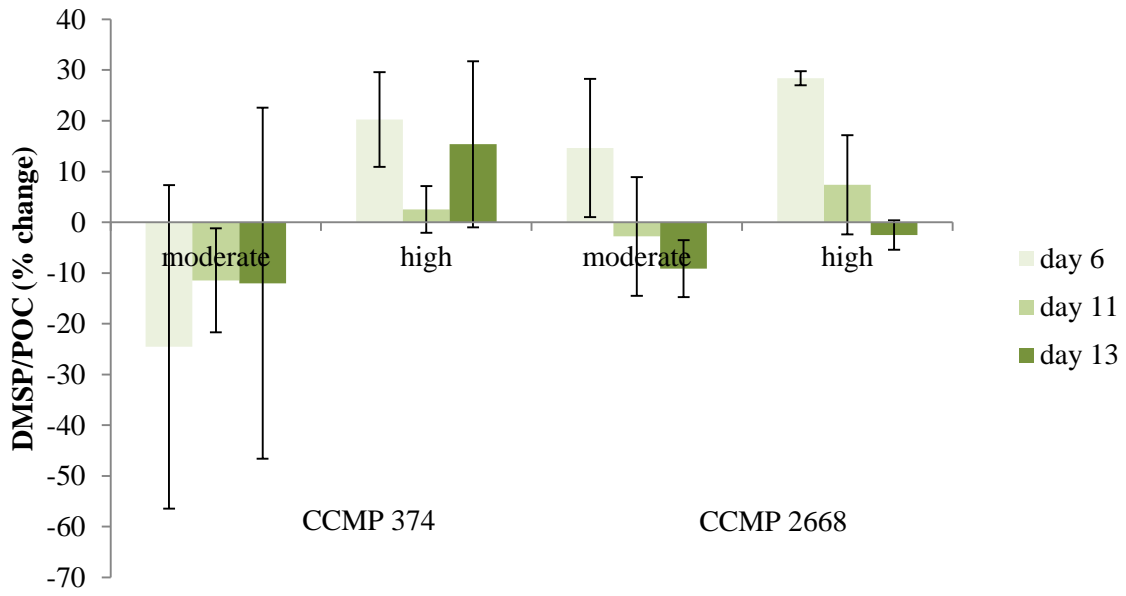


Figure 18. Percent change in DMSP/POC relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

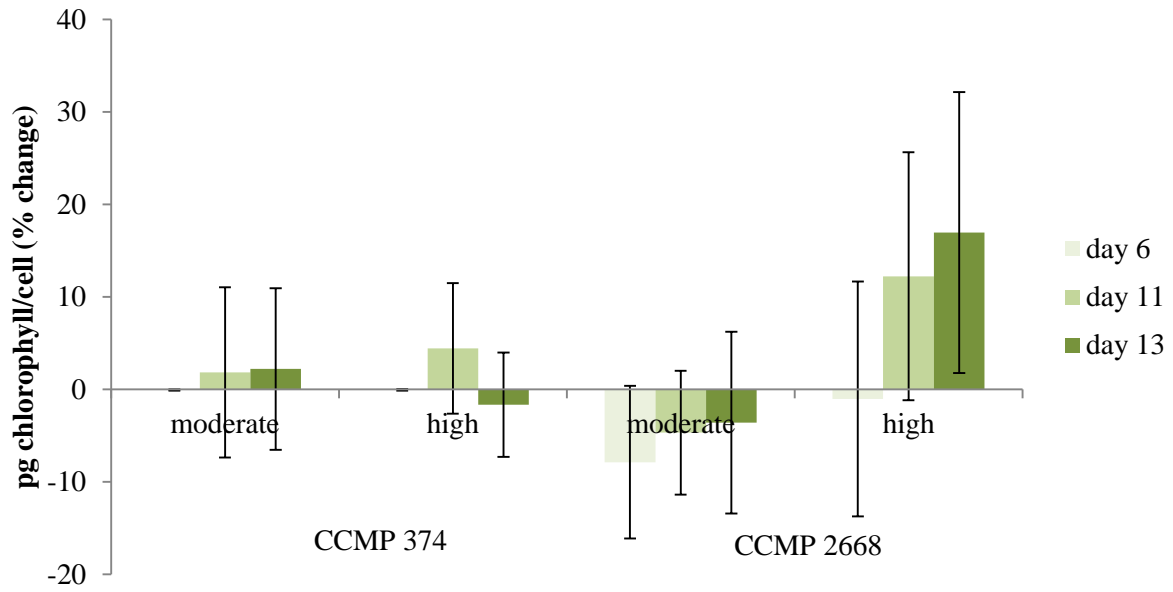


Figure 19. Percent change in chlorophyll/cell relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

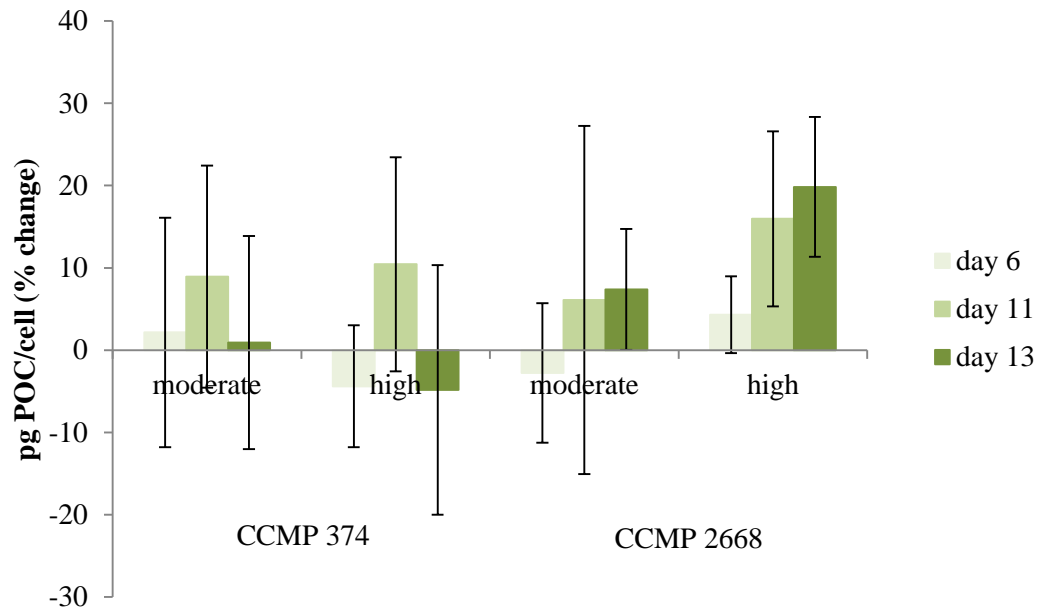


Figure 20. Percent change in POC/cell relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

in strain CCMP 2668. For strain CCMP 374, however, only DMSP cell<sup>-1</sup> increased with cell size (Figure 17). Therefore, not all cell parameters measured scaled up with cell size in strain 374. Figures that compare percent change in POC μm<sup>-3</sup>, POC:PON, PIC cell<sup>-1</sup>, DMSP μm<sup>-3</sup>, chlorophyll POC<sup>-1</sup>, and chlorophyll μm<sup>-3</sup> are located in the appendix.

## DISCUSSION

The largest physiological effect of elevated CO<sub>2</sub>, observed in both strain of the coccolithophore *Emiliana huxleyi*, was an increase in cell size. In addition, there was a decrease in calcification in the calcifying strain under elevated CO<sub>2</sub> conditions. In the calcifying strain, other cellular constituents examined, including organic carbon, DMSPP, and chlorophyll, scaled up with cell size to varying degrees. In the non-calcifying strain, DMSPP scaled up with cell size, but organic carbon and chlorophyll did not.

I hypothesized that cell size would be greater in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>. This hypothesis was based on the idea that carbon from carbon fertilization in elevated CO<sub>2</sub> may be utilized as increased biomass, yielding larger cells. My research showed that cell size was greater in elevated CO<sub>2</sub> in both strains of *Emiliana huxleyi*. However, even though cell size increased with elevated CO<sub>2</sub>, organic carbon per cell did not. Therefore, carbon from carbon fertilization may not be utilized as biomass. It is important to note, though, that even though it was not statistically significant, in the calcifying strain (CCMP 2668) organic carbon per cell had a tendency to increase in elevated CO<sub>2</sub>. Organic carbon per cell was still increasing at the end of the experiment, so this may have been statistically significant if the experiment had continued for a longer period of time (Figure 4b, Table 1). Thus, there is the potential for carbon from carbon fertilization to be utilized as biomass, at least in the calcifying strain (CCMP 2668). On the other hand, it is entirely possible that the algal cells were primarily increasing their size by increasing the volume of water within the cells. *Emiliana huxleyi* cells may have increased the volume of water inside their cells by changing the amount of osmolytes within the cell (see DMSPP below). It is also important to note that in the calcifying strain, calcification decreased in elevated CO<sub>2</sub>,

and therefore, the proportion of cell size occupied by inorganic carbon would likely have decreased. This means that even more of the cell than indicated by the net cell size increase must have been composed of some other constituent, such as water. It is possible, however, that the placement of coccoliths changed between CO<sub>2</sub> treatments. For example, coccoliths could have been arranged more loosely around the cell in elevated CO<sub>2</sub>, making the cells appear larger overall, but not increasing the size of the organic portion of the cells. In summary, calcification, coccolith placement, organic carbon content, and internal water volume may all have had a role in cell size changes.

Cell size was the most pronounced observed change (15-37% increase) in *Emiliana huxleyi* physiology in response to elevated CO<sub>2</sub>. Prey cell size is an important factor in the mechanical ability of zooplankton to feed (Hansen *et al.* 1994). The increase in cell size may make *Emiliana huxleyi* more available to some predators and less available to other predators (Hansen *et al.*, 1996). Some raptorial feeding predators, such as dinoflagellates that feed by a peduncle, and larger filter feeding predators, such as larger ciliates, may be able to increase their feeding rates on larger *Emiliana huxleyi* cells. Some other raptorial feeding predators, such as those that feed by phagocytosis, and small filter feeding predators, such as small ciliates, may no longer be able to feed on these larger prey cells. Grazing rates on *Emiliana huxleyi* influence whether nutrients are transported upward through successive trophic levels or are transported through the microbial loop or sink to the deep ocean as dead cells during and after bloom events. Successful grazing by particular species can also lead to ecosystem composition shifts (Bergquist *et al.* 1985). Several other studies found elevated CO<sub>2</sub> to influence *Emiliana huxleyi* cell size.



Barcelos e Ramos *et al.* (2009) found an initial decrease, followed by an increase in *Emiliana huxleyi* cell size over 26 h under elevated CO<sub>2</sub>. Iglesias-Rodriguez *et al.* (2008) found an increase in *Emiliana huxleyi* cell volume under elevated CO<sub>2</sub> conditions. My research showed a similar trend. The initial decrease in cell size in the Barcelos e Ramos *et al.* study was after only 14 h. This is a smaller time scale than used in my research and appeared to be only temporary. Engel *et al.* (2005) found an increase in coccosphere size of *Emiliana huxleyi* under low CO<sub>2</sub> conditions. The coccosphere size in the present-day (410 ppm) and the high (710 ppm) CO<sub>2</sub> treatment in the Engel *et. al.* study, however, were not significantly different. The treatment that yielded larger cells was the low (190 ppm) CO<sub>2</sub> treatment in comparison to present day and high CO<sub>2</sub> (Engel *et al.*, 2005). Since my research did not include this pre-industrial era CO<sub>2</sub> value, the trend cannot be accurately compared.

I also hypothesized that intracellular dimethylsulfoniopropionate (DMSPp) would increase in cells exposed to elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>. This hypothesis was based on the idea that a decrease in pH would be a stress to the algal cells, which could increase the amount of DMSPp. Stresses from pH could include a change in the speciation of necessary elements in seawater, an increase in energy consumption to maintain ion balance, and the inactivation of enzymes (Hinga, 2002). This research showed that DMSPp did not show a linear response to elevated CO<sub>2</sub> in the non-calcifying strain, and DMSPp increased in response to elevated CO<sub>2</sub> in the calcifying strain.

In the non-calcifying strain (CCMP 374), DMSPp was reduced in the moderate CO<sub>2</sub> level compared to the high CO<sub>2</sub> level, but DMSPp of the ambient CO<sub>2</sub> level was not significantly different from either the high or the moderate CO<sub>2</sub> levels. This trend was seen in DMSPp on a

per cell basis and a per unit organic carbon basis, but not on a per unit volume basis. This means that DMSPp scaled up with cell size, but organic carbon did not follow the same trend. This scaling discrepancy is supported by the result that the DMSPp per unit volume was not significantly different among all CO<sub>2</sub> treatments. The carbon from DMSPp was 3% to 6% of POC per cell. Due to its small contribution to POC, it is possible for DMSPp, a carbon-rich molecule, to have a differing trend than POC.

In the calcifying strain (CCMP 2668), DMSPp per cell increased in high CO<sub>2</sub> compared to moderate and ambient CO<sub>2</sub> treatments, but DMSPp per unit organic carbon did not increase. This means that DMSPp increased in the same manner as organic carbon, indicating that DMSPp content scaled up proportionally with cell size. Therefore, DMSPp is contributing to some extent to the increase in cell size.

This increase in DMSP could have caused the increase in cell size, rather than cell size increase causing an increase in DMSP. Since DMSP is an osmolyte (Kirst 1996; Vairavamurthy *et al.*, 1985), the increase in DMSP could have caused the cells to increase in size by bringing more seawater into the cells. This scenario could explain the increase in cell size and DMSP without a change in cellular POC and chlorophyll in the non-calcifying strain.

Two possibilities as to why DMSPp may increase in response to elevated CO<sub>2</sub> are the synthesis of DMSPp as excess photosynthesis carbon waste and as a stress response. DMSPp is a molecule that is rich in carbon, but does not contain nitrogen. One function of DMSPp may be as excess photosynthesis carbon waste (Stefels, 2000). This would generally occur when photosynthesis continues to occur, but protein synthesis is limited by nutrient limitation. In my study, nutrient limitation would not have occurred due to the semi-continuous method of

culturing. However, it is possible that the rate of photosynthesis increased beyond the rate of protein synthesis and excess photosynthesis carbon was created. Further studies should examine the rate of photosynthesis under elevated CO<sub>2</sub>. The other possibility for increased DMSPp is that it is a stress response to elevated CO<sub>2</sub>. An increase in DMSP has been shown to be caused by oxidative stress (Sunda *et al.*, 2002). An increase in CO<sub>2</sub> could be another stress to *Emiliana huxleyi*.

