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Abstract

Predictions of increasing levels of anthropogenic carbon dioxide (CO₂) and the specter of global warming have intensified research efforts to identify ways to sequester carbon. A number of novel avenues of research are being considered, including bioprocessing methods to promote and accelerate biosequestration of CO₂ from the environment through the growth of organisms such as coccolithophorids, which are capable of sequestering CO₂ relatively permanently.

Calcium and magnesium carbonates are currently the only proven, long-term storage reservoirs for carbon. Whereas organic carbon is readily oxidized and releases CO₂ through microbial decomposition on land and in the sea, carbonates can sequester carbon over geologic time scales. This proposal investigates the use of coccolithophorids — single-celled, marine algae that are the major global producers of calcium carbonate — to sequester CO₂ emissions from power plants. Cultivation of coccolithophorids for calcium carbonate (CaCO₃) precipitation is environmentally benign and results in a stable product with potential commercial value. Because this method of carbon sequestration does not impact natural ecosystem dynamics, it avoids controversial issues of public acceptability and legality associated with other options such as direct injection of CO₂ into the sea and ocean fertilization. Consequently, cultivation of coccolithophorids could be carried out immediately and the amount of carbon sequestered as CaCO₃ could be readily quantified. The significant advantages of this approach warrant its serious investigation. The major goals of the proposed research are to identify the growth conditions that will result in the maximum amount of CO₂ sequestration through coccolithophorid calcite production and to evaluate the costs/benefits of using coccolithophorid cultivation ponds to abate CO₂ emissions from power plants.

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Introduction

The objective of this project is to determine the efficacy of using coccolithophorid CaCO_3 production in CO_2 removal technology. This project will determine the methods and biological and chemical conditions needed to optimize the native ability of coccolithophorid algae to sequester CO_2 in the form of CaCO_3 . The initial task of the research was to identify the species, cell strain and the specific growth conditions (e.g., temperature, light intensity, salinity, nutrient concentrations, and carbonate chemistry) that maximize population growth rates and rates of calcification. Different coccolithophorid species (including the bloom-forming species, *Emiliania huxleyi* and *Gephyrocapsa oceanica*, the large-celled and low salinity tolerant *Pleurochrysis carterae*, and another large-celled species, *Coccolithus pelagicus*) were tested to determine which species and, if appropriate, cell line, has the maximum growth rate under specified conditions.

The second task of this project was to quantify the amount of CaCO_3 produced by cell lines under conditions that maximized both cell growth and calcification and therefore the amount of CO_2 sequestered.

The third task was to investigate the efficacy of utilizing CO_2 gas (e.g. from power plant emissions) to artificially weather waste concrete as a source of calcium and carbonate ions for coccolith production. This process was also employed to increase alkalinity in low salinity water to grow coccolithophore species/strains tolerant of low salinity conditions.

The information gained in this study can be incorporated into the design and construction of future algal ponds or bioreactors in follow-up research (not a part of this project) on CO_2 sequestration by coccolithophorids.

The final section of this report describes progress made towards fourth task, developing a method for separating calcareous coccoliths from cell organic matter such that the coccoliths may be recovered, while still retaining their fine microstructure for potential uses in the biomedical and/or industrial applications.

Experimental

Species and cell line selection

We obtained eight cell lines of the bloom-forming coccolithophorid *Emiliania huxleyi*, six of which calcify and two strains that do not, which were used to isolate experimental effects of calcification vs. cell growth. We also investigated two cell lines of the coccolithophorid *Gephyrocapsa oceanica*, both of which calcify, one cell line of the coccolithophorid *Pleurochrysis carterae*, and one strain of *Coccolithus pelagicus* (Table 1). We conducted multiple experiments to determine which growth media yields the highest growth rate for each of the cell lines. These experiments involved using 4-6 different media compositions. The various media compositions included different

nitrogen concentrations and sources (nitrate alone, ammonia alone, or both nitrate and ammonia), different concentrations and chemical forms of phosphate, various concentrations of trace elements, and high and low levels of chelating. In addition, we also used these cell lines to test the hypothesis that coccolithophorids have an absolute requirement for selenium, as reported in the literature (Danbara and Shiraiwa, 1999).

During the first year of the project, we made substantial progress in three different areas of the project: 1) cell growth as a function of media composition and macronutrient loading, 2) cell growth and calcification as a function of selenium enrichment, and 3) photosynthesis and calcification rates in various cell strains as a function of irradiance and media composition.

Based on results from the first year of the study, we identified coccolithophorid *Emiliana huxleyi*, cell strain CCMP 371 (Figure 1A-B) as the best candidate among available coccolithophorid cell strains for CO₂ sequestration technologies. Some of our work also suggested that *Coccolithus pelagicus* (Figure 1C) was also a possible candidate. However, we obtained this isolate later in the project and consequently it was not tested as thoroughly as CCMP 371.

Table 1. Coccolithophorid species and cell strains we maintained in our laboratory. We conducted experiments with all of these cultures during the first year of the project, except for two cell lines that were acquired later and are denoted with an asterisk.		
Species	Cell strain no.	Calcifying cells?
<i>Gephyrocapsa oceanica</i>	CCMP 2051	Yes
	CCMP 2054*	Yes
<i>Pleurochrysis carterae</i>	CCMP 645	Yes
<i>Emiliana huxleyi</i>	CCMP 371	Yes
	CCMP 372	Yes
	CCMP 373	No
	CCMP 374	Yes
	CCMP 1516	Yes
	CCMP 1949	Yes
	CCMP 2090	No
	CCMP 2112*	Yes
<i>Coccolithus pelagicus</i>	AC400*	Yes

Nutrient/media experiments

We first investigated differences between two types of growth media: 1) F/50 or F/2 media (Guillard, 1975) for calcifying and noncalcifying cultures, respectively, and 2) K media (Keller et al., 1987). K media contains nitrogen in the form of nitrate and

ammonium, phosphorus in the form of organic phosphate (β -glycerophosphate), EDTA as a chelator, and selenium. In contrast, F/50 and F/2 media contain nitrogen only as nitrate, inorganic phosphate, about 10 times less EDTA than K media, and no selenium. The only difference between F/50 and F/2 is that F/50 has less phosphate (1.46 μ M as opposed to 36.6 μ M in F/2); this low-phosphate condition seems to enhance calcification. In addition, we modified K media and F/50 (particularly changing the phosphate concentration) to determine if we could identify a growth media that would yield rapid cell growth rates as well as highly calcified cells.

Table 2. Major differences in the compositions of F/50 media and K media.		
	F/50 Media	K Media
Nitrogen	883 μ M Nitrate	883 μ M Nitrate & 50 μ M ammonia
Phosphate	1.46 μ M Inorganic phosphate	10 μ M Organic phosphate
EDTA	11.7 μ M	111 μ M
Selenium	None	10 μ M

Selenium and Phosphate experiments

A second part of our research into different nutrient requirements examined the hypothesis that coccolithophorids require selenium (as selenite) for growth. Many microalgae, including species of diatoms and dinoflagellates, have an obligate requirement for selenium (e.g., Price et al., 1987; Price and Harrison, 1987; Doblin et al., 1999). Recent studies (Danbara and Shiraiwa, 1999; Baines and Fisher, 2001) showed that growth rates of the coccolithophorids *Emiliania huxleyi* and *Gephyrocapsa oceanica* were increased with the addition of selenium.

We first examined the effect of selenium addition in CCMP 373, a noncalcifying coccolithophorid but a fast grower. In our treatment flasks, we added 1-2 nM selenite (H_2SeO_3) to F/2 media; the control flasks had only F/2 with no selenite. Within 4 days, we noticed markedly enhanced growth in flasks containing selenium compared to the control flasks (Figure 2). We next conducted a suite of experiments with calcifying cell lines to determine if the addition of selenium similarly increased growth rates. We specifically wanted to learn if selenium was the critical constituent of K media that yielded high growth rates in some cell lines.

Because low phosphate conditions are known to stimulate calcification in *E. huxleyi*, we conducted another set of experiments in which we decreased the phosphate concentration of K media to equal that of F/50; we called this modified media K/50. We then examined the effect of selenium addition in this modified media.

Photosynthesis-irradiance and calcification-irradiance experiments

The next component of our work focused on the measurement of rates of photosynthesis and calcification as a function of light intensity. While photosynthetic rates are fairly easy to measure, calcification rates are difficult to measure accurately. Modifying the micro-diffusion method of Paasche and Brubak (1994), we developed ^{14}C -uptake

methods for the accurate measurement of calcification rates in coccolithophorids over a range of cell densities and growth rates. Calcification–Irradiance (C-I) curves generally show more scatter than Photosynthesis–Irradiance (P-I) curves, particularly at low light intensities, low cell densities, and/or low calcification rates.

We measured the calcification and photosynthetic rates of various cell strains and species at different light intensities as a function of different nutrient loadings. We primarily conducted experiments at 18°C; the few experiments we conducted at 15°C or 20°C did not result in higher rates calcification. Rates of calcification and photosynthesis (the y-axis of each graph) are presented in units of ng C cell⁻¹ h⁻¹ except for *C. pelagicus*, which is presented in pg C cell⁻¹ h⁻¹. It is important to note that the range of both the y-axis and the x-axis varies between figures. P-I curves were estimated from iterative curve-fitting in the statistics software Systat (SPSS) using the following equation:

$$P_n = \frac{P_{\max}(I - I_c)}{K_I + (I - I_c)} \quad (\text{Equation 1})$$

Where P_n is the net photosynthesis as calculated by the gross photosynthesis minus respiration, $P_{\max}$ is the maximal value of photosynthesis, I is the light intensity, I_c is the compensation light intensity at which photosynthetic production equals respiratory losses, K_I is the half-saturation constant, or the light intensity when $P = P_{\max}/2$.

C-I curves were estimated similarly, substituting the net calcification rate for P_n , the maximal value for calcification for $P_{\max}$, and defining I_c as the compensation light intensity at which the calcification production equals the respiratory losses.

Batch culture experiments

Salinity

In other experiments, we grew *E. huxleyi* cell strains CCMP 371 and 374 in low-salinity (20 ppt) F/50 media in an attempt to ascertain the salinity tolerance of the cells. Selenium was also added to some of the low salinity flasks to determine if this enrichment would enhance growth in this low salinity seawater. We also used low salinity water in waste concrete weathering experiments described below. The primary goal was to reduce the cost of producing media while still achieving a high level of calcification.

Nitrate vs. Nitrate plus Ammonia

We then examined the effects of nitrogen source on cell growth and calcification in a batch culture experiment. Two treatments were investigated. The first treatment consisted of unmodified F/50 media containing only 883µM nitrate. The second media was investigated contained an 883µM total nitrogen loading, comprised of 833µM nitrate and 50µM ammonium. *Emiliania huxleyi* cell strain CCMP 371 was inoculated into three replicate flasks of each media. Flasks were kept in an environmental chamber at 18.5°C and a light/ dark cycle of 16 hours light/8 hours dark. Cell densities were

determined by direct counts and subsamples were taken for particulate calcium and measured by flame atomic absorption spectroscopy.

Different Nitrate Loadings

In other batch culture experiments, we investigated the effects of different nitrate concentrations on cell growth and calcification. *Emiliana huxleyi* cell strain CCMP 371 was grown in unmodified F/50 media which has a nitrate concentration of 0.88mM and in modified F/50 media in which the nitrate concentration was increased to the following values: 1.5 mM, 2.0 mM, and 2.5mM. Three replicates of each nitrate concentration were incubated at 18.5°C and a light/ dark cycle of 16 hours light/8 hours dark.

Closed-vessel experiments

For Phase II of the project in which we quantified the amount of dissolved CO₂ sequestered through coccolithophorid calcification, we manufactured a custom-made experimental vessel (Figure 3A) and developed a data acquisition program that allowed us to measure the effects of coccolithophorid growth and calcification on the CO₂ system parameters.

We conducted closed-vessel experiments to investigate the impacts of coccolithophorid growth on the CO₂ system parameters. The protocol consisted of inoculating 7 liters of various culture media with a small volume of *Emiliana huxleyi* cell strain CCMP 371. The culture was stirred continuously to keep cells uniformly suspended. This vessel was placed in a temperature- and light-controlled environmental chamber. We ran the initial experiment for 26 days, at a temperature of 18.5°C, and a light/dark cycle of 16 hours light/8 hours dark. Data from this experiment indicated that stationary phase of growth occurred in about 6-12 days however experiments were typically allowed to run for longer to monitor the long-term effects on the CO₂ system.

Throughout the closed-vessel experiments, temperature and electromotive force (EMF in mV) were recorded every hour using the data acquisition program we developed in our lab. Daily samples of the culture were taken for measurements of cell density, total alkalinity (TA), and total dissolved inorganic carbon (DIC). Total dissolved inorganic carbon in duplicate samples was measured using coulometry (Dickson and Goyet, 1994). TA was measured using a modified Gran titration technique. Because we could measure TA and DIC with a high degree of accuracy, the speciation of the CO₂ system could be calculated with good confidence. Subsamples for these measurements were taken without introducing additional atmospheric CO₂, thereby not altering the system. The sample volume removed for analyses was replaced with argon from a Mylar reservoir.

Figure 3B shows the experimental vessel with the pH and temperature probes, the sampling port outfitted with a 3-way stopcock, and the Mylar reservoir filled with argon. Cell densities were determined daily by direct count of samples taken using the sampling port.

In the second closed vessel experiment, we tested growth at a reduced nitrogen level (approximately half that of F/50) and with two sources of nitrogen: 400 µM nitrate and

50 μM ammonia. The cells grew very slowly and examination of the cells indicated weakly calcified, abnormally-shaped cells. We terminated the experiment at 10 days, hypothesizing that ammonia toxicity possibly caused the abnormal cell shape and slow cell growth.

The third closed vessel experiment utilized reduced nitrogen level of 450 μM nitrate (approximately half that of F/50) in the absence of ammonia. In addition to the samples taken for TA, DIC, and cell counts, samples for particulate calcium were also taken during this experiment.

The differences in nitrogen sources and concentrations in growth media used in the first three closed vessel experiments is described in Table 3.

Table 3. Experimental conditions of each closed vessel experiment investigating impacts of nitrogen source and concentration on coccolithophorid growth and calcification.				
	Nitrate	Ammonia	Total Nitrogen	Comments
Experiment 1	883 μM	None	883 μM	Unmodified F/50 media
Experiment 2	400 μM	50 μM	450 μM	Approximately 50% of nitrogen that is normally contained in F/50; two sources of nitrogen
Experiment 3	450 μM	None	450 μM	Approximately 50% of nitrogen that is normally contained in F/50; one source of nitrogen

To determine if the production rate and maximum yield of coccolithophorids could be increased from our earlier results, we conducted two more closed vessel experiments, designed specifically to directly compare growth and inorganic carbon uptake of CCMP 371 in F/50 and K media. Each vessel contained 7 liters of media (either F/50 or K) and was inoculated with a very small volume of *Emiliania huxleyi* cell strain CCMP 371 to give an initial cell density of 4×10^2 cells ml^{-1} . Like all of the previous closed vessel experiments, the cultures were continuously stirred to keep the cells suspended.

Dissolved inorganic carbon and alkalinity

Results from the previous series of experiments supported out previous hypothesis that the best candidate for CO_2 sequestration was *E. huxleyi* strain CCMP 371, particularly when grown in high-salinity (~ 31 ppt) F/50 media and containing only nitrate as a nitrogen source. Furthermore, the nitrate level in the aforementioned media would likely be in such excess that could easily be halved without the occurrence of nitrogen limitation. This would make the growth media even less expensive to produce than unmodified F/50 media and substantially less costly and easier to produce than K media.

The next area of research focused on the manipulation of the inorganic carbon pool. Although the recipe we used for making our artificial seawater is well-known and commonly used in other laboratories, we were dissatisfied with the total alkalinity and DIC levels that we were attaining. We therefore modified our artificial seawater recipe in an effort to increase total alkalinity and dissolved inorganic carbon. We suspected that we were losing a significant fraction of dissolved inorganic carbon due to outgassing during autoclaving. The newly modified seawater recipe was used in the next closed vessel experiments. Later experiments (described in Annual report #3) investigated the use of enriched natural seawater and we found it to have higher TA and DIC and was superior even to the modified our modified artificial seawater.

“High” and “Low” CO₂ closed-vessel experiments

To investigate the effects of differing inorganic carbon pools on the growth and inorganic carbon uptake in CCMP 371, we performed another set of closed-vessel experiments. One experiment utilized artificial seawater and in the other we used enriched natural seawater. We used two different methods to manipulate the inorganic carbon pool (described in Annual report #3) to investigate the effects of high and low initial CO₂ conditions. The effects of these conditions on CCMP 371 were studied.

Artificial weathering of waste concrete

To ascertain the efficacy of utilizing waste concrete as a possible source of calcium and carbonate ions for coccolithophores, we conducted a series of experiments in which various size fractions of waste concrete were placed in porous bags and suspended in 0.5 liters of distilled water. In a typical experiment, we used a sledgehammer to break waste concrete into smaller pieces. These pieces were then put through a series of wire mesh sieves to separate the particles by size. Subsamples of all size class were weighed and put into individual porous bags. A single bag was placed into a 500-ml glass jar containing deionized water. Experimental treatment jars were bubbled with CO₂ gas for 1 hour each day for a total of 11 days. Control jars containing similar size-fractionated waste concrete were not bubbled with CO₂ gas. The pH was measured in each jar on a daily basis. At the end of each experiment, the bags were removed and the remaining waste concrete was dried and weighed. The water from each bottle was filtered onto pre-weighed GF/F filters to capture any small particles of waste concrete that might have escaped through the porous bag.

Because we found that the CO₂ treatment was effective in dissolving waste concrete and consequently increasing TA in deionized water, we devised an experiment designed to modify growth media using this technique. We used seawater of two different salinities (20‰ and 33‰) in 2-L jars. To each replicate jar (n=3) we added porous bags containing waste concrete broken into the most efficient size fraction (≤ 1 mm) and then bubbled bottles with concentrated CO₂ gas for 48 hours. Three replicate bottles for each salinity treatment were also prepared with similar porous bags of waste concrete and bubbled with compressed air as a control. After the bubbling of CO₂ gas and compressed air, subsamples were taken for total alkalinity measurements determined by potentiometric Gran titration (Dickson and Goyet, 1994).

Total alkalinity measurements confirmed that the artificial weathering of waste concrete by CO₂ gas also resulted in seawater with substantially higher alkalinity; we then sterile filtered the different media types and inoculated replicate flasks with *Emiliania huxleyi* CCMP 371 that had been previously acclimated to the appropriate salinity (i.e., 20‰ or 33‰). Flasks were kept in a growth chamber at 17° C in a light: dark cycle of 16 hours light: 8 hours dark. After 10 days, cell counts were conducted on replicate samples and the percentage of calcified cells in each treatment was recorded.

Cell digestion and coccolith detachment

The final phase of this project was to establish a method for harvesting coccoliths by separating them from organic material. The primary goal was to collect the coccoliths in such a way as to preserve the surface microstructure of the calcareous coccoliths which may have a variety of industrial uses.

We used a procedure modified from Paasche et al., 1996. First, we grew cultures in f/50 media (Guillard, 1975) under conditions described in previous reports. Subsequently, cell cultures were subsampled and centrifuged at 1000 rpm for 10 minutes. The resulting pellet was resuspended in 3 ml of supernatant. A 1% solution of Triton X-100 in 0.05 M NaHCO₃ was added to the resuspended pellet. Two drops of commercial NaOCl (ca. 8%) was added. The flask was then placed on rotating shaker at 100 excursions min⁻¹ for 30 minutes. The experimental control consisted of a subsample centrifuged and resuspended in the same manner, but not exposed to detergent. Both light microscopy and subsequent SEM analysis were used to establish the efficiency of the procedure.

This procedure was performed on cultures of *E. huxleyi* CCMP371 and *Coccolithus pelagicus* strain AC400. *C. pelagicus* is a much larger cell that produces large coccoliths that lack the fine microstructure of coccoliths of *E. huxleyi*. A time series was performed wherein subsamples were exposed to the detergent for time periods from 15 minutes to 24 hours to ascertain the amount of time required to maximize digestion of the organic component of the sample while minimizing the potential for dissolution or damage to the coccoliths.

Results and Discussion

Nutrient/media experiments

Our results indicate that growth rates and degree of calcification vary widely among cell lines and species. No single growth media resulted in high growth rates and high percentages of calcified cells for all cell lines. For example, the strain of *Gephyrocapsa oceanica* we tested in the first year of the project did not grow well in any of the media and was consequently dismissed as a potential candidate for CO₂ sequestration. Most promising were several calcifying strains of *E. huxleyi*. Even they demonstrated mixed results. For example, most showed faster growth rates in K media, but the percentage of calcified cells was less in K media than in F/50 media, most likely due to enhanced calcification resulting from phosphate starvation. One strain of *Emiliania huxleyi*, cell

strain CCMP 371, consistently grew well, with $\geq 98\%$ cells calcified, in both K and F/50 media. *Emiliania huxleyi* strain CCMP 371 developed multiple layers of coccoliths in both types of media. In other calcifying cell lines, the results were mixed. For example, when *E. huxleyi* strain CCMP 1516 was grown in F/50 and K media, 28% of cells were calcified in F/50 but only 7% of cells were calcified in K media. Moreover, the cell size in CCMP 1516 was significantly smaller in K media than in F/50. Noncalcifying strains of *E. huxleyi* (CCMP 373 and 2090) grew better in K media than the F/2 media. The most consistently high percentage of calcified cells and high growth rates were exhibited by CCMP 371 and it was therefore considered as one of the better candidates for further study.

Selenium and Phosphate experiments

Selenium enrichment seemed to have a much greater effect on non-calcifying strains of *E. huxleyi*. In fact, no cell strain or species of calcifying coccolithophorids responded to the addition of selenium as markedly as CCMP 373 shown in Figure 2.

Typical results of selenium enrichment experiments are illustrated in Figure 4. As the slopes of the curves in Figures 4A, 4B, 4C and 4D show, cell growth rates of calcifying strains in K media (which contains selenium) vs. K media without selenium (i.e., K minus Se) did not significantly differ. Occasionally, the maximum cell density of these calcifying strains of *E. huxleyi* was about one half order of magnitude greater in K media than in K without the selenium (e.g., Figures 4A and 4C), but this was not a consistent result. For example, with an initial cell density of 1×10^4 cells ml^{-1} , CCMP 371 and 1516 reached stationary growth phase ($\sim 10^6$ cells ml^{-1}) in about 6 days, whereas CCMP 1949 and 374 (Figures 4A and 4C) reached stationary phase in about 3 days. We maintained and used CCMP 1949 and 374 in subsequent experiments, however only about 30-40% of cells in these two cell lines were calcified, considerably less than the percentage ($\geq 98\%$) of calcified cells in CCMP 371. The increase in growth seen in the strain *Emiliania huxleyi* CCMP1949 and CCMP 374 with the addition of selenium did not appear to offset the very low percentage in calcified cells and therefore did not make either strain a good candidate for CO_2 sequestration.