DMS, which is derived from DMSP, is an important climatic gas involved in the formation of cloud condensation nuclei (Charlson *et al.*, 1987). Due to the non-linear relationship between DMSPp and pCO<sub>2</sub>, as well as the variety of factors affecting cell DMSP content, it is unclear how intracellular DMSP will change in future high-CO<sub>2</sub> conditions. Marine production of DMS is a significant part of the sulfur cycle. If the production of DMS changes, sulfur cycling will be greatly impacted, as well as the formation of clouds. *Emiliana huxleyi* is not the only producer of DMSP. There are other marine microalgal and macroalgal species that produce DMSP (Keller *et al.*, 1989), and marine bacteria can also convert DMSP to DMS (Kiene, 1990). These species must also be examined to gain a clearer picture of how DMS production might change in the future. If DMS production increases, it could provide a negative feedback to global warming.

Dissolved DMSP is a chemical signal which may deter predators without damage to the *Emiliana huxleyi* cell. Dissolved DMSP has been shown to decrease grazing rates (Strom *et al.*, 2003b). Since DMSPp is increased under elevated CO<sub>2</sub>, at least in the calcifying strain, there is the potential for grazing rates on *Emiliana huxleyi* to decrease, assuming increased particulate

DMSP translates into increased dissolved DMSP. This change in grazing rates could change the path of nutrient transport and the occurrence and extent of bloom events.

Wingenter *et al.* (2007) found that dimethyl sulfide (DMS) concentrations in mesocosms dominated by *Emiliana huxleyi* increased under elevated CO<sub>2</sub> conditions. In contrast, this research focused on intracellular DMSP; however the trend in DMSPp was similar to that found in DMS in the Wingenter *et al.* study.

I also hypothesized that calcification would decrease in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>. This hypothesis was based on the idea that calcium carbonate formation would be energetically less favorable in a lower pH environment (a lower carbonate saturation state). Under lower pH conditions, coccoliths would be more susceptible to dissolution, and the creation of coccoliths would require more energy. My research showed that calcification decreased in elevated CO<sub>2</sub>, as indicated by the PIC:POC ratio. It is expected that under normal conditions, changes in PIC and POC should parallel each other. In my study, however, there was a tendency for organic carbon to increase in response to elevated CO<sub>2</sub>, while inorganic carbon did not. Due to the changes in cell size, the PIC:POC ratio is a better indicator of the process of calcification than PIC cell<sup>-1</sup>. Although PIC cell<sup>-1</sup> did not change in response to CO<sub>2</sub> treatment, the process of calcification did change, since elevated CO<sub>2</sub> resulted in less inorganic carbon per unit organic carbon. It is important to note that the change in PIC:POC is being driven by both the increase in POC cell<sup>-1</sup> in the high CO<sub>2</sub> treatment and the decrease PIC in the moderate CO<sub>2</sub> treatment. The cells are getting larger, but have proportionally less PIC in elevated CO<sub>2</sub> than in ambient CO<sub>2</sub>.

Reduced calcification of *Emiliana huxleyi* in projected future high-CO<sub>2</sub> conditions could alter biogeochemical cycling of carbon. Calcium carbonate from coccoliths is an important global carbon sink. These coccoliths remove carbon from the dissolved inorganic carbon pool in seawater and can sink as a precipitate when the algal cell dies. Even if the algal cell is eaten, some predatory species will excrete the coccoliths as a precipitate (pellet) (Harris, 1994). The end of a coccolithophore bloom due to consumption of nutrients can result in significant carbon export to the deep ocean and into sedimentary rocks (Buitenhuis *et al.*, 2001). If less calcium carbonate is being incorporated into coccoliths, less carbon is going to be exported to the deep ocean. This will cause a change in the export fluxes of carbon. This is particularly important in *Emiliana huxleyi*, because large marine sedimentary deposits have been formed by recurrent coccolithophore blooms (Witty, 2011), and *Emiliana huxleyi* is a cosmopolitan coccolithophore.

One possible advantage to coccoliths is defense against predation, although the reason for coccolith production is unknown, and several theories exist (Young, 1994). Coccoliths could particularly have potential advantage against predators with a cell piercing feeding mode, such as a peduncle. In a future high-CO<sub>2</sub> ocean, grazing rates on *Emiliana huxleyi* have the potential to increase as calcification is decreased. If grazing rates increase, more nutrients may be kept in surface waters and transported to successive trophic levels. This could also impact the size and recurrence of *Emiliana huxleyi* blooms.

My finding of decreased calcification is similar to results from previous studies. Decreased PIC:POC in *Emiliana huxleyi* under elevated CO<sub>2</sub> conditions was found by Riebesell *et al.* (2000), Zondervan *et al.* (2001), Barcelos e Ramos *et al.* (2009) and Engel *et al.* (2005). Iglesias-Rodriguez *et al.* (2008) found no change in PIC:POC in *Emiliana huxleyi* under

elevated CO<sub>2</sub> conditions, but found an increase in both inorganic and organic carbon. The Iglesias-Rodriguez *et al.* study had a short time scale of only 1.5 d to 3 d. There is a possibility that this was not a sufficient amount of time for the algal cells to adjust their inorganic and organic carbon. However, the Barcelos e Ramos *et al.* study had a similar experimental time scale (26 hours) to the Iglesias-Rodriguez *et al.* study. Therefore, the difference in calcification trends could be a strain difference.

I also hypothesized that chlorophyll content would not be affected by elevated CO<sub>2</sub>. This hypothesis was based on the idea that chlorophyll content is influenced mainly by light intensity and nutrient availability. My research showed that chlorophyll content was not affected by elevated CO<sub>2</sub> in the non-calcifying strain (CCMP 374), and chlorophyll content did not linearly change with elevated CO<sub>2</sub> in the calcifying strain (CCMP 2668).

Chlorophyll content was not affected by elevated CO<sub>2</sub> in the non-calcifying strain (CCMP 374). Although not significant, there was a tendency for high CO<sub>2</sub> cells to have less chlorophyll per unit volume. This means that even though the cells grew larger in high CO<sub>2</sub>, they did not increase the amount of chlorophyll in proportion to that cell volume increase. Therefore, chlorophyll was not a major component of the increase in cell size. This is another indicator that the algal cells may be increasing their size by increasing their intracellular water volume.

Chlorophyll content per cell in the calcifying strain (CCMP 2668) was lower in moderate CO<sub>2</sub> than in high CO<sub>2</sub>, but neither moderate nor high CO<sub>2</sub> was different from ambient CO<sub>2</sub>. This non-linear response of chlorophyll content to elevated CO<sub>2</sub> indicates that there was more than one process governing the cell chlorophyll response to CO<sub>2</sub>. For example, there may be a

balance between the amount of light reaching the cell surface through the coccoliths and the production of chlorophyll from an increased cell size. Since calcification decreased in elevated CO<sub>2</sub>, more light may have penetrated the cell surface, which could have caused a decrease in cell chlorophyll content. However, the cells were also larger in elevated CO<sub>2</sub>, which may have caused an increase in cell chlorophyll content. The balance of these two processes could result in slightly lower chlorophyll content in moderate CO<sub>2</sub> and slightly higher chlorophyll content in high CO<sub>2</sub>, but no significant difference from ambient CO<sub>2</sub>. Since chlorophyll per unit organic carbon was not significantly different, that suggests that chlorophyll was scaling with the cell size increase in the same manner that organic carbon was scaling with the cell size increase.

I also hypothesized that growth rates would be higher in elevated CO<sub>2</sub> relative to ambient CO<sub>2</sub>. This hypothesis was based on the idea that carbon from carbon fertilization in elevated CO<sub>2</sub> may be utilized as increased biomass. Increased biomass can be achieved through a higher density of cells (i.e. higher growth rate) or through an increase in carbon content of individual cells. My research showed that growth rate (the rate of cell division) was not affected by CO<sub>2</sub> in either *Emiliana huxleyi* strain. This indicates that excess carbon may be utilized in some other fashion. Carbon can also be utilized by being incorporated into cell size or by being excreted from the cell as dissolved organic carbon. Another aspect to consider is that the process of semi-continuous culturing could affect the growth rate. The process of dilution to maintain steady-state exponential growth could be a larger influence on growth rate than the influence of CO<sub>2</sub> treatment, and therefore mask any subtle differences in growth rate due to CO<sub>2</sub> concentration.

Previous studies have showed mixed responses of *Emiliana huxleyi* growth rates to changes in pCO<sub>2</sub>. Clark and Flynn (2000) examined the growth rate of *Emiliana huxleyi* under

various dissolved inorganic carbon (DIC) concentrations with a constant pH, and did not see an influence of DIC on growth rate. This is in agreement with my findings. Barcelos e Ramos *et al.* (2009), however, observed a slight decrease in growth rate of *Emiliana huxleyi* with elevated CO<sub>2</sub>. The difference in trends between my study and the Barcelos e Ramos *et al.* study could be due to the CO<sub>2</sub> manipulation method and the experimental time scale. Barcelos e Ramos *et al.* used acid and base addition to manipulate the pH and carbon chemistry, and their experimental time scale was 2 h to 26 h. Iglesias-Rodriguez *et al.* (2008) also found a decrease in growth rate of *Emiliana huxleyi* under elevated CO<sub>2</sub> conditions. The difference in trends between the Iglesias-Rodriguez *et al.* study and my study could be due to strain differences.

The thresholds for change were different for each parameter studied and for each *Emiliana huxleyi* strain. There was a high CO<sub>2</sub> threshold for the calcifying strain (CCMP 2668) for changes in organic carbon, DMSPP, and chlorophyll, and for the non-calcifying strain (CCMP 374) for cell size. There was a moderate CO<sub>2</sub> level threshold for the calcifying strain (CCMP 2668) for changes in calcification and cell size. This is important when forecasting the response of *Emiliana huxleyi* to elevated CO<sub>2</sub> in the future. If future CO<sub>2</sub> conditions are on the lower end of the expected range, then only some of these changes will be seen.

Although careful consideration was taken to minimize the biological effect on pCO<sub>2</sub>, there is an indication that algal biomass had a small effect. Seawater pCO<sub>2</sub> concentrations in my experiments were lower than atmospheric pCO<sub>2</sub> concentrations in the incubation chambers. The difference between the atmospheric and dissolved pCO<sub>2</sub> may have been due to photosynthesis. The difference between atmospheric and dissolved concentrations was larger in high CO<sub>2</sub> than in moderate CO<sub>2</sub>. This indicates that more CO<sub>2</sub> may have been drawn out of the water per algal



cell in high CO<sub>2</sub> compared to moderate CO<sub>2</sub>. That would imply that carbon fertilization occurred, raising the question of where this carbon was partitioned. The carbon was not incorporated into biomass through increased growth rate (cells ml<sup>-1</sup>) and not significantly incorporated into biomass through increased cell size (by an increase in organic carbon per cell). The carbon may have been incorporated into the dissolved organic carbon pool in the form of organic exudates. Since algal cells cannot cease photosynthesis when exposed to light, there may have been an increase in carbon fixation under elevated CO<sub>2</sub>, and this extra carbon may have been exuded from the cells as waste. Although it is unlikely that nutrients were depleted in the seawater, the rate of photosynthesis may have increased more than the rate of nutrient acquisition, which could lead to an increase in production of these carbon-rich exudates. According to Borchard and Engel (2012), transparent exopolymer particles and dissolved carbohydrates were increased in higher CO<sub>2</sub> and higher temperature in experiments with *Emiliana huxleyi*. Further studies need to be done to examine the influence of elevated CO<sub>2</sub> on photosynthetic rates and dissolved organic carbon release.

### **Conclusion**

The largest influence of CO<sub>2</sub> on *Emiliana huxleyi*, inclusive of both strains, was the increase in cell size under elevated CO<sub>2</sub> conditions. This has the potential to change food web dynamics. The second largest impact of CO<sub>2</sub> on *Emiliana huxleyi* was the decrease in calcification seen in the calcifying strain. This has the potential to change food web dynamics and biogeochemical cycling of carbon. The other effects of CO<sub>2</sub> on *Emiliana huxleyi* were the non-linear change in DMSP in the non-calcifying strain, the increase in DMSP under elevated

CO<sub>2</sub> in the calcifying strain, and the non-linear change in chlorophyll in the calcifying strain.

These strain-specific differences in physiological effects resulting from elevated CO<sub>2</sub> reinforce the importance of strain-specific differences in experimental and field studies. Many studies do not report the strains they used, which may be very important in determining the significance of responses. The variety of responses to future oceanic conditions is enhanced by this strain-specific variability.

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## APPENDIX

### Preliminary chamber effect experiment

The effect of the chamber was analyzed by comparing growth rates of *Emiliana huxleyi* strain CCMP 2668 over 8 d in batch culture across all chambers. Growth rates were not affected by chambers. Growth rates (average  $\pm$  1 SD) were  $0.80 \pm 0.03$  d<sup>-1</sup> averaged across all chambers (Figure Ap1).

The conclusion was that the chamber that a culture was grown in did not affect the culture.

### Preliminary volume experiment

This experiment analyzed the effect of culture volume on pCO<sub>2</sub> and growth rate of *Emiliana huxleyi* strain CCMP 2668 for 7 d in batch culture in high CO<sub>2</sub>. Growth rates based on cell concentration via flow cytometry were not affected by culture volume (Figure Ap2). Growth rates based on cell concentration via flow cytometry (average  $\pm$  1 SD) were  $0.85 \pm 0.05$  d<sup>-1</sup> across all culture volumes. Growth rates based on in vivo fluorescence were higher in the 500 ml culture volume than in the 900 ml culture volume (ANOVA with Tukey's post-hoc comparison; p-value 0.013) (Figure Ap3). Growth rates based on in vivo fluorescence in the 900 ml culture volume were 4.5% less than those in the 500 ml culture volume. Fluorescence per cell was not affected by culture volume, but was affected by time (Figure Ap4). Fluorescence per cell (average  $\pm$  1 SD) was  $7.38 \times 10^{-6} \pm 1.84 \times 10^{-7}$  RFU cell<sup>-1</sup> across all culture volumes. Fluorescence per cell increased over time.