We found that the addition of selenium in K/50 increased the growth rate as well maximum cell density at stationary phase relative to K/50 without selenium. Figure 4E presents the data for CCMP 371. Comparison of Figure 4D and 4E reveals that, although the stimulatory growth effect of selenium was more pronounced in phosphate-limited culture, the growth rate and maximum cell density obtained was greater in the K media (which contains more phosphate than the K/50 media).

Photosynthesis-irradiance and calcification-irradiance experiments

Examples of P-I and C-I curves are shown in Figures 5-7. Nonlinear regression analysis revealed that the data were a good fit with the model (i.e., equation 1) with r^2 values ranging from 0.73 to 0.97. The same model was used to fit the C-I data and the majority of the r^2 values were greater than 0.6. Figure 8 shows the P-I/ C-I curves for *Coccolithus*

pelagicus strain AC400. Note that this was the only isolate tested in which calcification exceeded photosynthesis.

We did see an effect with selenium enrichment in CCMP 371 grown in K medium. Both growth and calcification seemed to be enhanced with the addition of selenium to K media. Figures 9 and 10 compare the calcification rate vs. irradiance and photosynthetic rate vs. irradiance of CCMP 371 grown in K media with cells grown in K media without selenium.

Preliminarily, the data from the P-I and C-I experiment indicated that CCMP 371 grown in K media (which contains selenium) demonstrated the highest rate of calcification relative to other cell lines of *E. huxleyi* and other species. When grown under these conditions, CCMP 371 had a mean P/C ratio of 1.36, which indicates a high rate of calcification. Subsequent closed-vessel experiments did not yield similar results with respect to selenium enrichment, however. Most of the data by this point in the project indicated that CCMP 371 was the best candidate for further CO₂ sequestration and was the subject of the majority of the subsequent experiments.

Batch culture experiments

Salinity

The addition of selenium to low salinity (20 ppt) and normal salinity (31 ppt) F/50 media did not enhance the growth rates of CCMP cell strains 371 or 374. Consistent with our previous results, however, selenium-enhanced F/50 media did result in a slightly higher value of the maximum cell density attained, compared to the maximum cell density attained in F/50 media without selenium. However, this was not always the case and the differences in cell density were not considered significant enough to pursue the matter further.

An unexpected result of the low salinity experiments was the appearance of inordinately heavily calcified cells. While only 25 to 50% of the total numbers of cells were calcified, those cells which were calcified had multiple layers of coccoliths, often resulting in coccospheres up to twice the diameter of typical coccospheres (Figure 11). Attempts to replicate the results of this experiment were unsuccessful.

Nitrate vs. Nitrate plus Ammonia

By day 6 of the experiment, there was a slightly higher total cell density achieved in the unmodified F/50 media (containing nitrate as the sole nitrogen source). However, analysis showed that this difference was not statistically significant (t-test, $P > 0.05$).

A significant difference was seen in particulate calcium production. During the mid-logarithmic growth phase, the per cell particulate calcium content in the unmodified F/50 media was over 40% higher than in the media containing both nitrate and ammonia as nitrogen sources (Figure 12).

Different nitrate loadings

No appreciable difference was observed in cultures of CCMP 371 grown in F/50 media with increasing nitrate levels. While there seemed to be no toxic effect from increasing the nitrate levels to over four times that of unmodified F/50 (2.5mM), there also did not seem to be any advantage to it either. Indeed, later experiments in which the nitrate level was decreased to half that of normal F/50 levels (from 883 μ M to ~ 450 μ M) did not appear to have a detrimental effect on growth. This suggested that this media contains nitrate far in excess of what is required by the cells for normal growth.

Closed vessel experiments

Our first closed vessel experiment was quite successful with respect to our vessel design and data-acquisition software. Some of the results of the first 19 days of experiment with respect to changes in cell density, TA and DIC are shown in Figure 13. Because we inoculated the vessel with such a small amount of cells, the exponential growth curve included a short lag, then the classic logarithmic and stationary phases.

Values of pH (calculated from EMF measurements) similarly increased with population growth. When cell density appeared to reach stationary phase at 5.7×10^6 cells ml⁻¹, we spiked the culture with additional nutrients, which stimulated a second pulse of growth. Subsequently, the cell density doubled then stabilized. The highest density of discarded coccoliths during the experiment was 3×10^8 coccoliths ml⁻¹, approximately an order of magnitude higher than the stationary phase cell density, indicating that coccoliths were continuously being produced and shed into the ambient water. Carbon limitation was suspected when the cultures reached stationary phase the second time, prompting us to allow the vessel exposure to the ambient atmosphere.

During the period of exponential cell growth, pH increased dramatically, over 0.6 pH units during the 26 days of the experiment (data shown in Quarterly progress report #7). Values of pH oscillated on a diel basis. As shown in Figure 14, pH increased during light hours and decreased during the dark. It is interesting to note that over the course of all of the closed vessel experiments, CCMP 371 was subjected to pH ranges from approximately 7.6 to nearly 8.7, sometimes experiencing changes of nearly a full pH unit over the course of a single experiment. This indicates a wide range of pH tolerances and a high degree of adaptability to changes in pH.

Ensuing analysis of the seawater subsamples showed marked changes in total alkalinity (TA) and total dissolved inorganic carbon (DIC) changed with increasing cell density. As shown in Figure 13, the DIC steadily declined with increasing cell numbers. The TA decreases with increased cell density until about Day 8. This decrease is due to the precipitation of calcium carbonate by the growing population of *E. huxleyi* cells. Our results indicated that there was an increase in TA around day 8, but this was attributed to dissolution of coccoliths in the subsamples and not considered indicative of conditions in the vessel. Subsequent modifications were made to the sample preservation protocol in an effort to minimize this effect. While our efforts did tend to minimize this effect, it was still evident, to a lesser degree, in later experiments. This experiment also demonstrated

that our data acquisition software was functional and continuous monitoring of the conditions within the vessel was possible.

The third closed vessel experiment, performed with nitrate as the sole nitrogen source was more successful than the second. Daily changes in cell density, alkalinity and dissolved inorganic carbon (DIC) are graphed in Figure 15B. Comparison of the two graphs in Figure 15 reveals that when nitrate was supplied at half the concentration of unmodified F/50 media, the time to reach stationary phase was shorter (6 days versus 8 days) and maximum cell density attained at 8 days was slightly higher (Figure 15B) than when the nitrate concentration was twice as high (Figure 15A), suggesting the possibility of a mild toxic effect from high nitrate levels. We cannot say conclusively if this result was a function of the decrease in nitrate concentration, however, because the higher levels of DIC and TA in the revised laboratory-made seawater used in the third experiment (Fig. 15B) may have had a stimulatory effect on cell growth. Although the initial content of DIC was lower in the first experiment (Figure 15A), after 8 days, DIC was depleted to a lower value in the experiment with half the normal nitrate concentration (Figure 15B).

As expected, particulate calcium increased with the increasing cell density (Figure 16). However, as the cells reached stationary phase, particulate calcium continued to increase. This increase was attributed to the cells continuing to produce and shed coccoliths into the ambient water. Early after inoculation, the ratio of particulate calcium per cell was $\sim 81 \text{ pg}$. When the cell density reached stationary phase ($\sim 10^6 \text{ cells ml}^{-1}$), this number dropped to approximately $2.6 \text{ pg Ca cell}^{-1}$ (Figure 17). The data suggest that the ratio of particulate calcium to organic material is highest in lag phase through the early stage of logarithmic growth. These data also demonstrate that decreases in TA impair calcification and tend to allow only moderate levels of organic growth.

The fourth closed vessel experiment, directly comparing F/50 and K media yielded results that showed very little difference in either cell growth or dissolved inorganic carbon uptake. Figure 18 shows that stationary phase cell densities and growth rates were comparable. Furthermore, DIC uptake was also similar and total DIC was nearly exhausted in both vessels by day 12. The similarities between growth of CCMP 371 in K media and F/50 strongly suggested that there was no particular advantage to using the more complex and expensive K media over F/50.

Cell growth in F/50 at high pH and low pH

There was a definite effect of increasing the pCO_2 on *Emiliania huxleyi* strain CCMP 371. Higher DIC (and consequently lower pH) resulted in a longer lag phase with respect to growth. As expected, the increase in pCO_2 resulted in a lower rate of CaCO_3 production. This effect was observed in both artificial and natural seawater. A more complete description of the results is included in Annual report #3.

Artificial weathering of waste concrete

The results of the experiments indicated that all size fractions of waste concrete experienced significant dissolution under the experimental conditions compared to

control samples which were not bubbled with CO₂ gas. The greatest amount of dissolution occurred in the fine size fraction that was $\leq 0.5\text{mm}$. Over the course of experiments, nearly 20% of the initial mass of waste concrete in the fine size fraction dissolved. However, even coarse sizes of waste concrete, ranging from 1.1 to 5.0 mm, showed about 14% dissolution by weight. The finer particles of waste concrete had higher surface area to volume ratios, and thus would be expected to dissolve at faster rates than the coarse particles. These experiments demonstrate that pre-crushed, waste concrete is readily dissolved by pure CO₂ gas.

The increase in TA achieved in both high and low salinity bottles containing waste concrete treated with CO₂ was dramatic (Table 4). Both treatments exhibited over a 300% increase in TA. By contrast, the compressed air treatments resulted in TA increases of approximately 30%, clearly demonstrating that the increase in dissolution was not an artifact of the bubbling alone.

Table 4. Mean values for total alkalinity in seawater samples containing waste concrete bubbled with either CO ₂ or compressed air		
	33‰ Salinity	20‰ Salinity
Bubbled with CO ₂ gas for 48 h	7083	7285
Bubbled with compressed air for 48 h	2777	2107

Growth experiments with CCMP 371 grown in the high alkalinity water indicated that this cell line was very tolerant of the dramatic increase in TA. Although stationary phase cell densities were not quite as high as those normally achieved over a 10 day incubation (10^5 vs. 10^6 cells ml⁻¹ typical of F/50 media), a high percentage of the cells were calcified and the cells were calcified heavily.

Cell digestion and coccolith detachment

The modified Triton X-100 treatment method we used to solubilize the cells and detach the coccoliths was quite successful. Light microscopy revealed that the procedure resulted in a nearly homogeneous suspension of coccoliths exhibiting little or no clumping (Figure 19A). Samples treated without detergent were composed primarily of intact cells and clumps of cells retaining their coccoliths (Figure 19B). Attempts to reduce the time needed to produce a coccoliths suspension were unsuccessful. Reducing the exposure time from 30 to 15 minutes resulted in incomplete digestion of the organic material in the sample. This may have been due to the high cell density of the sample (10^7 cells ml⁻¹).

The time series that was performed to ascertain if longer exposure to the detergent would result in a more complete digestion of the organic component of the samples suggested

that there is an optimum time period for best results. Samples of *E. huxleyi* CCMP 371 and *Coccolithus pelagicus* strain AC400 were used in this experiment. SEM analysis showed that coccoliths of CCMP 371 that were exposed to the detergent for time periods not exceeding 30 minutes showed little damage or dissolution (Figure 20). The larger and more robust coccoliths of *C. pelagicus* exhibited very little damage from the digestion and harvesting procedure (Figure 21). However, when exposed to the digestion matrix for longer periods of time, dissolution and damage were evident in coccoliths of CCMP 371 (Figure 22). Samples not exposed to detergent showed that the organic component of the sample persisted. SEM analysis revealed the presence of what appears to be noncalcifying cells (Figure 23). It is unclear whether these cells lacked coccoliths in culture or the coccoliths were somehow lost during centrifugation.

Conclusions

The results from this project strongly suggest that due to their high rates of calcification and/or tolerances to a wide range of environmental conditions, two coccolithophore isolates stand out among the 10 examined as the most promising candidates for future use in CO₂ abatement projects. *E. huxleyi* strain CCMP 371 was the most thoroughly examined has consistently demonstrated a high degree of calcification and adaptability to a wide range of environmental conditions. *Coccolithus pelagicus* strain AC400 is also a good candidate due to its high per cell CaCO₃ content and apparent lack of photoinhibition. It would therefore be as suitable as CCMP 371 for shallow, outdoor ponds or similar facilities. It should be mentioned, however, that *C. pelagicus* typically does not tend to “overproduce” excess coccoliths and shed them into the ambient water.

Considering all of the data, it is our belief that there are optimum conditions for *E. huxleyi* strain CCMP 371 to be effectively employed in CO₂ abatement projects. For the most effective use of this organism, it should be maintained in a chemostatic apparatus such that logarithmic growth is kept constant by a steady influx of media replacing what is lost by regular harvesting of a portion of the cells. The best and incidentally most inexpensive media should consist of natural seawater with a salinity of approximately 31-33ppt enriched with no more than 400µM nitrate, 1.5µM phosphate, and F/50 levels of trace metals.

Although natural seawater is preferable due to its higher alkalinity and DIC, satisfactory results can be obtained using artificial seawater if projects employing this organism are inland, away from sources of natural seawater. Lower salinities (~20ppt) can also be tolerated by CCMP 371. This would be advantageous if artificial seawater would have to be made as this would reduce the production cost.

Using CO₂ to artificially weather concrete is effective and greatly enriches the media with both calcium and carbonate ions for calcification. While this process is more effective when the concrete is crushed into particles ~1mm in diameter, however, sufficient dissolution can be achieved with a larger size fraction.

Calcareous coccoliths can be effectively harvested by using small amounts of detergent to solubilize the organic fraction of this organism and coccoliths can subsequently recovered by filtration. However, further research and development is necessary to devise a scaled-up apparatus to accomplish this.

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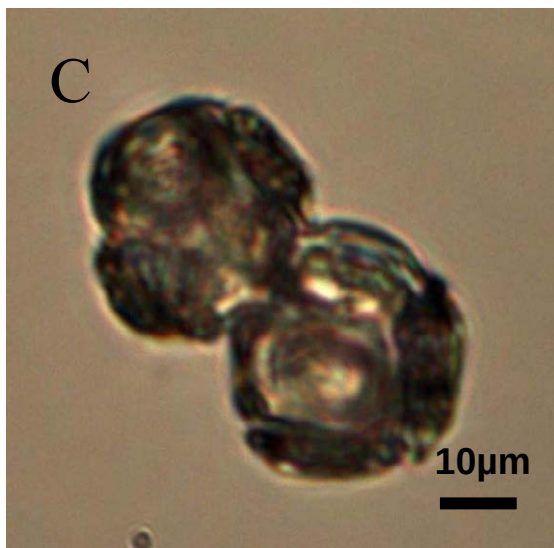
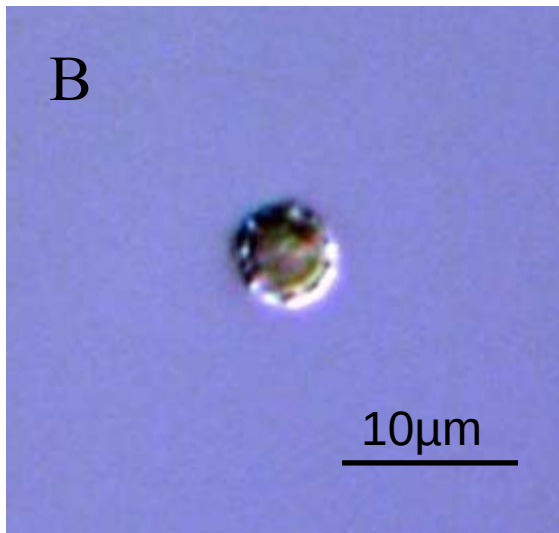
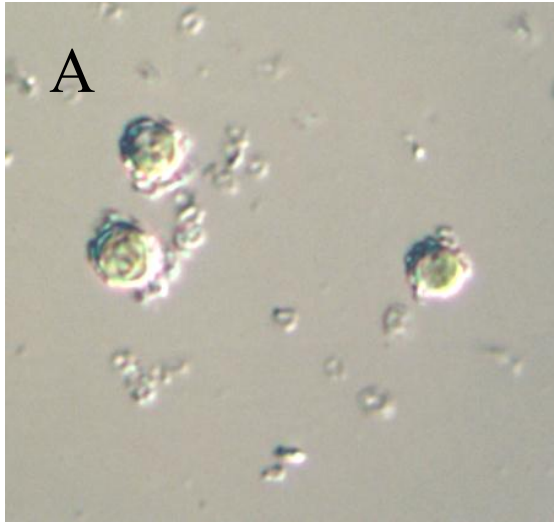


Figure 1 (A) Nomarski photomicrograph of *Emiliana huxleyi* strain CCMP 371. Cell diameter = 5-6.5 μm . Note cells are covered with multiple layers of coccoliths. Discarded coccoliths are also visible. (B) Cell at lag phase to early logarithmic growth phase is completely covered by 1-2 layers of coccoliths. (C) Well calcified cells of *Coccolithus pelagicus* strain AC400. Note the size discrepancy between *C. pelagicus* and *E. huxleyi*.



Figure 2. Flasks of *Emiliana huxleyi* noncalcifying strain CCMP 373. The flask on the right side of the photo contains cells grown in F/2 media enriched with selenium. The flask on the left contains no selenium. This cell strain grew faster and reached greater maximum cell densities in selenium-enriched media. Selenium enrichment had less of an effect on calcifying strains of this species.



Figure 3. (A) Custom-manufactured vessel with ports for continuous monitoring of temperature, mV, and oxygen by a computer-controlled data acquisition system. (B) The vessel during an experiment showing the Mylar argon reservoir and the sampling port. Note the turbidity of the water due to the high cell density

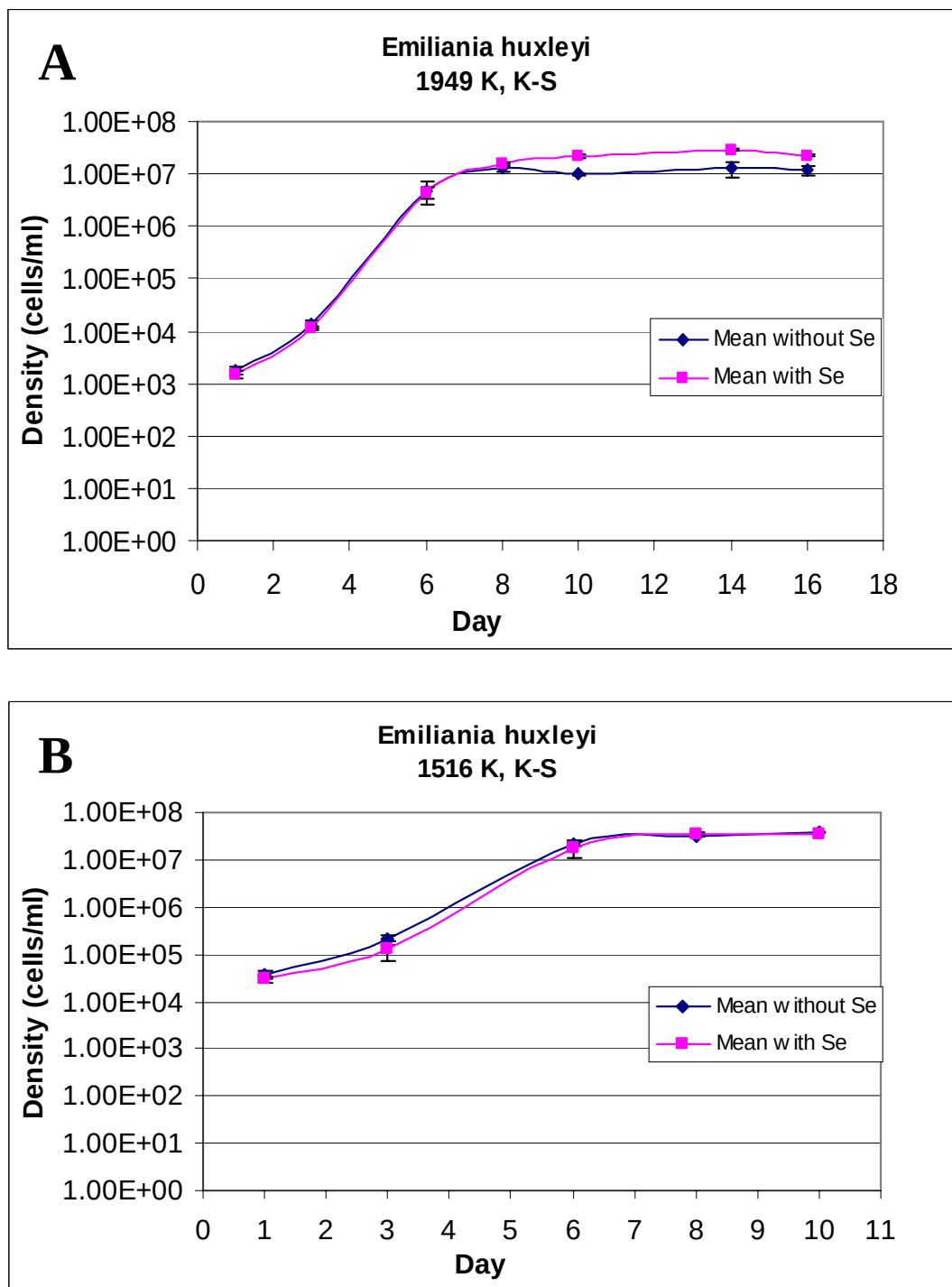


Figure 4. Cell growth in different calcifying cell lines of *Emiliana huxleyi* as a function of selenium enrichment (A-D) and phosphate-limitation (E). **(A)** *E. huxleyi* strain CCMP 1949 in K media with selenium compared to cells growing in K media without selenium. **(B)** *E. huxleyi* strain CCMP 1516 in K media with selenium compared to cells growing in K media without selenium.