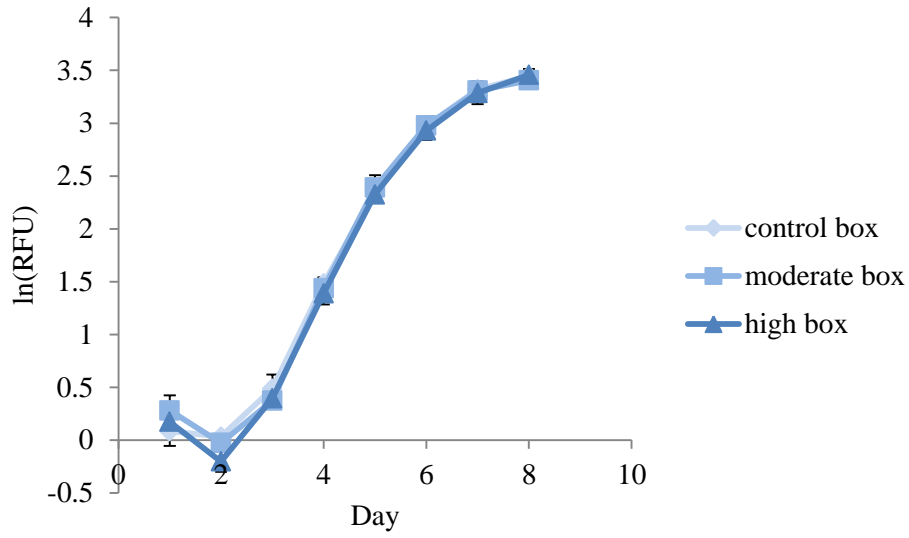


Figure Ap1. Growth curves of cultures during preliminary chamber effect experiment. Error bars represent  $\pm 1$  SD (n=3).

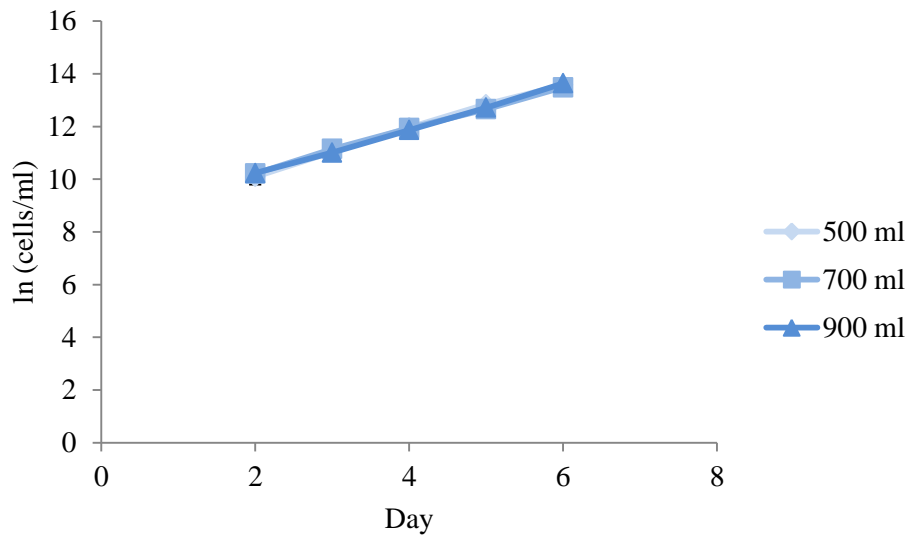


Figure Ap2. Growth curves of cultures based on cell concentrations via flow cytometry during preliminary volume experiment. Error bars represent  $\pm 1$  SD (n=3).

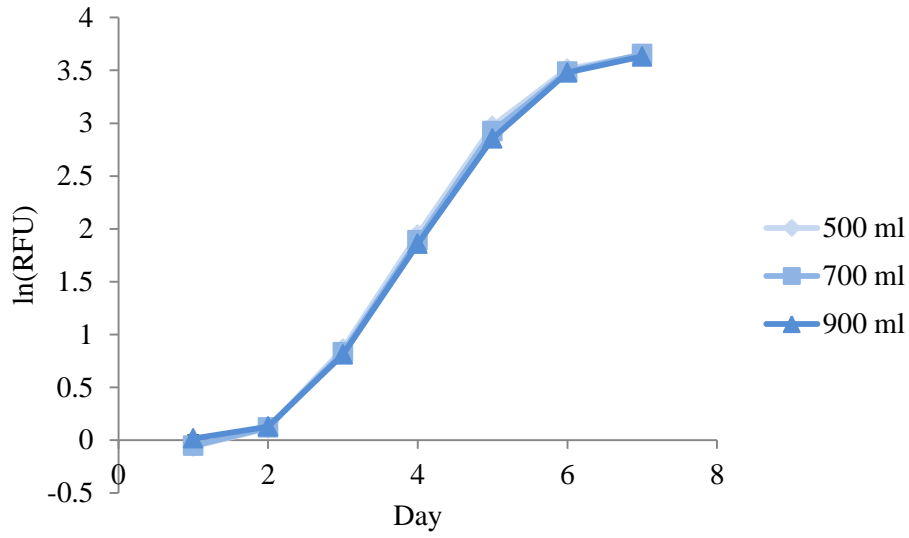


Figure Ap3. Growth curves of cultures based on in vivo fluorescence during preliminary volume experiment. Error bars represent  $\pm 1$  SD (n=3).

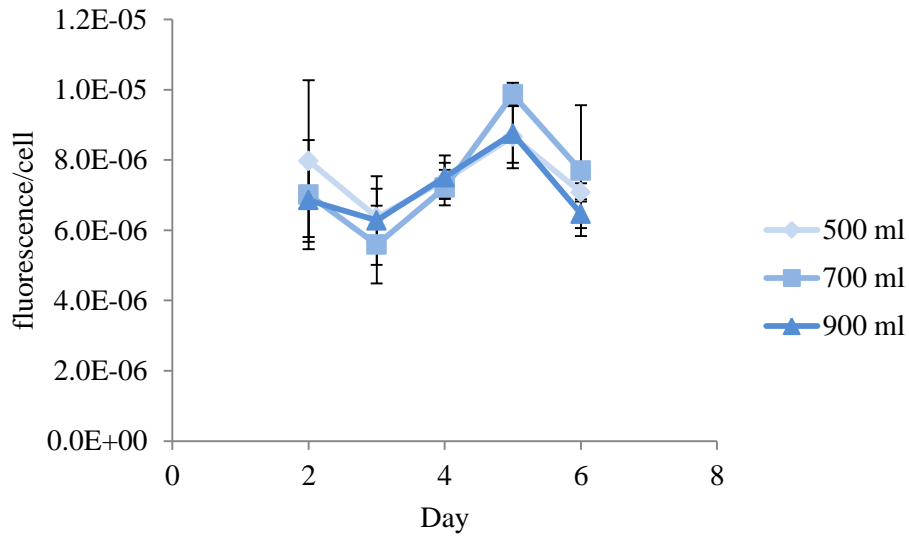


Figure Ap4. Fluorescence per cell during preliminary volume experiment. Error bars represent  $\pm$  1 SD (n=3).

pH had a significant interaction between time and volume (Figure Ap5). pH in the 900 ml culture was 2.7% higher than in the 500 ml culture volume and 1.1% higher than in the 700 ml culture volume at the end of the experiment. pCO<sub>2</sub> was affected by culture volume (Figure Ap6). pCO<sub>2</sub> was higher in the 500 ml culture volume than in the 700 ml or 900 ml culture volumes (ANOVA with Tukey's post-hoc comparison; p-value 0.005 and <0.001 respectively). pCO<sub>2</sub> was higher in the 700 ml culture volume than in the 900 ml culture volume (ANOVA with Tukey's post-hoc comparison; p-value 0.029). pCO<sub>2</sub> in the 500 ml culture volume was 45.4% higher than in the 900 ml culture volume and 26.5% higher than in the 700 ml culture volume. pCO<sub>2</sub> in the 700 ml culture volume was 25.8% higher than in the 900 ml culture volume.

The conclusion was that gas exchange was not as sufficient in maintaining the target pCO<sub>2</sub> with increased culture volume. The 500 ml culture volume had the most favorable surface area to volume ratio for sustaining target pCO<sub>2</sub>. This may or may not have an effect on growth rate, which can be noted from the discrepancy between trends in growth rate based on in vivo fluorescence and cell concentration via flow cytometry. There is a change in fluorescence per cell over the time course of the experiment, which may lead to the discrepancy between growth rates based on in vivo fluorescence and cell concentration via flow cytometry.

### **Preliminary batch culture experiment**

This primary reason for this experiment was to compare algal cell density to pH over time in batch culture in three pCO<sub>2</sub> conditions using separate cultures of *Emiliana huxleyi* strains CCMP 374 and CCMP 2668 to determine the appropriate target cell density for semi-continuous experiments. Biomass measurements were compared between using in vivo

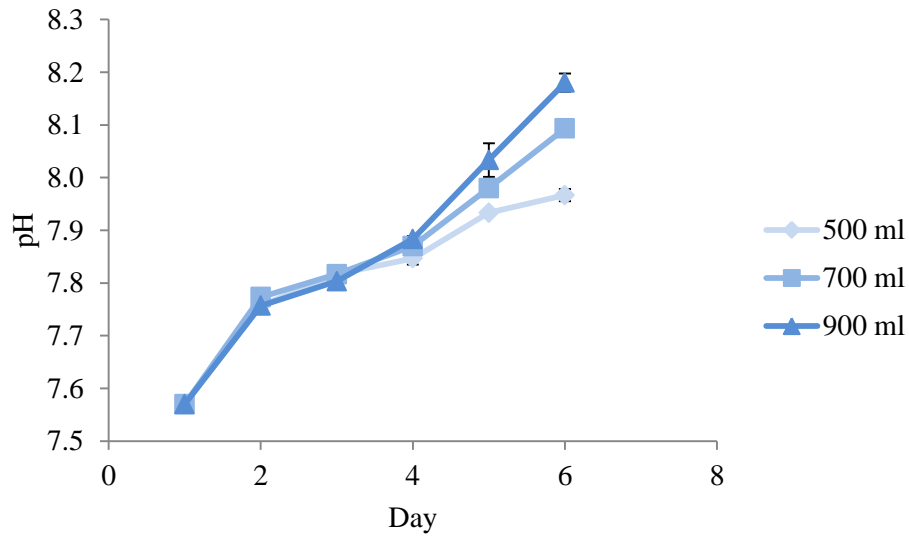


Figure Ap5. pH of cultures during preliminary volume experiment. Error bars represent  $\pm 1$  SD (n=3).

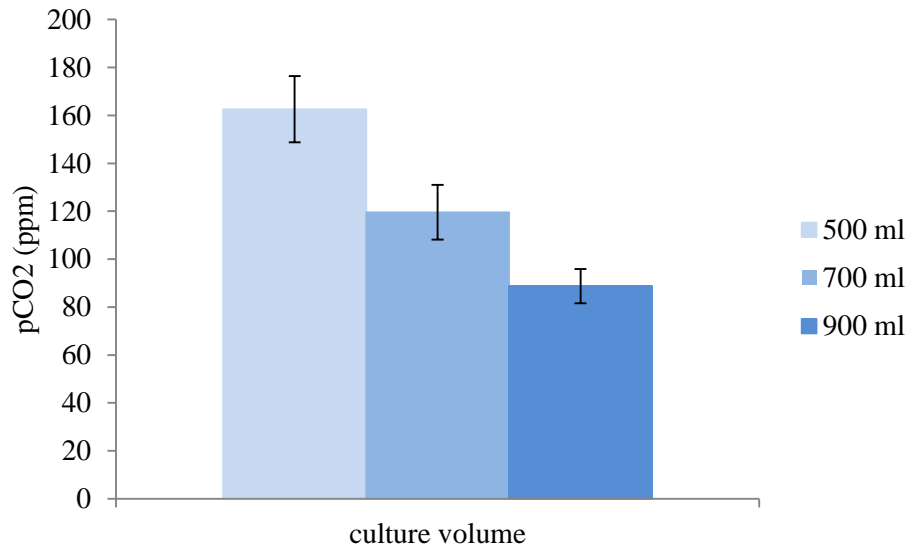


Figure Ap6. pCO<sub>2</sub> of cultures at the end of the preliminary volume experiment. Error bars represent ± 1 SD (n=3).

fluorescence and cell concentration via flow cytometry. Cellular carbon and nitrogen were also examined.

Growth rates based on cell concentration via flow cytometry for CCMP 374 were not affected by CO<sub>2</sub> (Figure Ap7). Growth rates (average  $\pm$  1 SD) were  $0.854 \pm 0.01 \text{ d}^{-1}$  across all CO<sub>2</sub> treatments. Growth rates based on cell concentration via flow cytometry for CCMP 2668 were lower in high CO<sub>2</sub> than in ambient CO<sub>2</sub> (ANOVA with Tukey's post-hoc comparison; p-value 0.022) (figure Ap7). Growth rates were 14.3% lower in high CO<sub>2</sub> than in ambient CO<sub>2</sub>.

Growth rates based on in vivo fluorescence for CCMP 374 were lower in high CO<sub>2</sub> than in ambient CO<sub>2</sub> (ANOVA with Tukey's post-hoc comparison; p-value 0.035) (Figure Ap8). Growth rates were 7.7% lower in high CO<sub>2</sub> than in ambient CO<sub>2</sub>. Growth rates based on in vivo fluorescence for CCMP 2668 were not affected by CO<sub>2</sub> (Figure Ap8). Growth rates (average  $\pm$  1 SD) were  $1.05 \pm 0.03 \text{ d}^{-1}$  across all CO<sub>2</sub> treatments.

Organic carbon per cell for CCMP 374 during the exponential phase of growth was not affected by CO<sub>2</sub> (Figure Ap9). Organic carbon per cell (average  $\pm$  1 SD) was  $8.61 \pm 1.43 \text{ pg POC cell}^{-1}$ . Organic carbon per cell for CCMP 374 during the stationary phase of growth was higher in high CO<sub>2</sub> than in ambient or moderate CO<sub>2</sub> (ANOVA with Tukey's post-hoc comparison; p-value 0.016 and 0.004 respectively) (Figure Ap10). Organic carbon per cell in high CO<sub>2</sub> was 13.7% higher than in ambient CO<sub>2</sub> and 17.6% higher than in moderate CO<sub>2</sub>. Nitrogen per cell for CCMP 374 during the stationary phase of growth was higher in ambient CO<sub>2</sub> than in moderate CO<sub>2</sub> (ANOVA with Tukey's post-hoc comparison; p-value 0.037) (Figure Ap11). Nitrogen per cell was 31.1% higher in ambient CO<sub>2</sub> than in moderate CO<sub>2</sub>.