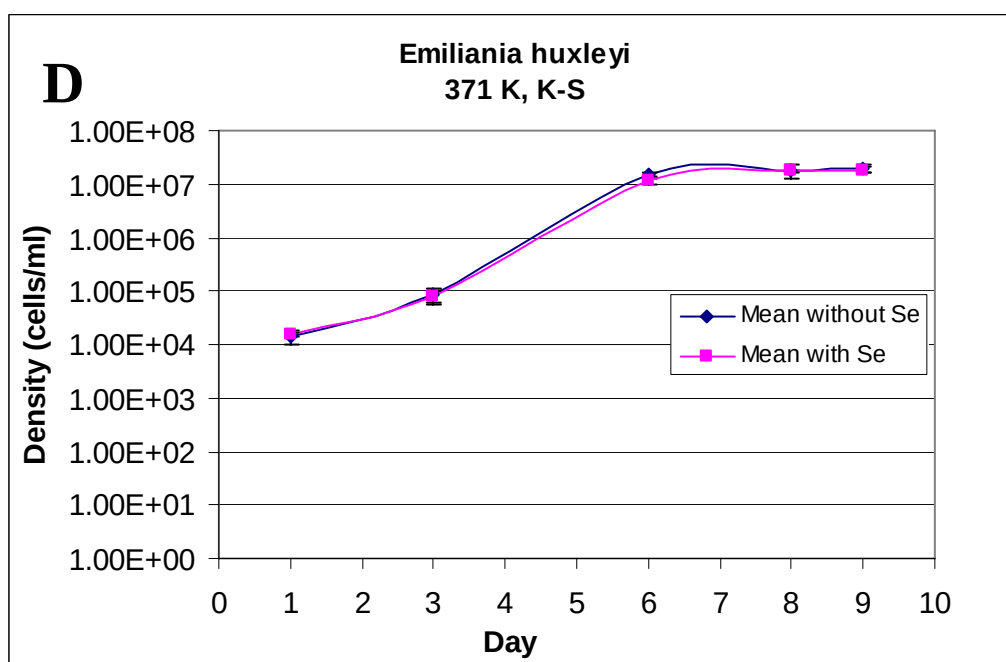
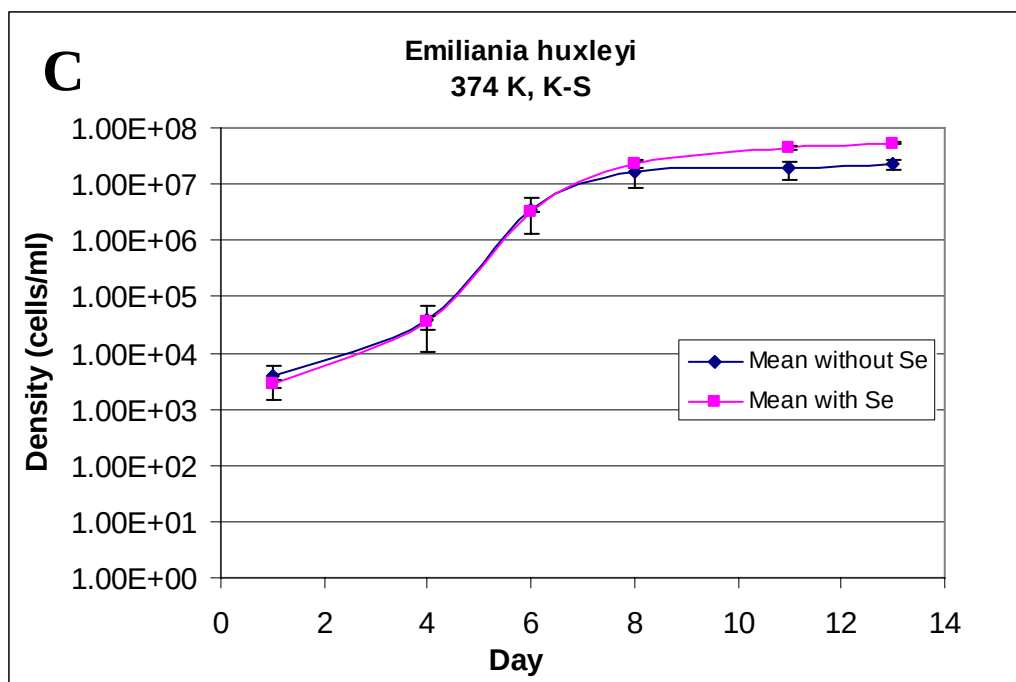


Figure 4. Cell growth in different calcifying cell lines of *Emiliana huxleyi* as a function of selenium enrichment (A-D) and phosphate-limitation (E). **(C)** *E. huxleyi* strain CCMP 374 in K media with selenium compared to cells growing in K media without selenium. **(D)** *E. huxleyi* strain CCMP 371 in K media with selenium compared to cells growing in K media without selenium

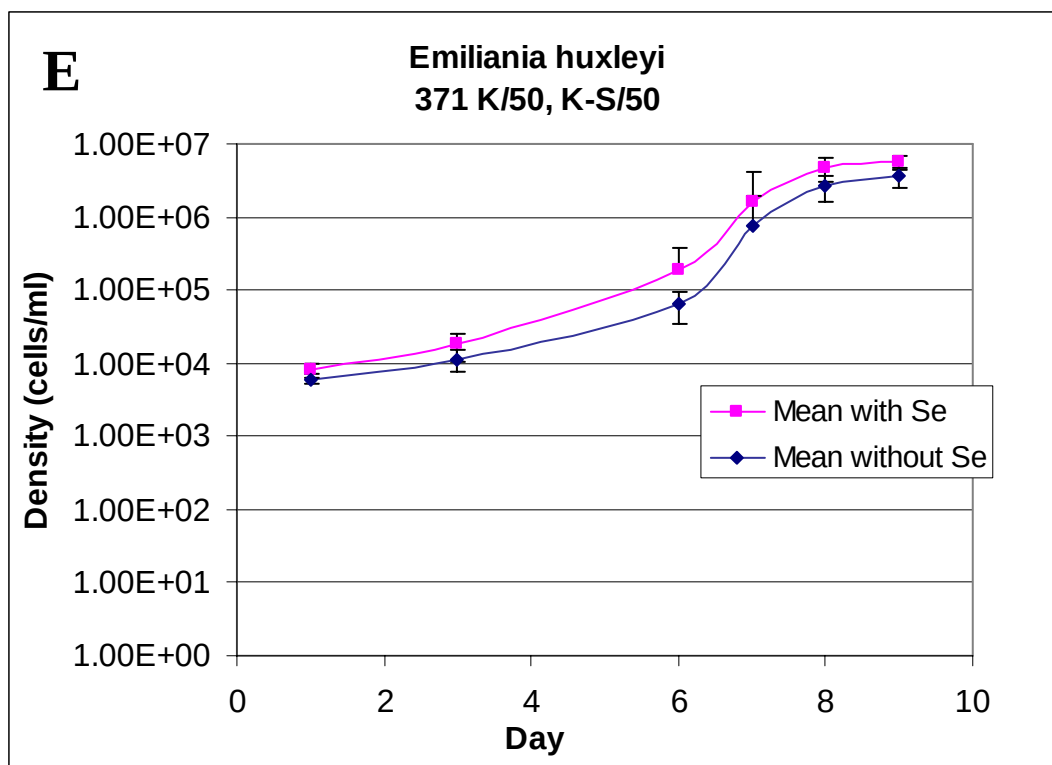


Figure 4. Cell growth in different calcifying cell lines of *Emiliana huxleyi* as a function of selenium enrichment (A-D) and phosphate-limitation (E). **(E)** *E. huxleyi* strain CCMP 371 in phosphate-limited K/50 media with selenium compared to cells growing in phosphate-limited K/50 media without selenium

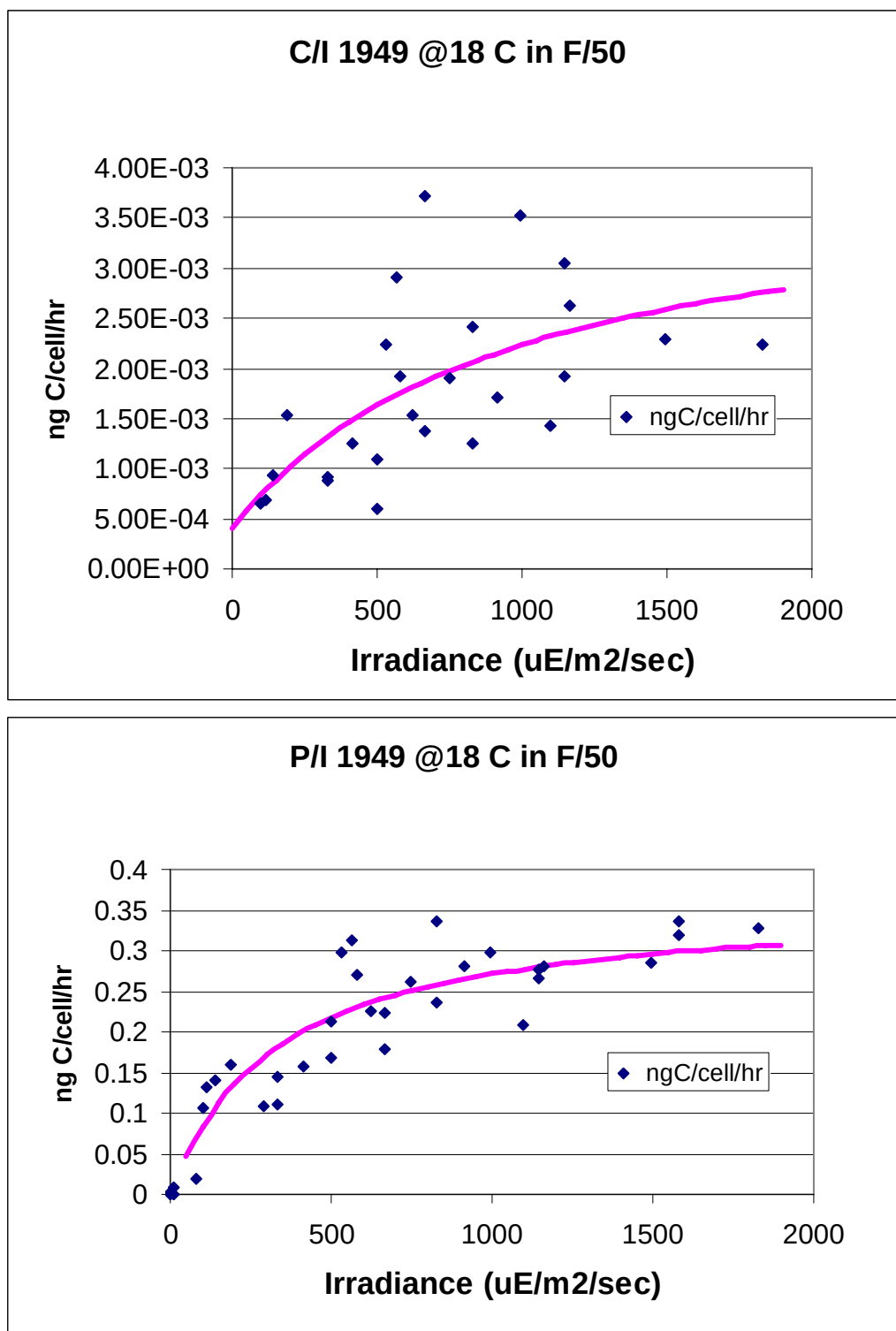


Figure 5. Calcification rate (top) and rate of photosynthesis (bottom) versus irradiance in *Emiliania huxleyi* strain CCMP 1949 at 18°C in F/50 media.

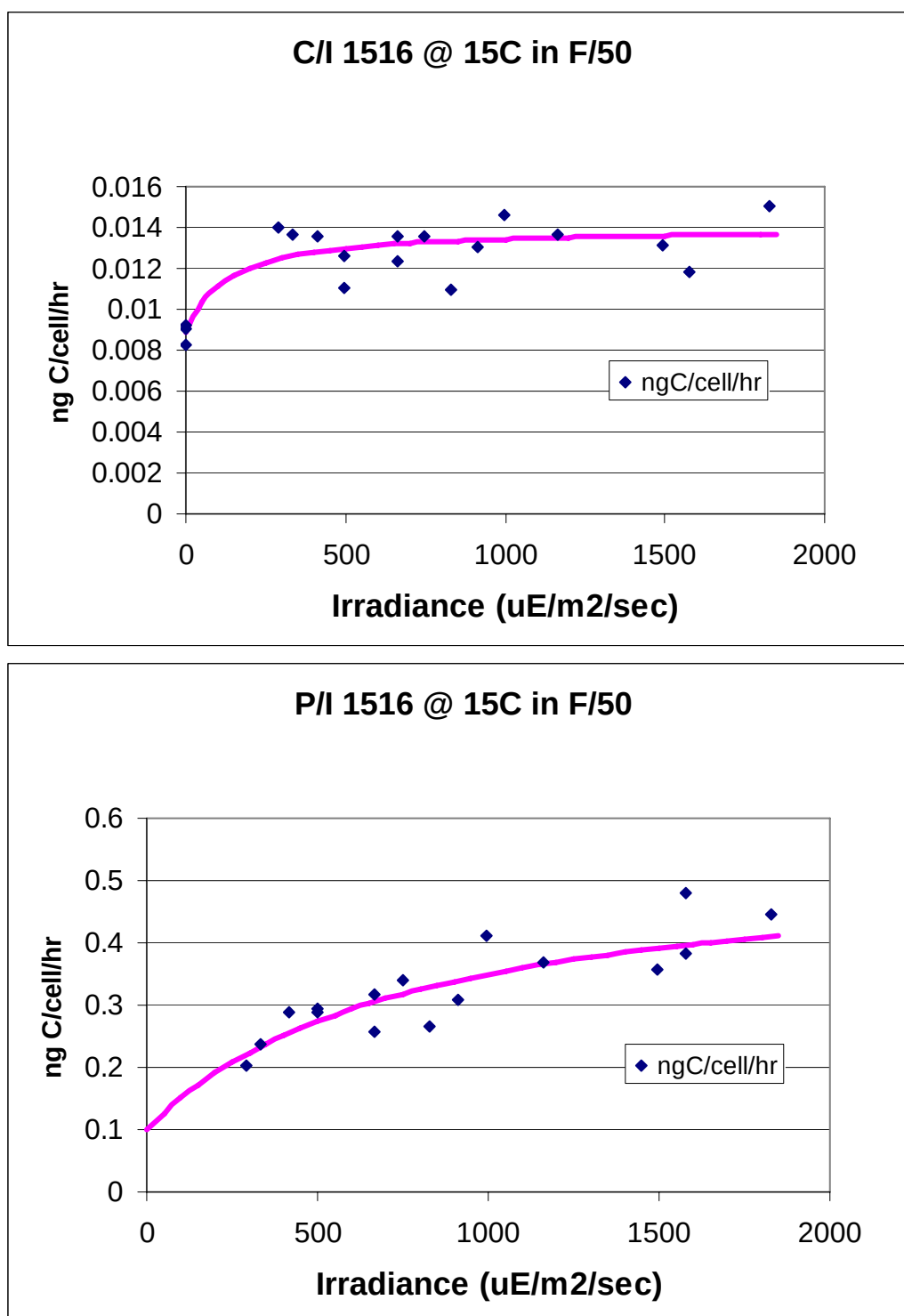


Figure 6. Calcification rate (top) and rate of photosynthesis (bottom) versus irradiance in *Emiliania huxleyi* strain CCMP 1516 at 15°C in F/50 media.

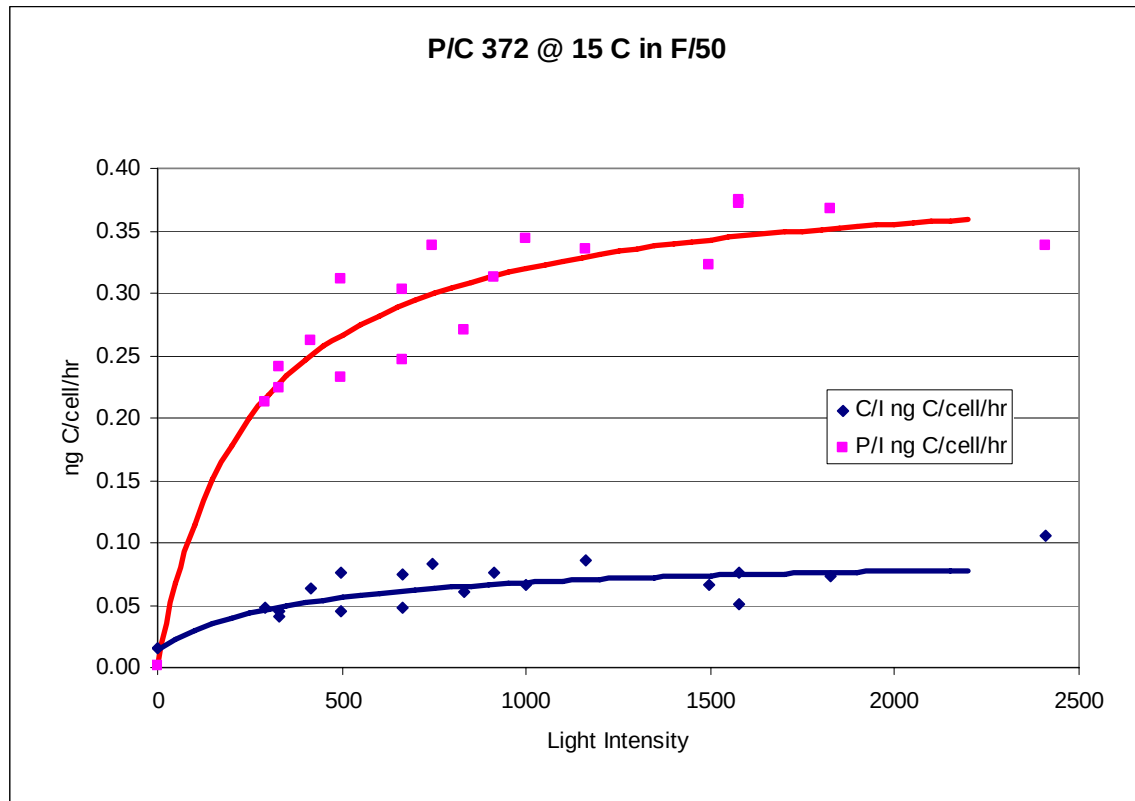


Figure 7. Calcification rate (diamonds in blue) and rate of photosynthesis (squares in pink) versus irradiance in *Emiliania huxleyi* strain CCMP 372 at 15°C in F/50 media.

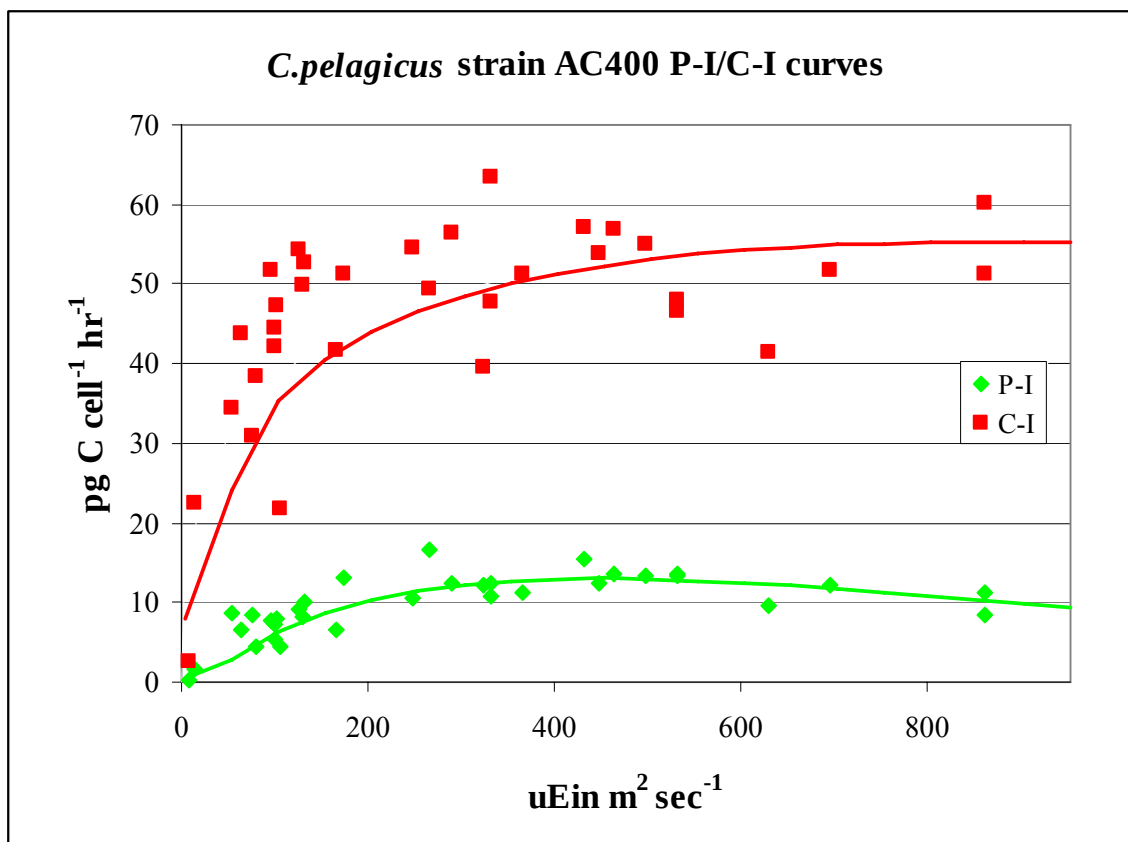


Figure 8. Photosynthesis and calcification as a function of light intensity in the coccolithophorid *Coccolithus pelagicus* strain AC400 grown in F/2 media. Note that unlike similar curves for *E. huxleyi*, calcification actually exceeds photosynthesis.