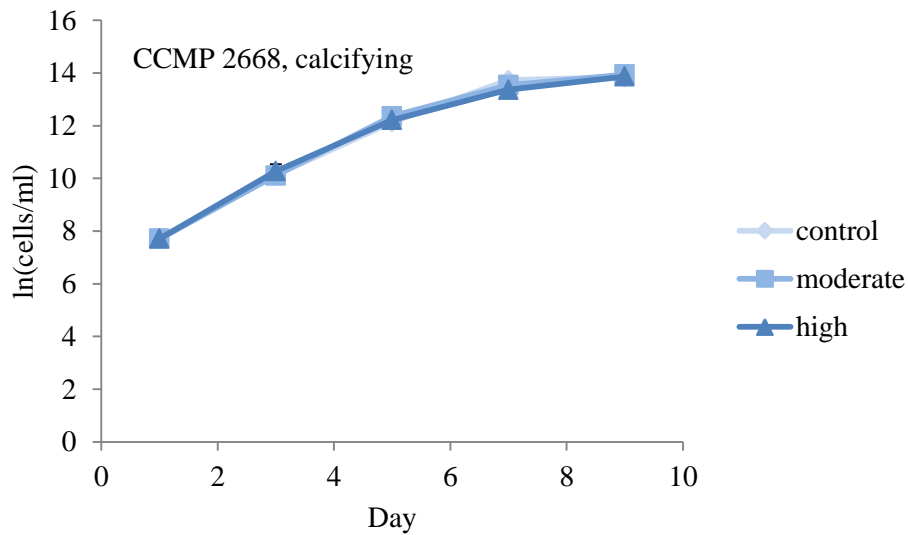
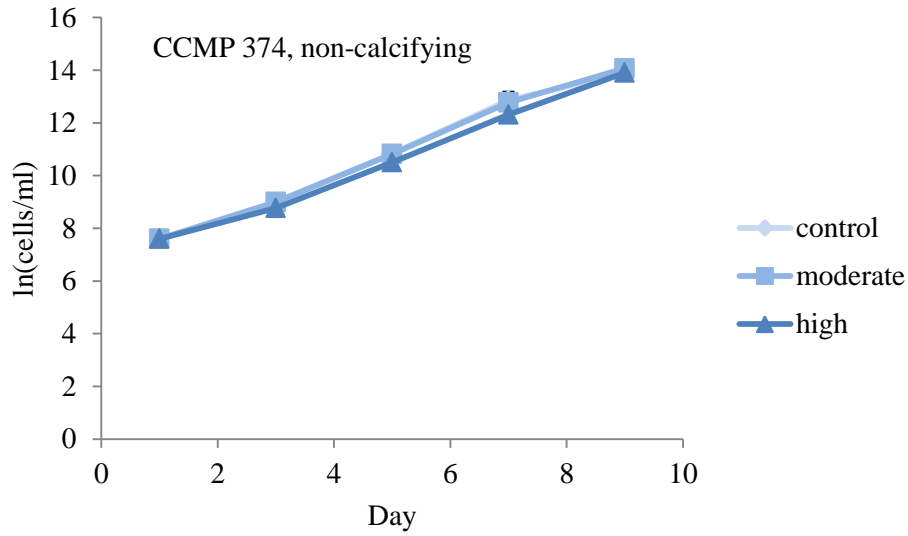


Figure Ap7. Growth curves based on cell concentration via flow cytometry during preliminary batch culture experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

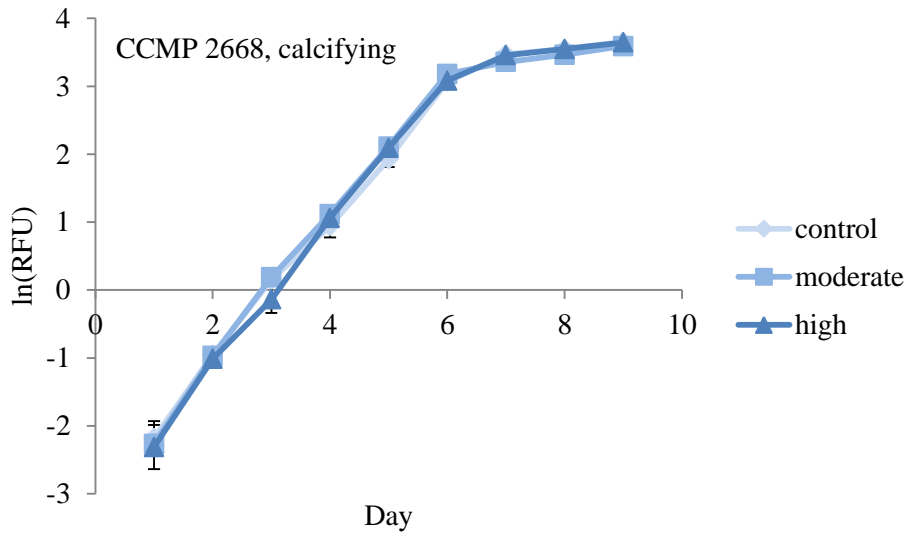
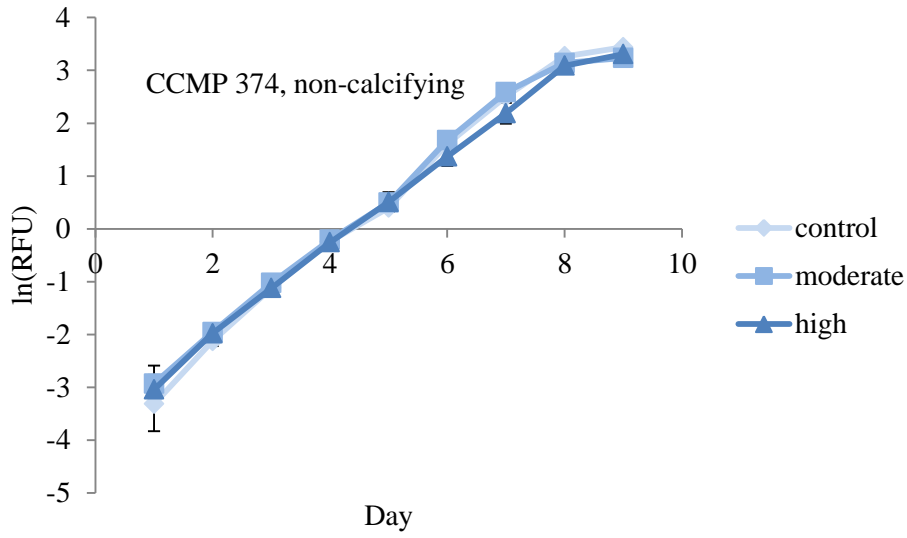


Figure Ap8. Growth curves based on in vivo fluorescence during preliminary batch culture experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

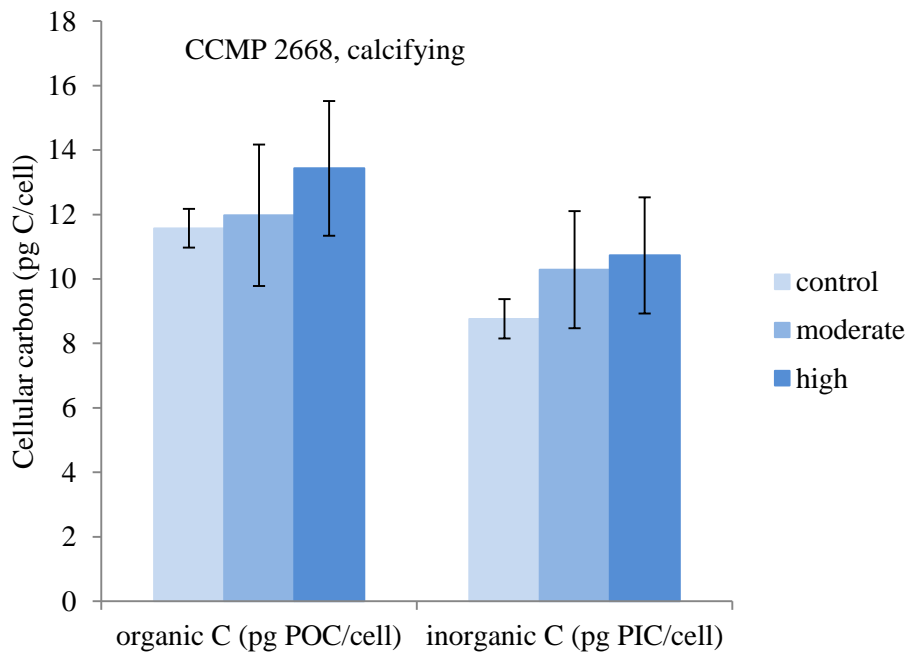
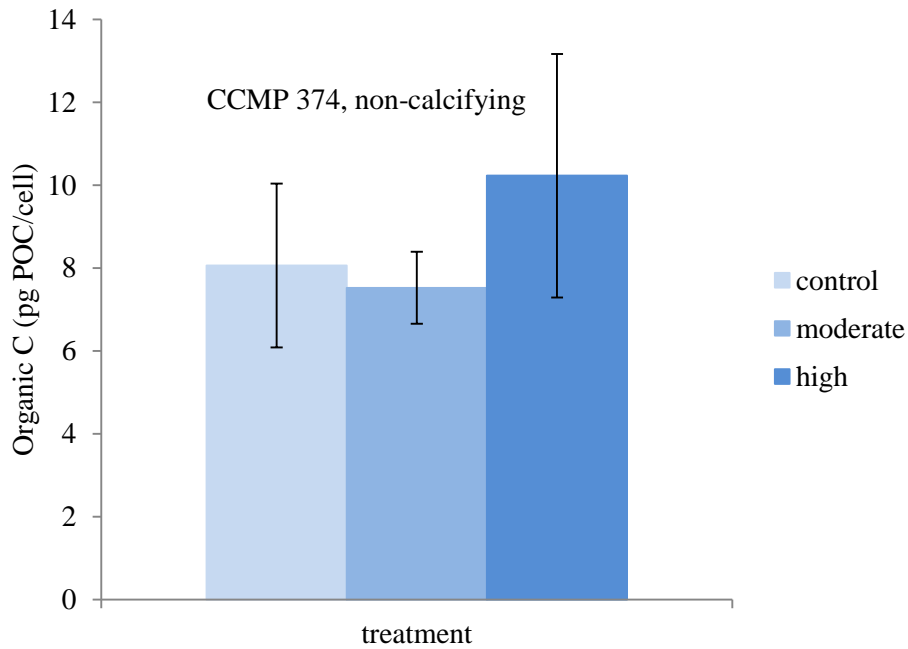


Figure Ap9. Organic and inorganic carbon per cell during exponential phase of preliminary batch culture experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

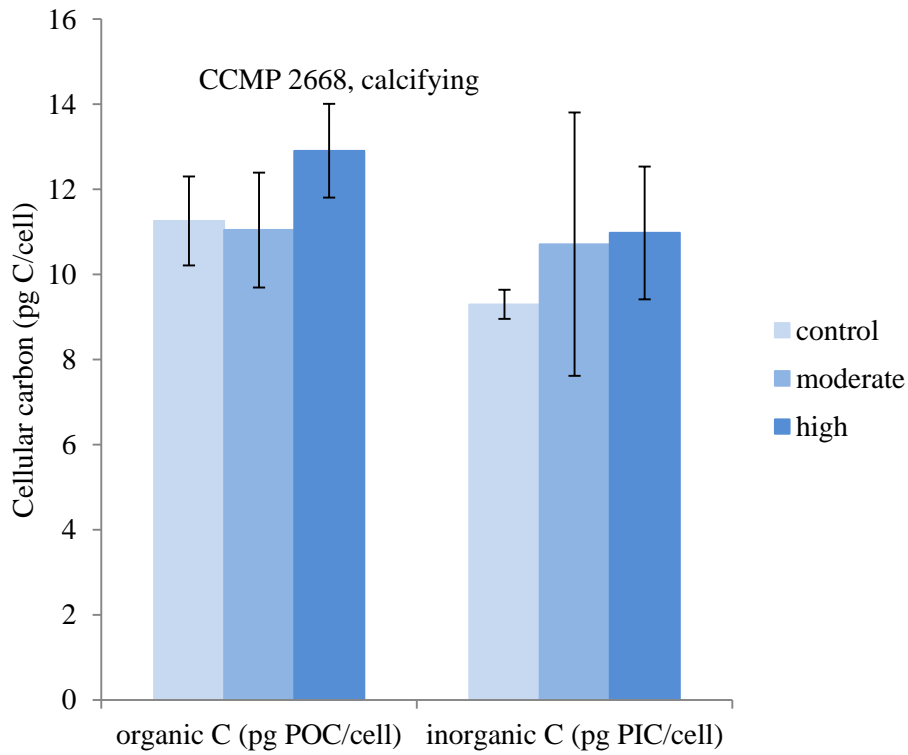
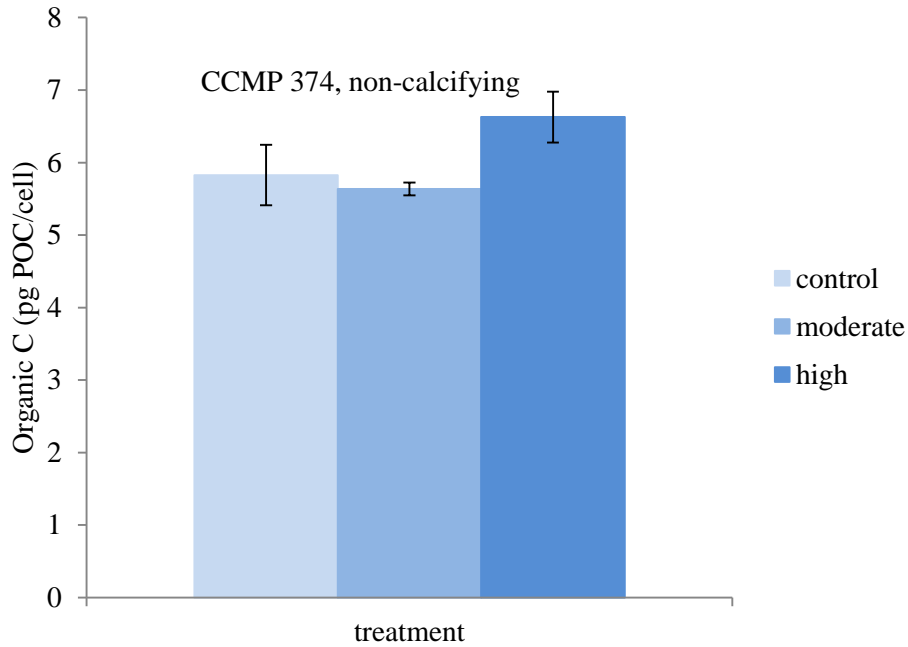