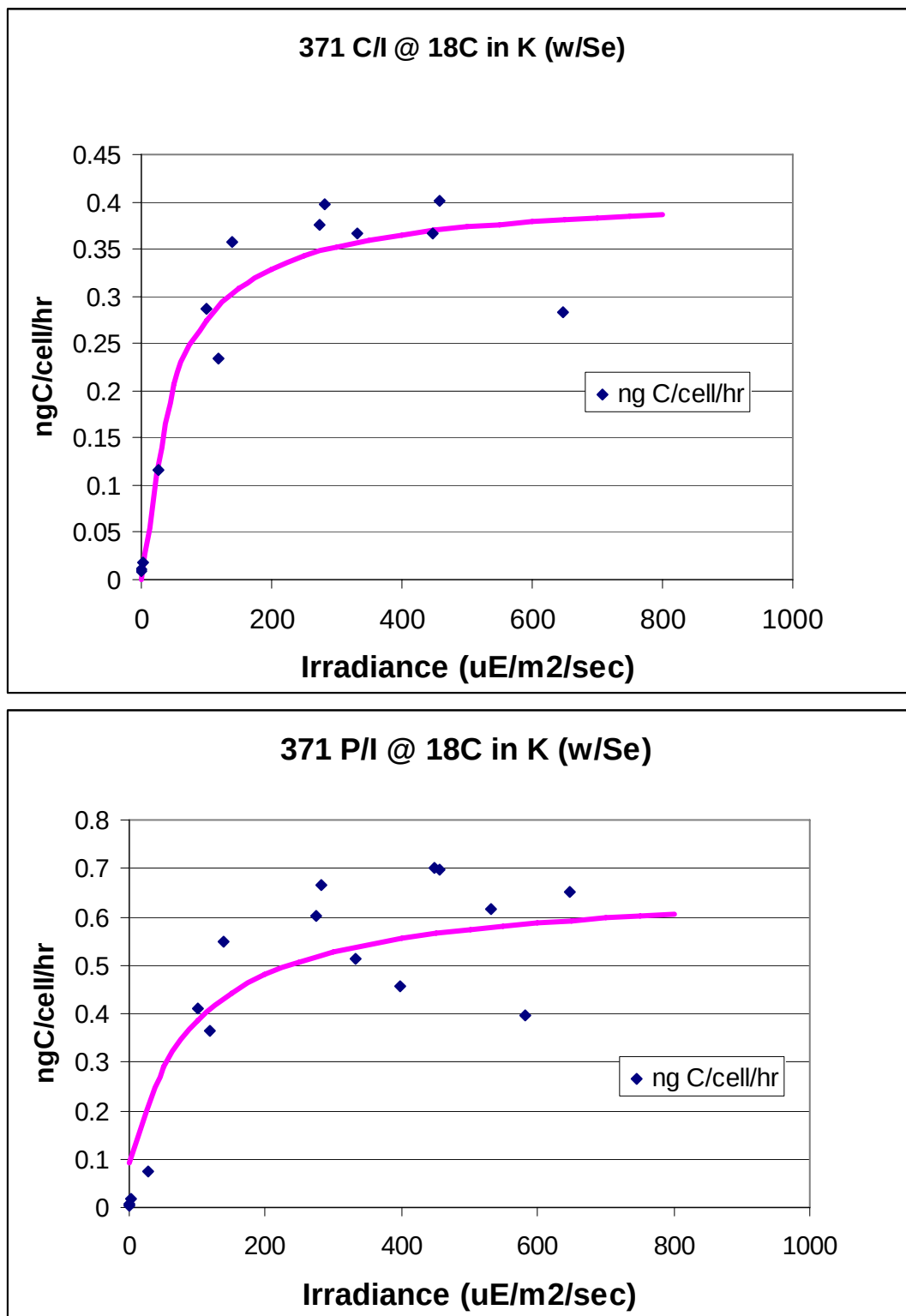


Figure 9. Calcification rate (top) and rate of photosynthesis (bottom) versus irradiance in *Emiliania huxleyi* strain CCMP 371 at 18°C in K media, which contains selenium.

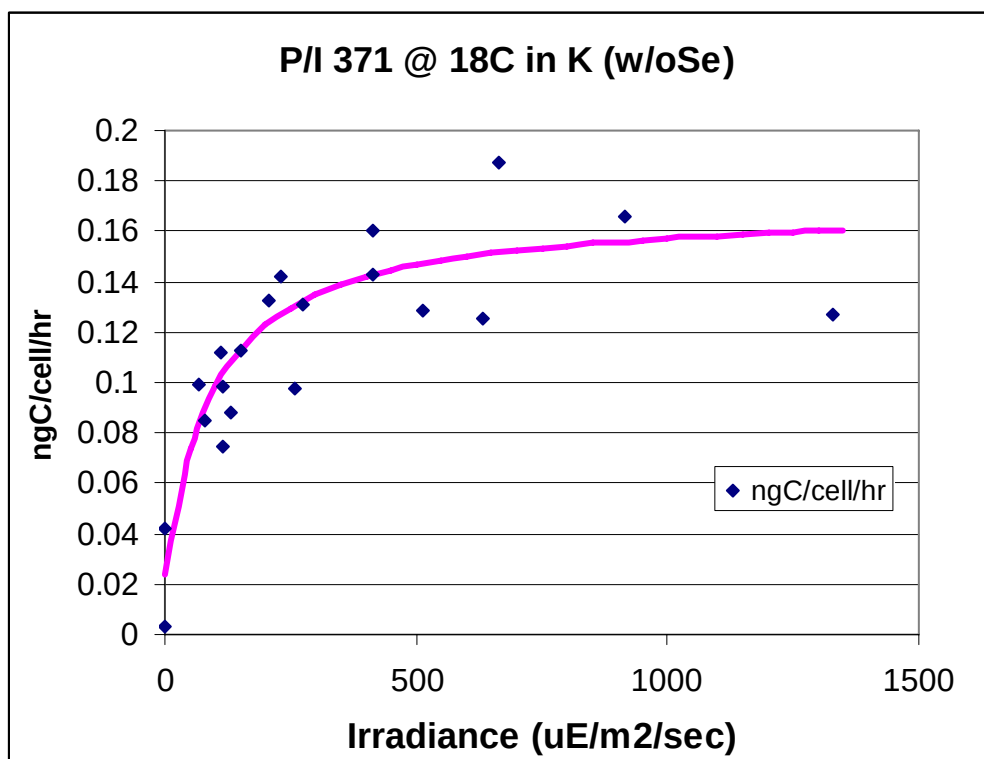
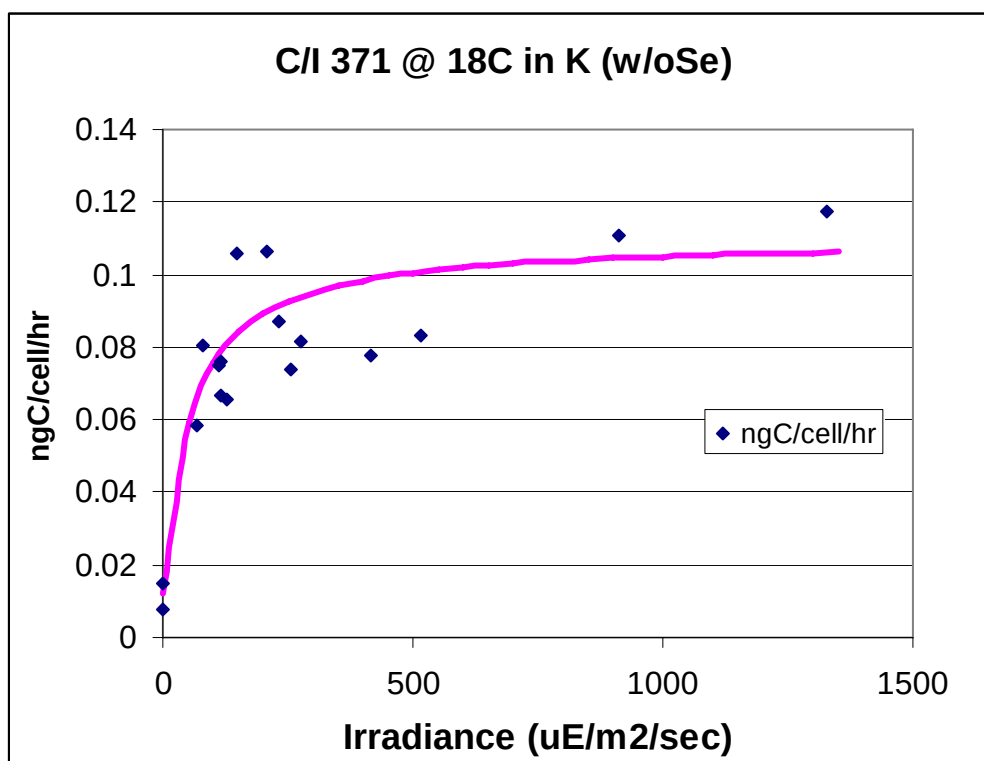


Figure 10. Calcification rate (top) and rate of photosynthesis (bottom) versus irradiance in *Emiliana huxleyi* strain CCMP 371 at 18°C in K media without selenium.

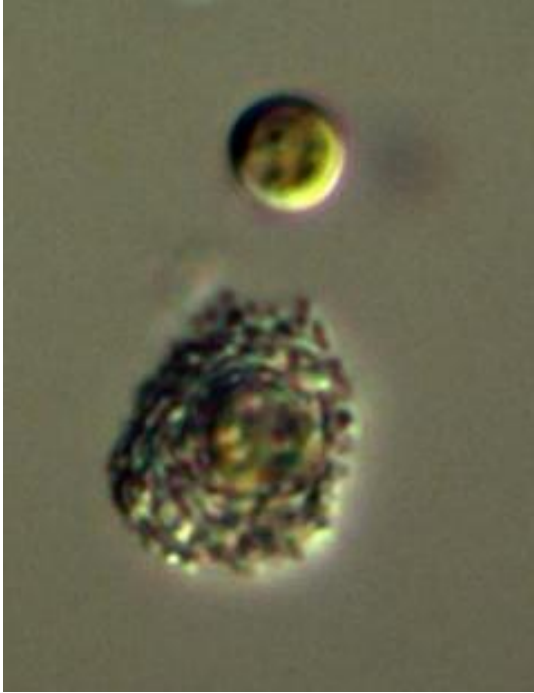
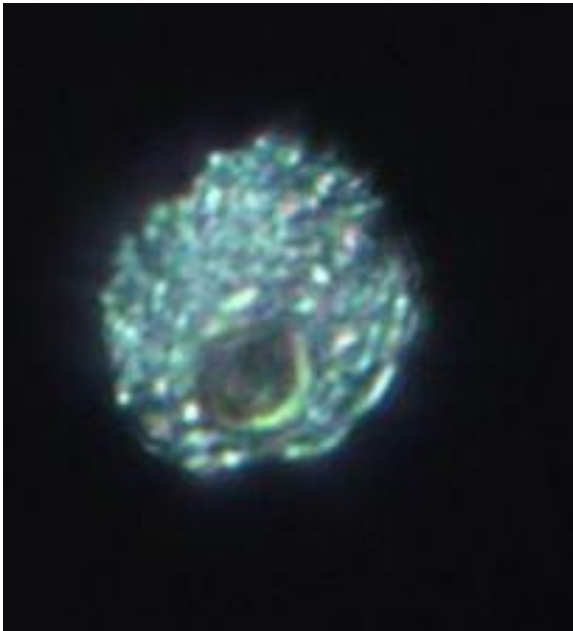


Figure 11. (a) Nomarski photomicrograph of *E. huxleyi* strain CCMP371 grown in low-salinity (20ppt) F/50 media. The top cell completely lacks coccoliths and the bottom cell exhibits multiple layers of coccoliths. (B). Polarized-light photomicrograph of another heavily calcified cell from the same low-