Figure Ap10. Organic and inorganic carbon per cell during stationary phase of preliminary batch culture experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

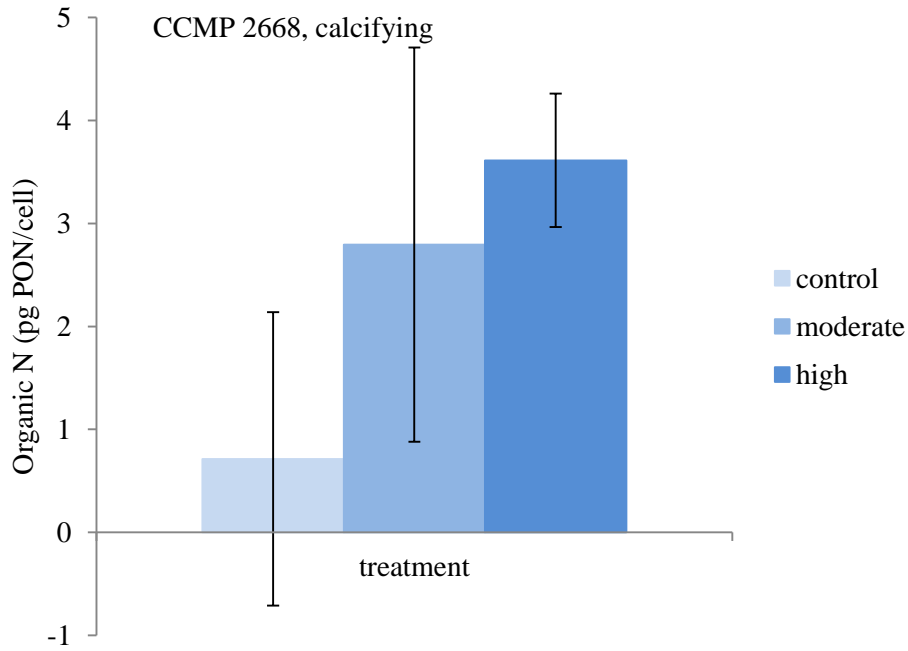


Figure Ap11. Cellular nitrogen per cell during exponential phase of preliminary batch culture experiment for CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

Organic and inorganic carbon per cell for CCMP 2668 during the exponential phase of growth was not affected by CO<sub>2</sub> (Figure Ap9). Organic carbon per cell (average  $\pm$  1 SD) was  $12.33 \pm 0.98$  pg POC cell<sup>-1</sup>. Inorganic carbon per cell (average  $\pm$  1 SD) was  $9.93 \pm 1.03$  pg PIC cell<sup>-1</sup>. Nitrogen per cell for CCMP 2668 during the exponential phase of growth was higher in high CO<sub>2</sub> than in ambient CO<sub>2</sub> (ANOVA with Tukey's post-hoc comparison; p-value 0.044) (Figure Ap11). Nitrogen per cell was 80.3 % higher in high CO<sub>2</sub> than in ambient CO<sub>2</sub>. Organic and inorganic carbon per cell and nitrogen per cell for CCMP 2668 during the stationary phase of growth were not affected by CO<sub>2</sub> (Figure Ap10 & Figure Ap12). Organic carbon per cell (average  $\pm$  1 SD) was  $11.74 \pm 1.02$  pg POC cell<sup>-1</sup>. Inorganic carbon per cell (average  $\pm$  1 SD) was  $10.33 \pm 0.90$  pg PIC cell<sup>-1</sup>. Nitrogen per cell (average  $\pm$  1 SD) was  $0.92 \pm 0.10$  pg PON cell<sup>-1</sup>.

pH begins to increase at approximately day 6 for CCMP 374 (Figure Ap13). pH changes much less dramatically for CCMP 2668, but mild changes begin at approximately day 6 (Figure 13). Day 6 consists of approximately 100,000 cells ml<sup>-1</sup> for CCMP 374 and approximately 400,000 cells ml<sup>-1</sup> for CCMP 2668.

The conclusion was that 100,000 cells ml<sup>-1</sup> is the best target cell density to be able to minimize biological influence on pH. Trends in growth rate do not agree between calculation methods based on cell concentration via flow cytometry and in vivo fluorescence. This could be due to changes in chlorophyll between CO<sub>2</sub> treatments and due to changes in chlorophyll over time.

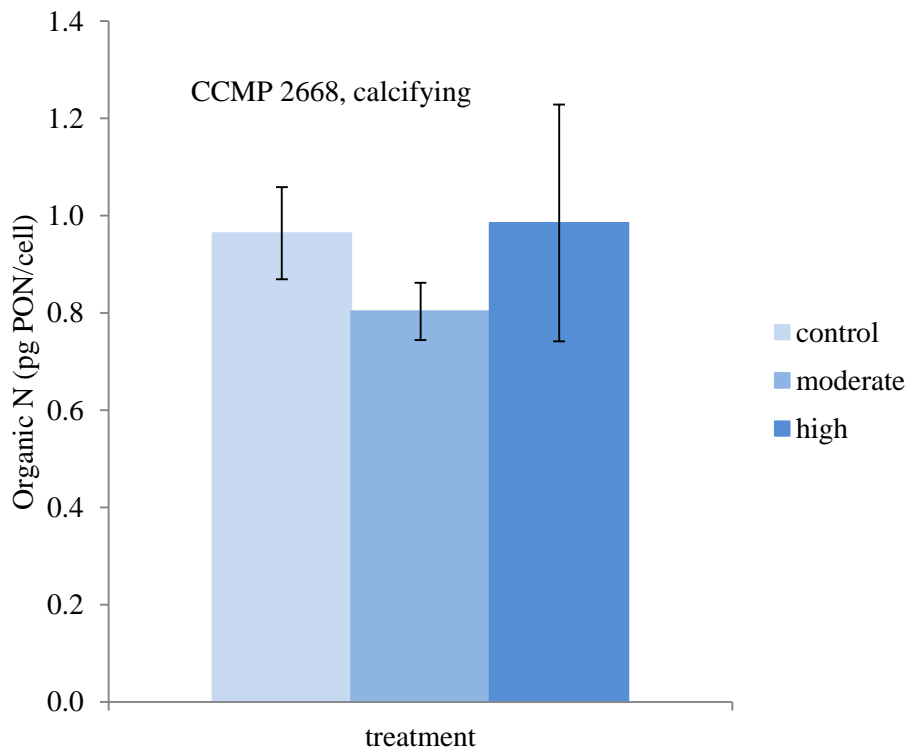
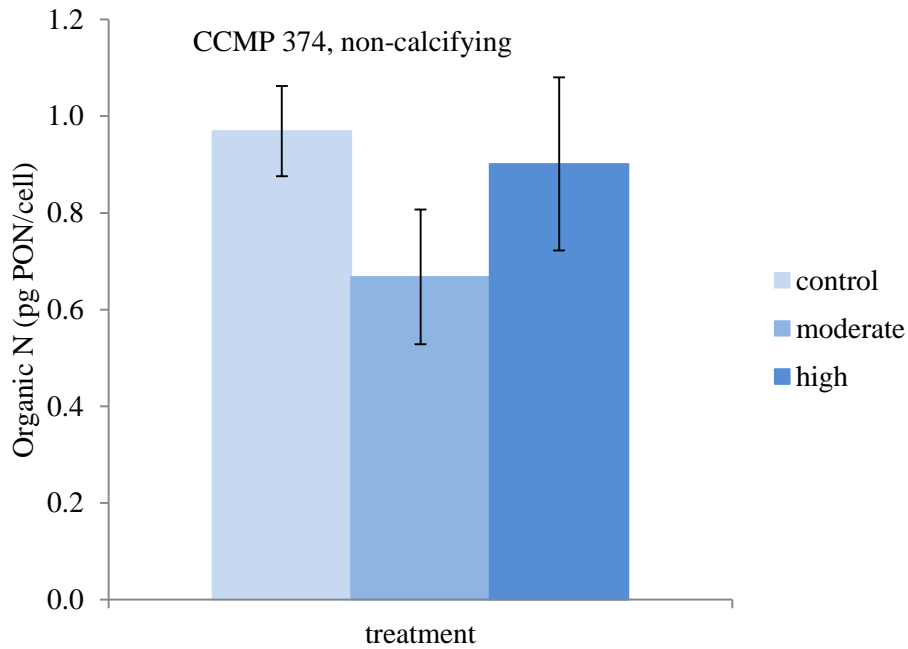


Figure Ap12. Cellular nitrogen per cell during stationary phase of preliminary batch culture experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=4).

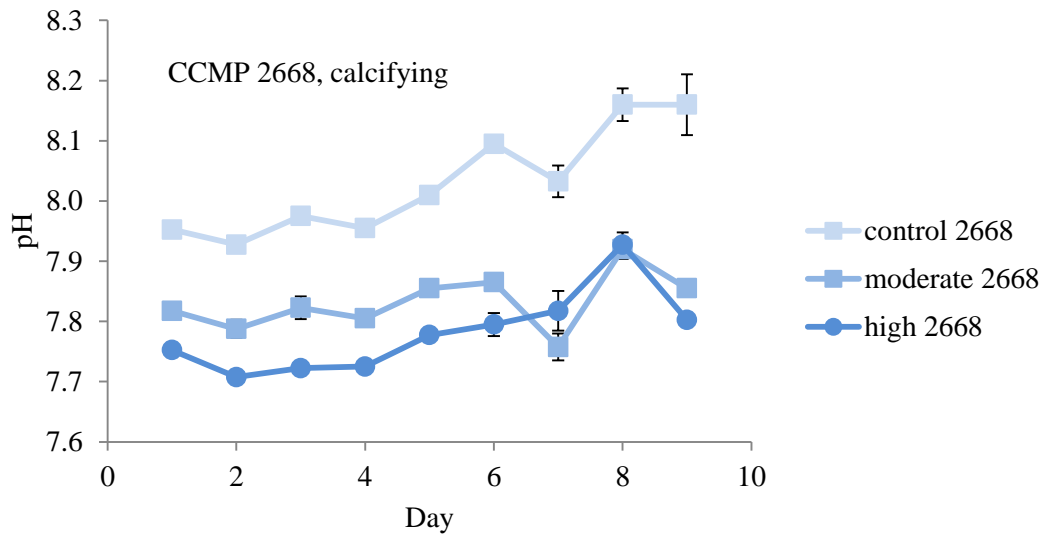
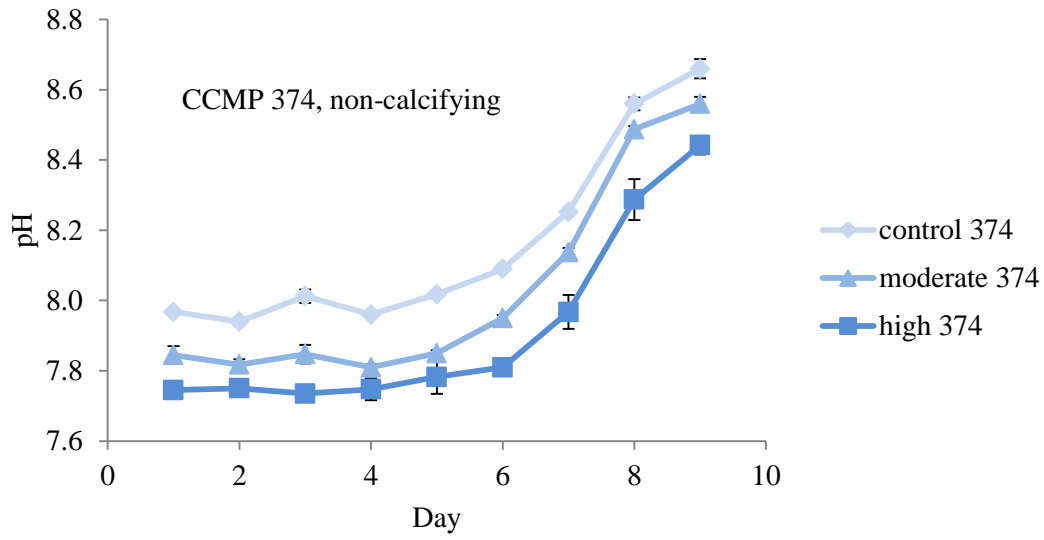


Figure Ap13. pH during preliminary batch culture experiment for CCMP 374 and CCMP 2668.

Error bars represent  $\pm 1$  SD (n=4).



Organic carbon increased in high CO<sub>2</sub> for CCMP 374 during stationary phase, but not during exponential phase. Organic and inorganic carbon were not affected by CO<sub>2</sub> for CCMP 2668 during stationary or exponential phase. Cellular nitrogen had a non-linear trend for CCMP 374 in stationary phase. Cellular nitrogen was increased in high CO<sub>2</sub> for CCMP 2668 during exponential phase, but not during stationary phase. These changes in cellular chemistry indicate an importance in differences between strains and between culture growth conditions.

### **Preliminary DMSPp experiment**

This preliminary experiment compared different DMSPp sampling methods. Filtration methods using gravity, hand pump, and low vacuum filtration were compared, and filtration volumes of 10 ml, 25 ml, and 50 ml were compared (Table Ap1).

For both strains, filtration volume did not affect DMSPp (Figure Ap14). For CCMP 374, there was no statistical difference in filtration method (Figure Ap14). However, there was close to a statistical difference between the vacuum and hand pump methods (ANOVA with Tukey's post-hoc comparison; p-value 0.061).

The conclusion was that 25 ml gravity filtration should be used for the rest of my study. This volume was the best to use given constraints of the gas chromatograph sensitivity and the amount of culture volume available to use for DMSPp sampling. Since there was no difference among filtration methods, gravity filtration was the simplest to use.

Table Ap1. *Emiliana huxleyi* strain, filtration method, filtration volume, and number of replicates filtered during the preliminary DMSPP experiment.

<b>Strain</b>	<b>Filtration Method</b>	<b>Volume (ml)</b>	<b>Replicates</b>
CCMP 2668	gravity	10	2
CCMP 2668	gravity	25	3
CCMP 2668	gravity	50	2
CCMP 374	gravity	10	2
CCMP 374	gravity	25	4
CCMP 374	gravity	50	2
CCMP 374	hand pump	25	2
CCMP 374	low vacuum	25	2

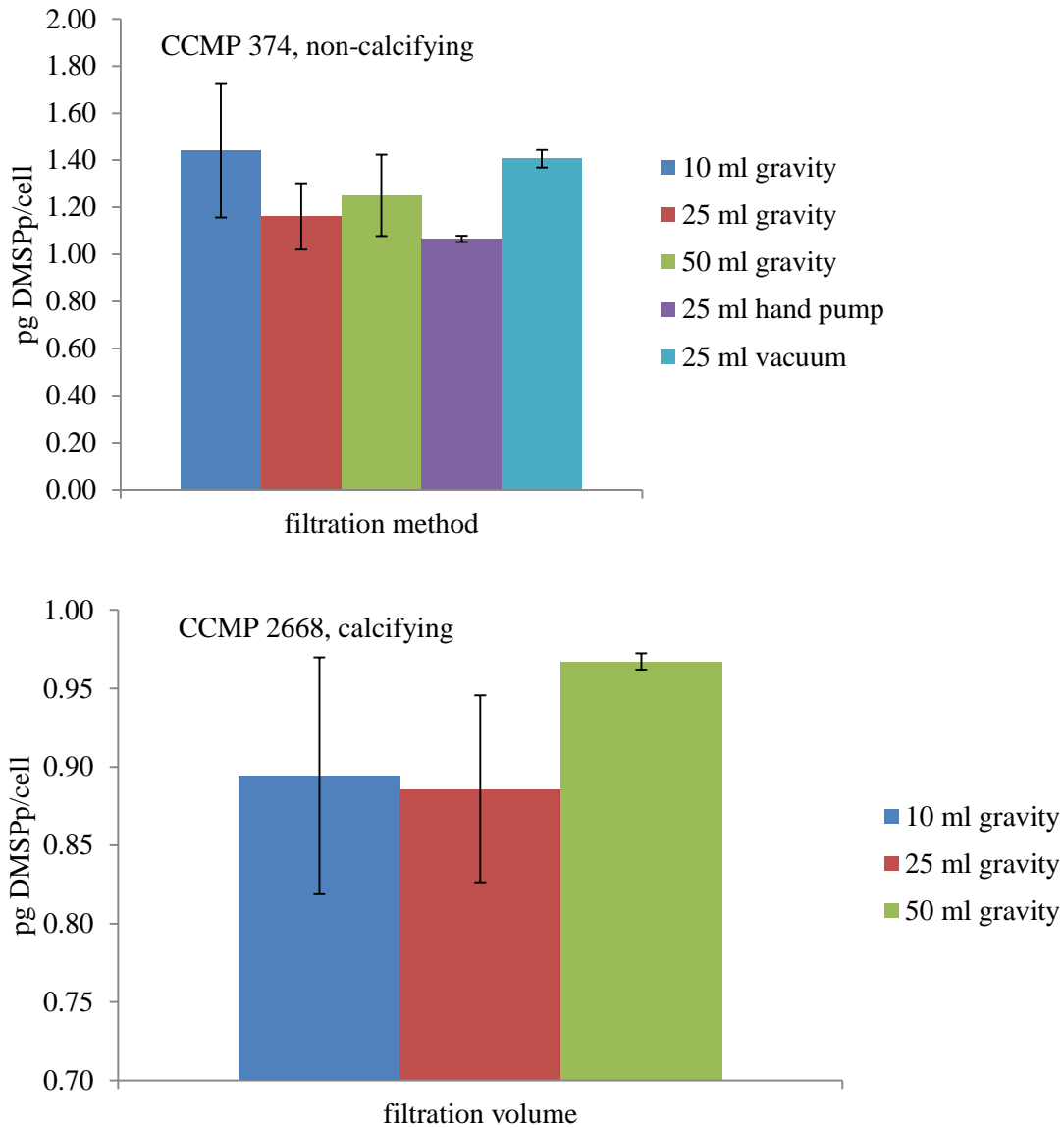


Figure Ap14. DMSPP after different filtration methods and volumes during preliminary DMSPP experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD.

### **Preliminary semi-continuous experiment**

This preliminary experiment focused on perfecting the semi-continuous culturing methods, including whether to use growth rates based on in vivo fluorescence or cell concentrations via flow cytometry to calculate dilution volumes. Cellular carbon, nitrogen, chlorophyll *a*, and DMSPP were also examined.

Growth rates based on cell concentration via flow cytometry for either strain were not affected by CO<sub>2</sub> treatment (Figure Ap15). Growth rates (average  $\pm$  1 SD) for CCMP 374 were  $0.91 \pm 0.30 \text{ d}^{-1}$  across all treatments and time points. Growth rates (average  $\pm$  1 SD) for CCMP 2668 were  $1.00 \pm 0.10 \text{ d}^{-1}$  across all treatments and time points.

Growth rates based on in vivo fluorescence for either strain were not affected by CO<sub>2</sub> treatment (Figure Ap16). Growth rates (average  $\pm$  1 SD) for CCMP 374 were  $0.84 \pm 0.11 \text{ d}^{-1}$  across all treatments and time points. Growth rates (average  $\pm$  1 SD) for CCMP 2668 were  $1.00 \pm 0.11 \text{ d}^{-1}$  across all treatments and time points.

Organic carbon per cell was not affected by CO<sub>2</sub> treatment for either strain (Figure Ap17). Organic carbon per cell for CCMP 374 was  $15.13 \pm 2.37 \text{ pg POC cell}^{-1}$  across all treatments and time points. Organic carbon per cell for CCMP 2668 was  $20.12 \pm 3.31 \text{ pg POC cell}^{-1}$  across all treatments and time points. PIC:POC was not affected by CO<sub>2</sub> treatment (Figure Ap18). PIC:POC was  $0.60 \pm 0.06 \text{ pg PIC pg POC}^{-1}$  across all treatments and time points. POC:PON was not affected by CO<sub>2</sub> for either strain (Figure Ap19). POC:PON for CCMP 374 was  $10.18 \pm 6.16 \text{ pg POC pg PON}^{-1}$  across all treatments and time points. POC:PON for CCMP 2668 was  $11.11 \pm 4.85 \text{ pg POC pg PON}^{-1}$  across all treatments and time points.

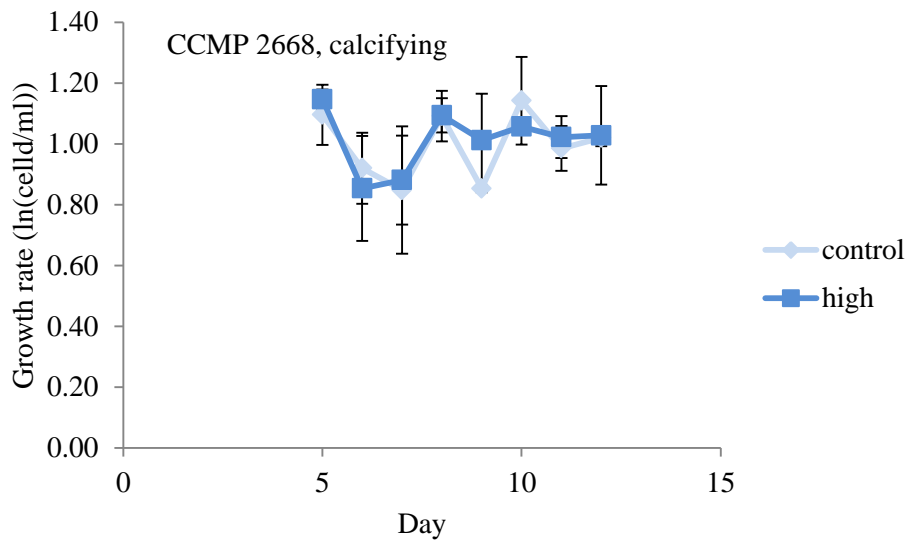
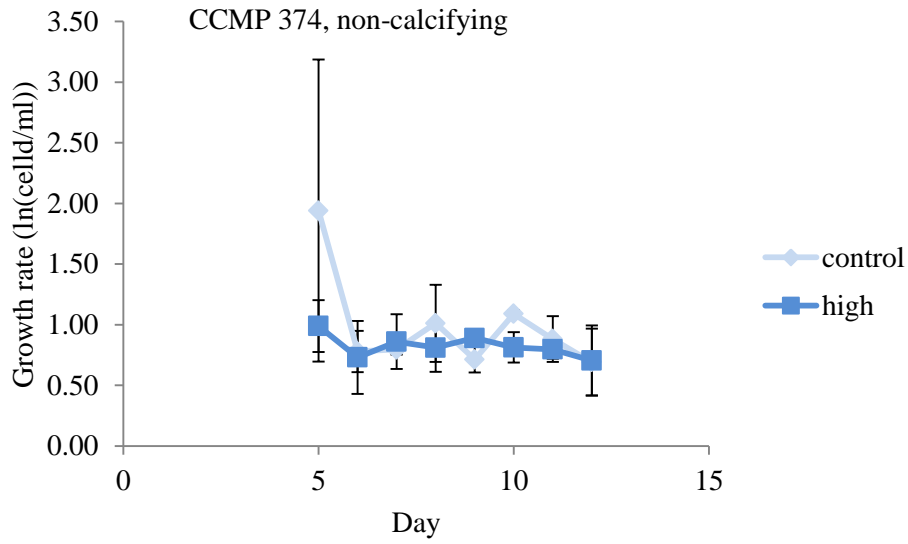


Figure Ap15. Growth rates based on cell concentration via flow cytometry of cultures during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm$  1 SD (n=2).

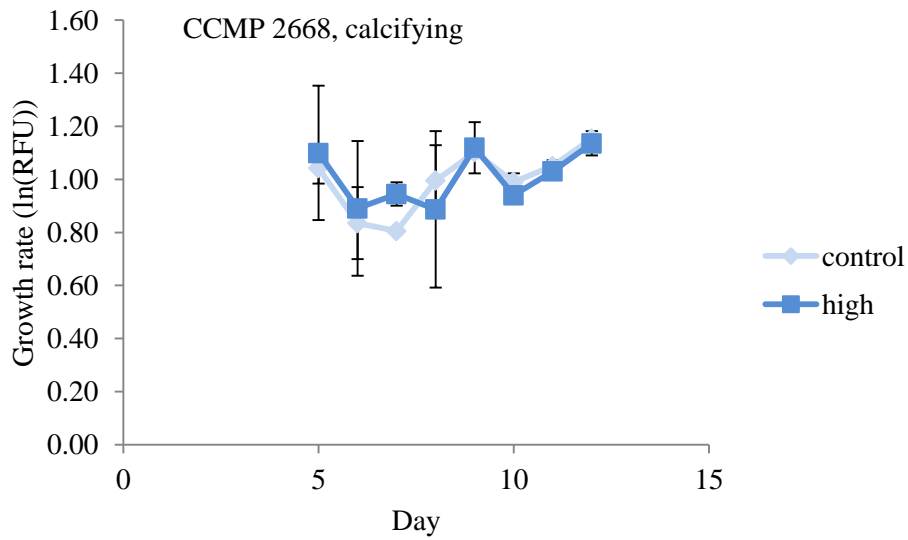
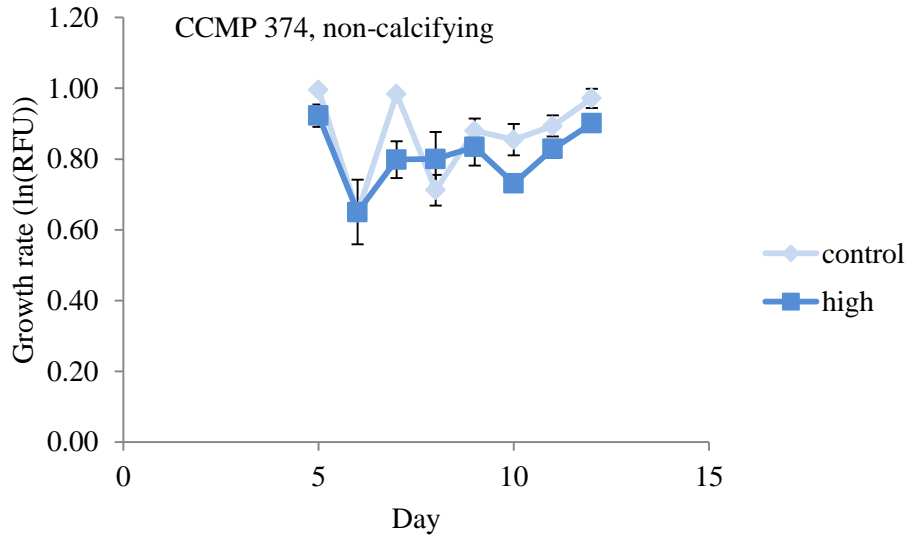


Figure Ap16. Growth rates based on in vivo fluorescence of cultures during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).

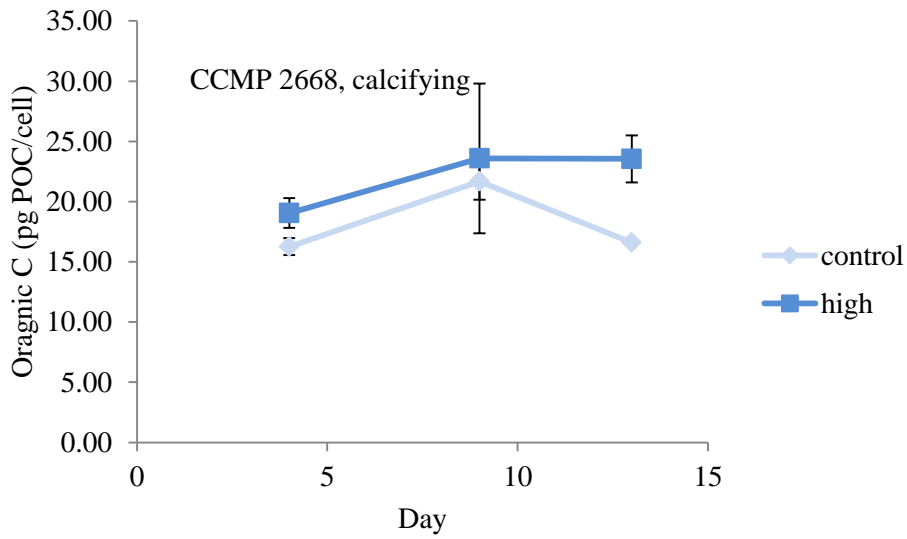
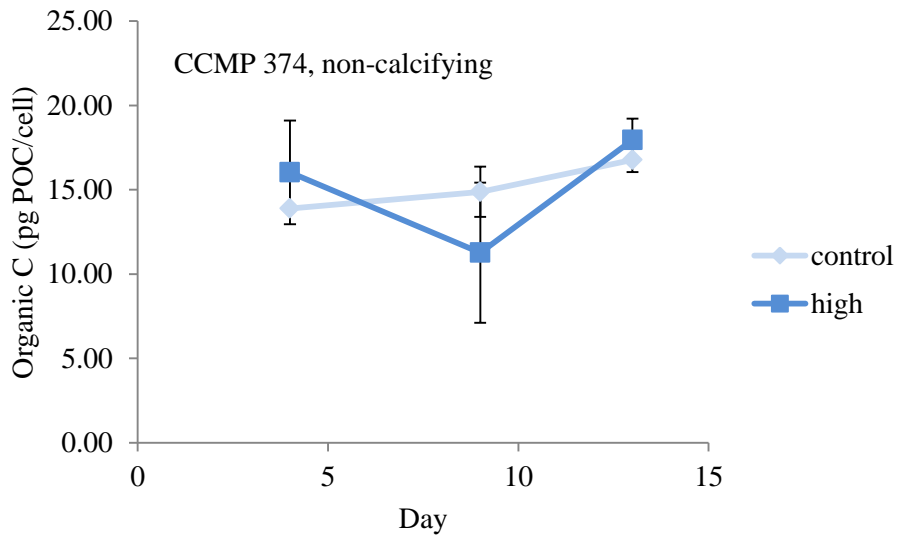


Figure Ap17. Organic carbon per cell during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).

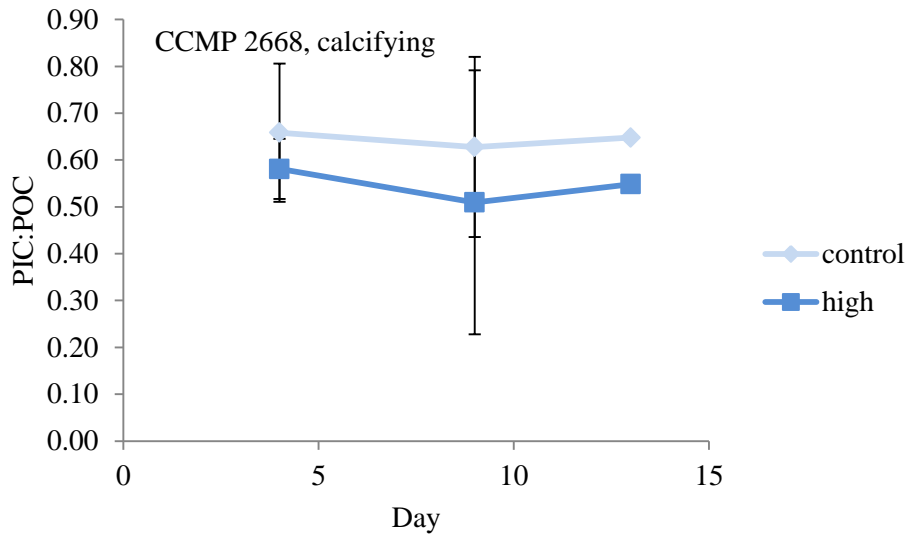


Figure Ap18. PIC:POC during preliminary semi-continuous experiment CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).



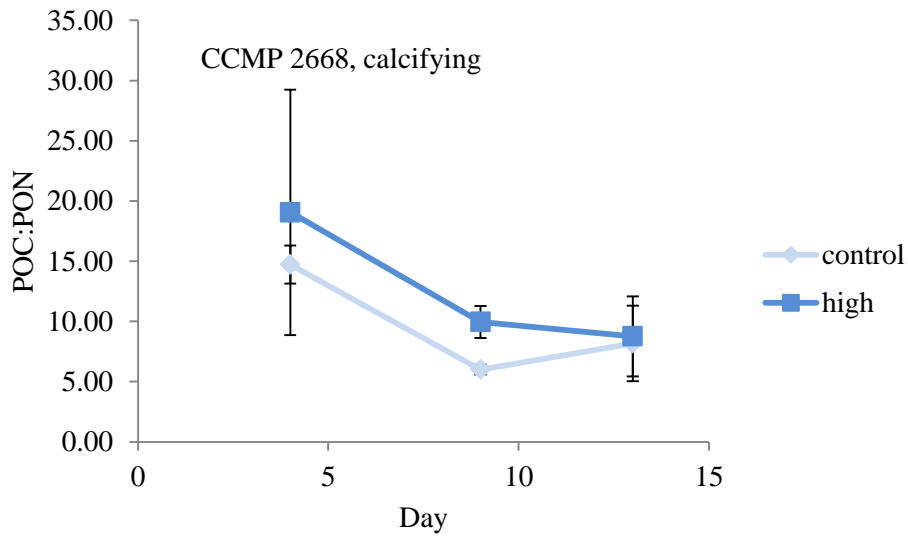
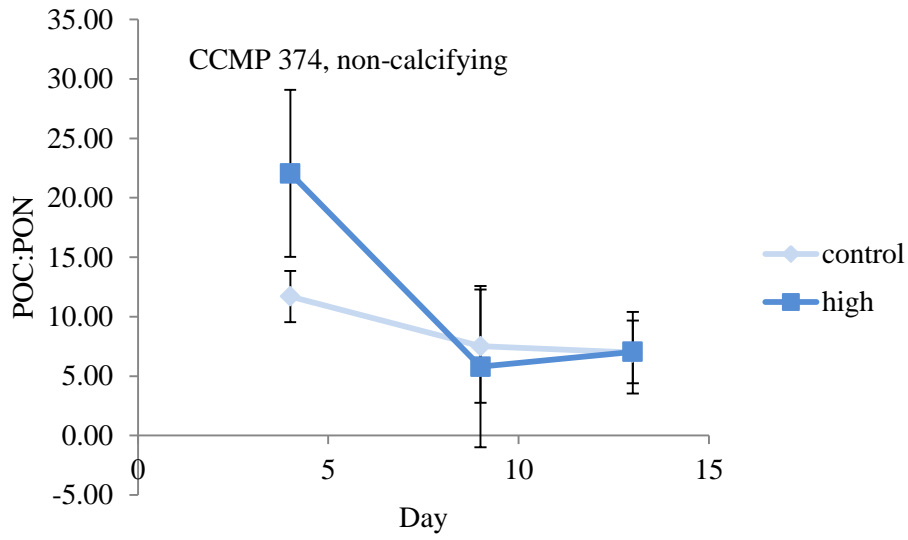


Figure Ap19. POC:PON during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).

Chlorophyll per cell was not affected by CO<sub>2</sub> treatment for either strain (Figure Ap20). Chlorophyll per cell was  $0.070 \pm 0.008$  pg chlorophyll cell<sup>-1</sup> for strain CCMP 374 and  $0.095 \pm 0.018$  pg chlorophyll cell<sup>-1</sup> for strain CCMP 2668 across all treatments and time points.

DMSP per cell was not affected by CO<sub>2</sub> treatment for either strain (Figure Ap21). DMSP per cell was  $0.14 \pm 0.03$  pg DMSP cell<sup>-1</sup> for strain CCMP 374 and  $0.13 \pm 0.03$  pg DMSP cell<sup>-1</sup> for strain CCMP 2668 across all treatments and time points.

The conclusion was that the method of semi-continuous culturing was successful in maintaining the target cell density. Growth rates based on cell concentrations via flow cytometry were not very different from growth rates based on in vivo fluorescence. However, since chlorophyll per cell changed over time, it was more reliable to use growth rates based on cell concentration via flow cytometry for future semi-continuous experiments. Dilution volumes used during this preliminary experiment were based on the growth rates based on cell concentration via flow cytometry, and proved to be successful in maintaining cell stable cell densities. Cellular constituents, such as organic carbon, nitrogen, chlorophyll, and DMSP, may have had too few replicates to reveal any treatment effects.

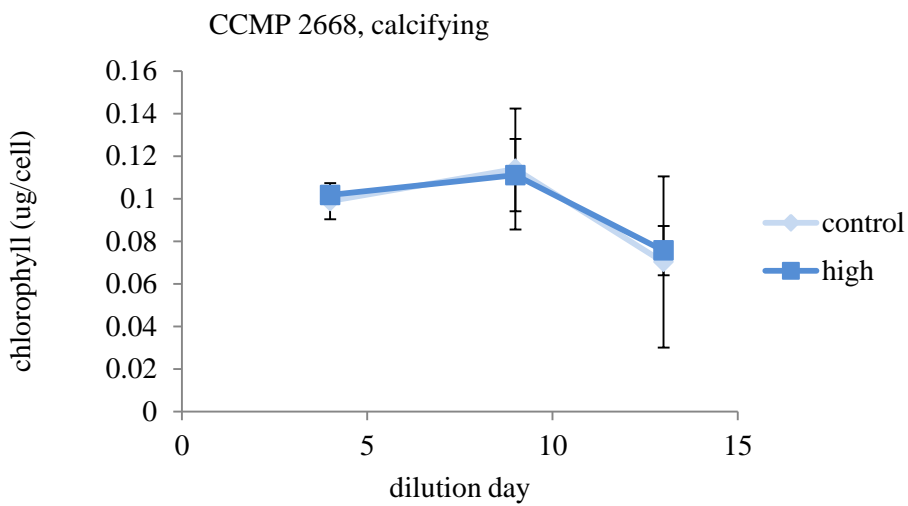
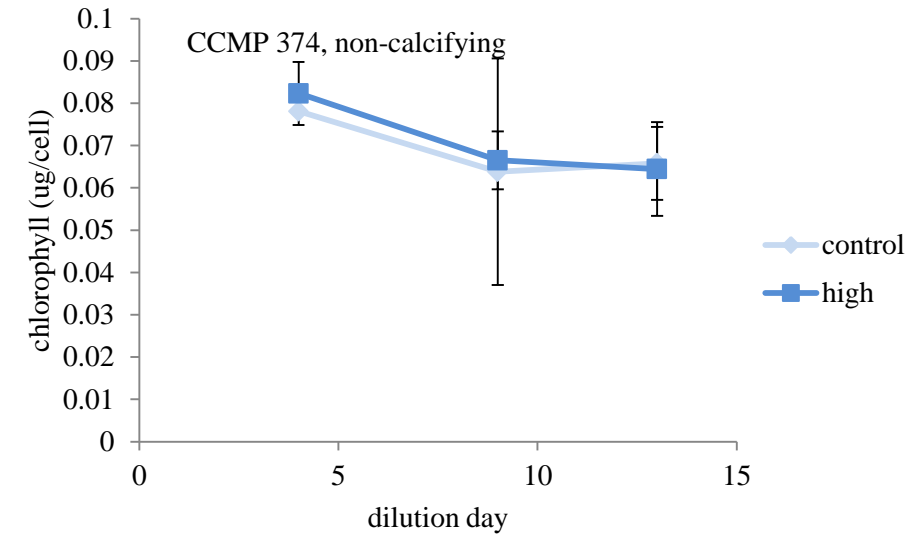


Figure Ap20. Chlorophyll per cell during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).

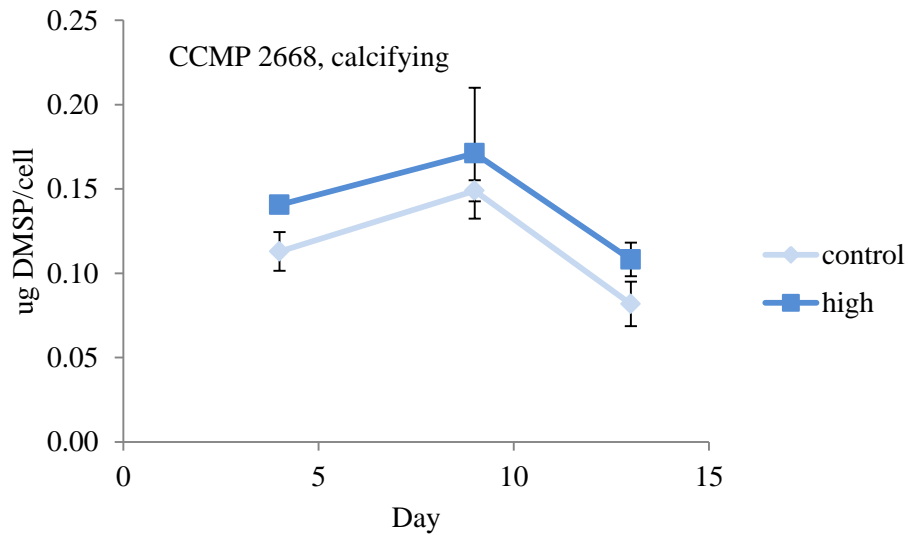
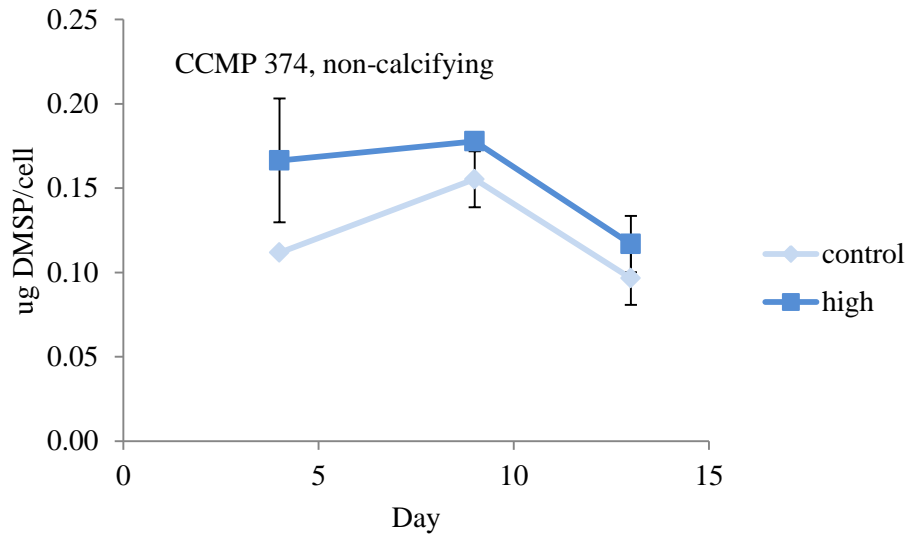


Figure Ap21. DMSP per cell during preliminary semi-continuous experiment for CCMP 374 and CCMP 2668. Error bars represent  $\pm 1$  SD (n=2).

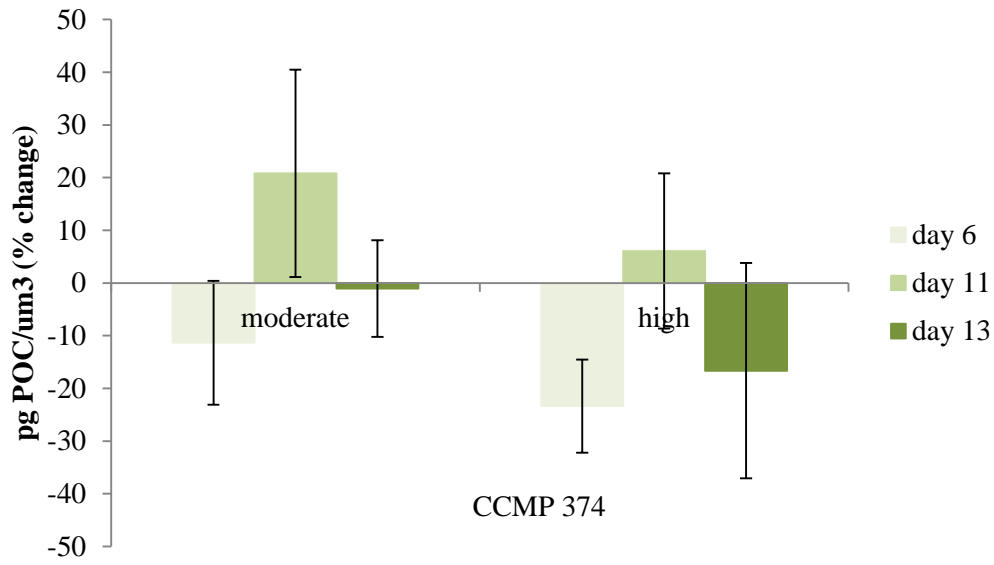


Figure Ap22. Percent change in POC/ $\mu\text{m}^3$  relative to ambient  $\text{CO}_2$ -grown cells for *Emiliana huxleyi* grown in moderate and high  $\text{CO}_2$  conditions. Error bars represent  $\pm 1$  SD (n=5).

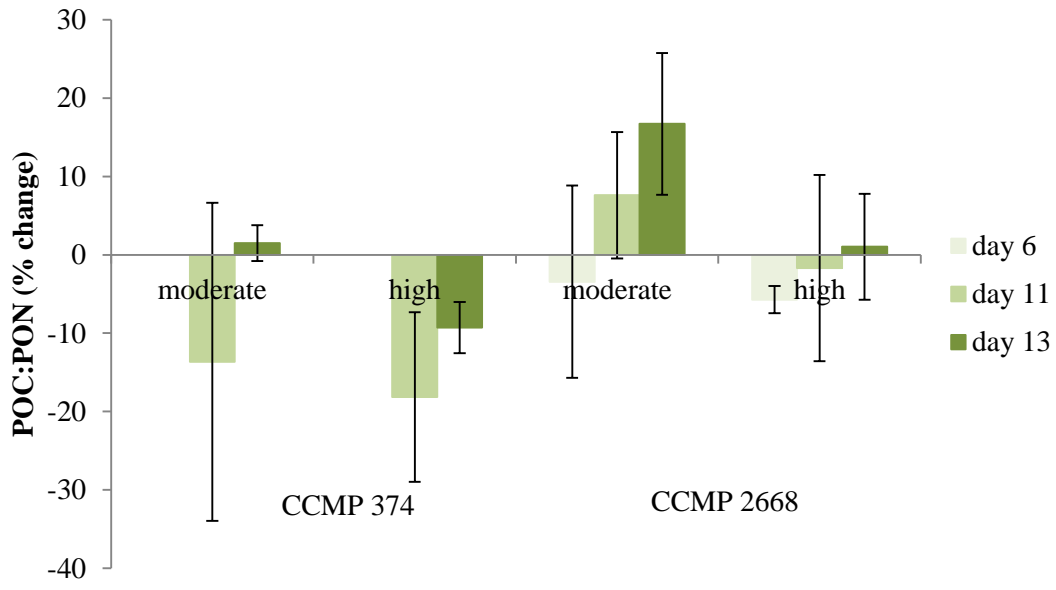


Figure Ap23. Percent change in POC:PON relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

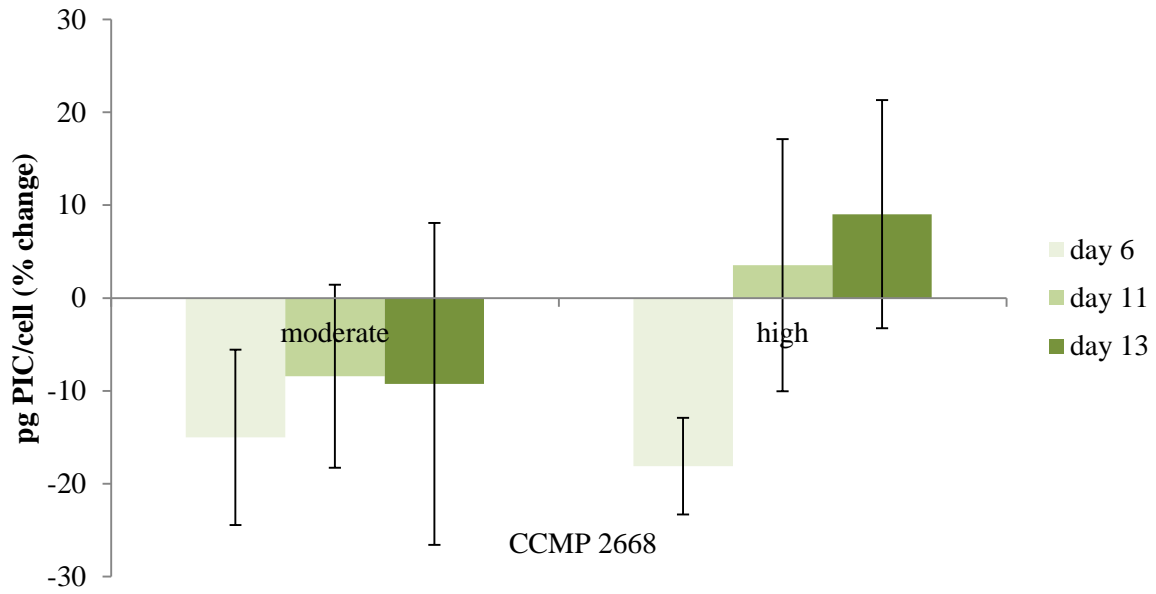


Figure Ap24. Percent change in PIC/cell relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent  $\pm 1$  SD (n=5).

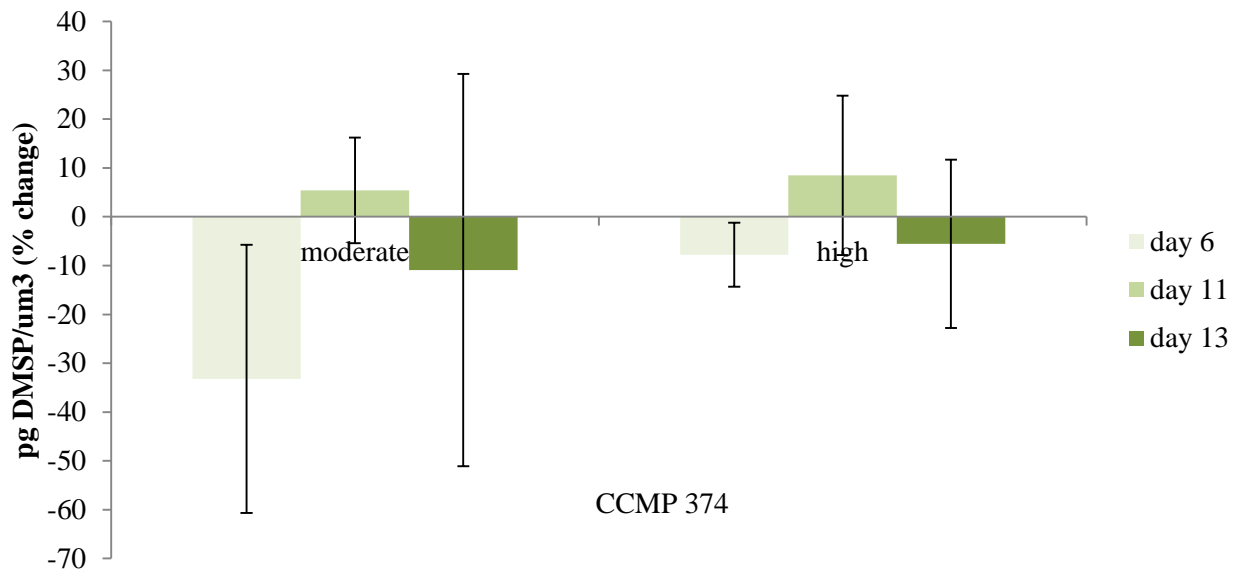


Figure Ap25. Percent change in DMSP/ $\mu\text{m}^3$  relative to ambient  $\text{CO}_2$ -grown cells for *Emiliana huxleyi* grown in moderate and high  $\text{CO}_2$  conditions. Error bars represent  $\pm 1$  SD (n=5).



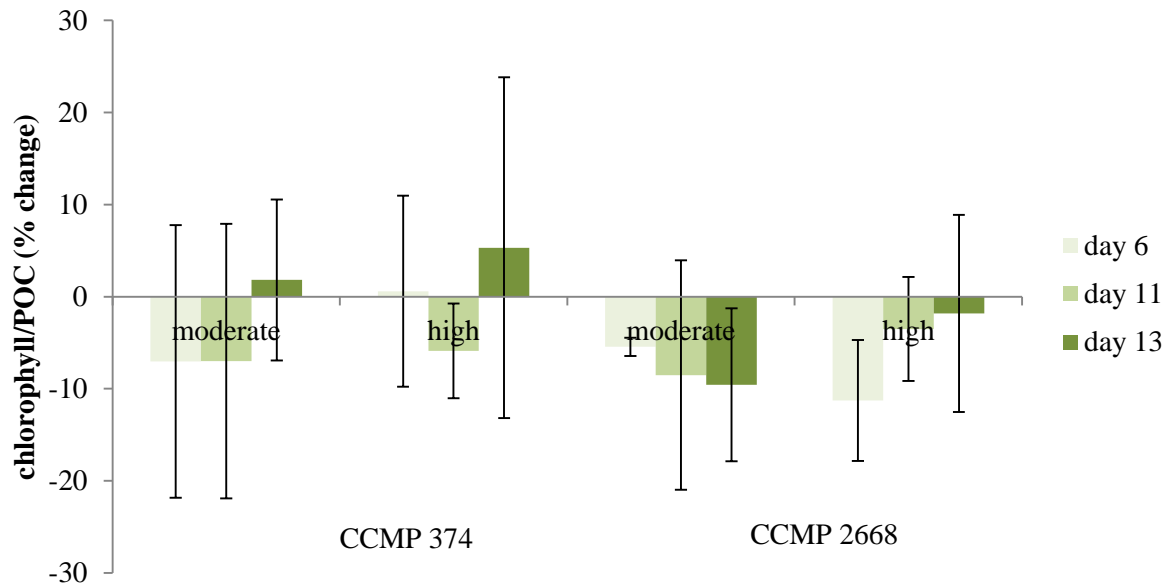


Figure Ap26. Percent change in chlorophyll/POC relative to ambient CO<sub>2</sub>-grown cells for *Emiliana huxleyi* grown in moderate and high CO<sub>2</sub> conditions. Error bars represent ± 1 SD (n=5).

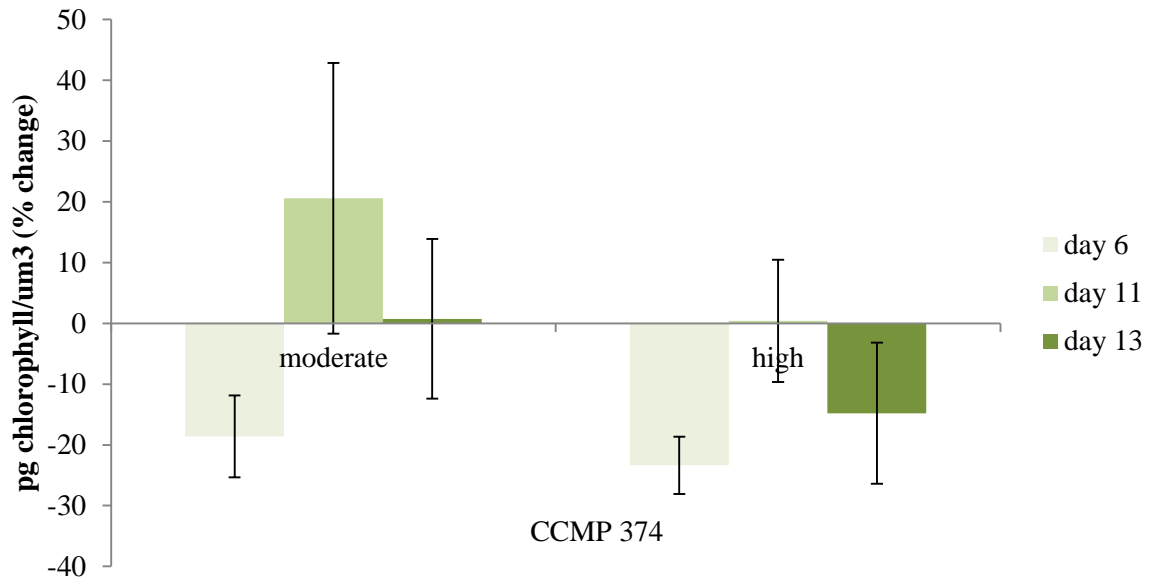


Figure Ap27. Percent change in chlorophyll/ $\mu\text{m}^3$  relative to ambient  $\text{CO}_2$ -grown cells for *Emiliania huxleyi* grown in moderate and high  $\text{CO}_2$  conditions. Error bars represent  $\pm 1$  SD (n=5).