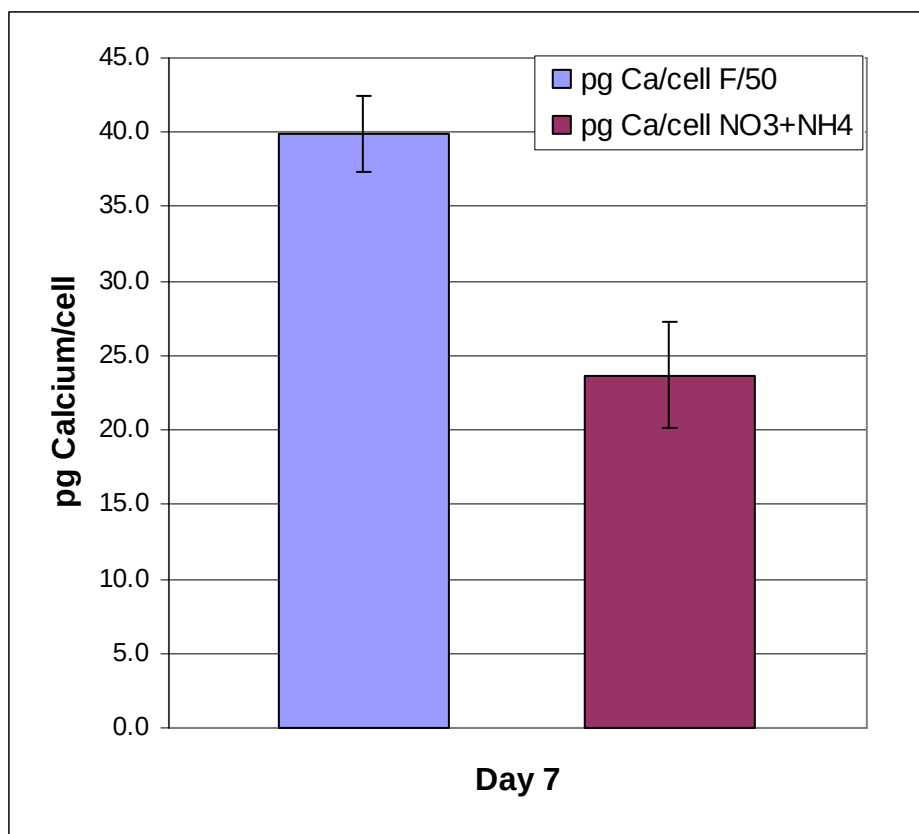


Figure 12. Differences in the ratio of particulate calcium to cell density (pg Ca/cell) in cultures of *Emiliana huxleyi* CCMP 371 grown in unmodified F/50 media (blue bars) containing 883 μM nitrate and modified F/50 media (magenta bars) containing two sources of nitrogen, 833 μM nitrate plus 50 μM ammonia. The graph represents day 7 of batch culture experiment in which cell densities were in early to mid logarithmic growth phase. Error bars = 1SD.

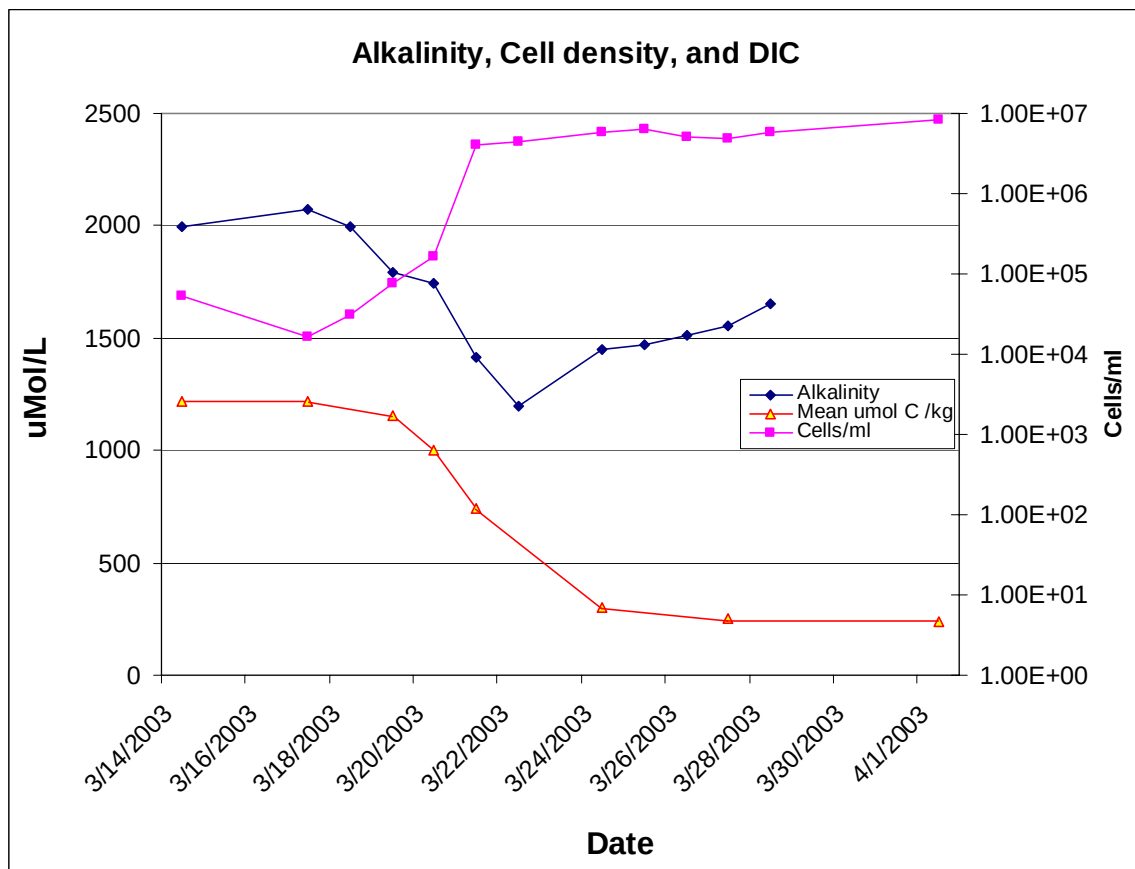


Figure 13. Changes in cell density (pink line), total alkalinity (blue line), and total dissolved inorganic carbon (orange line) over the first 19 days of the first closed vessel experiment.

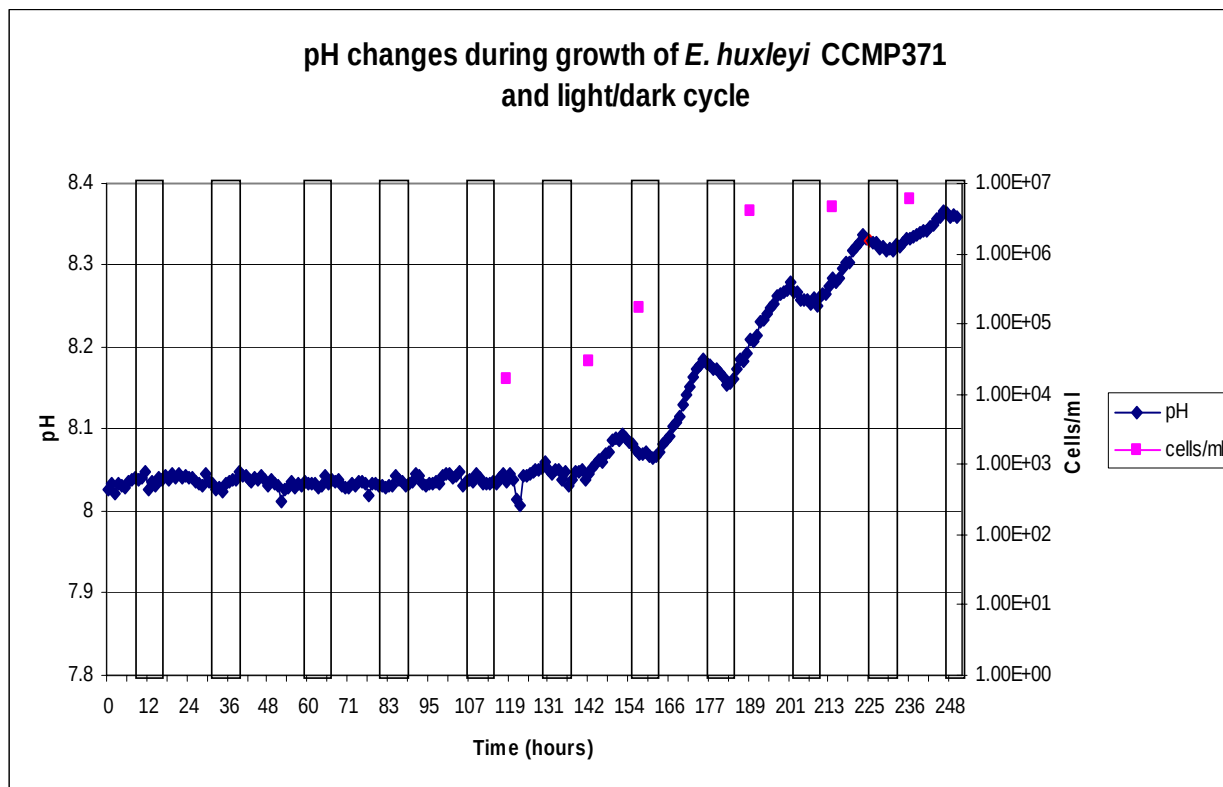


Figure 14. First 10 days of closed vessel experiment illustrating the relationships among pH (left Y-axis; blue line), cell density (right Y-axis; pink squares) and light/dark cycle (16 hours light/8 hours dark). Dark hours denoted by shaded bars. Note the pH decrease dark hours.

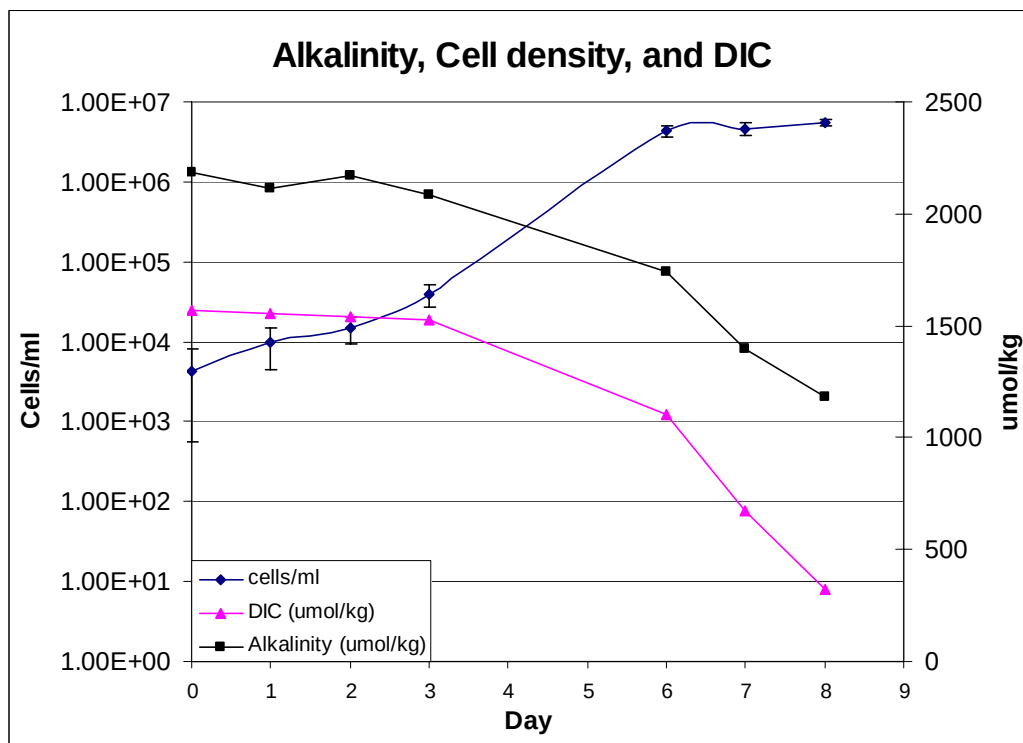
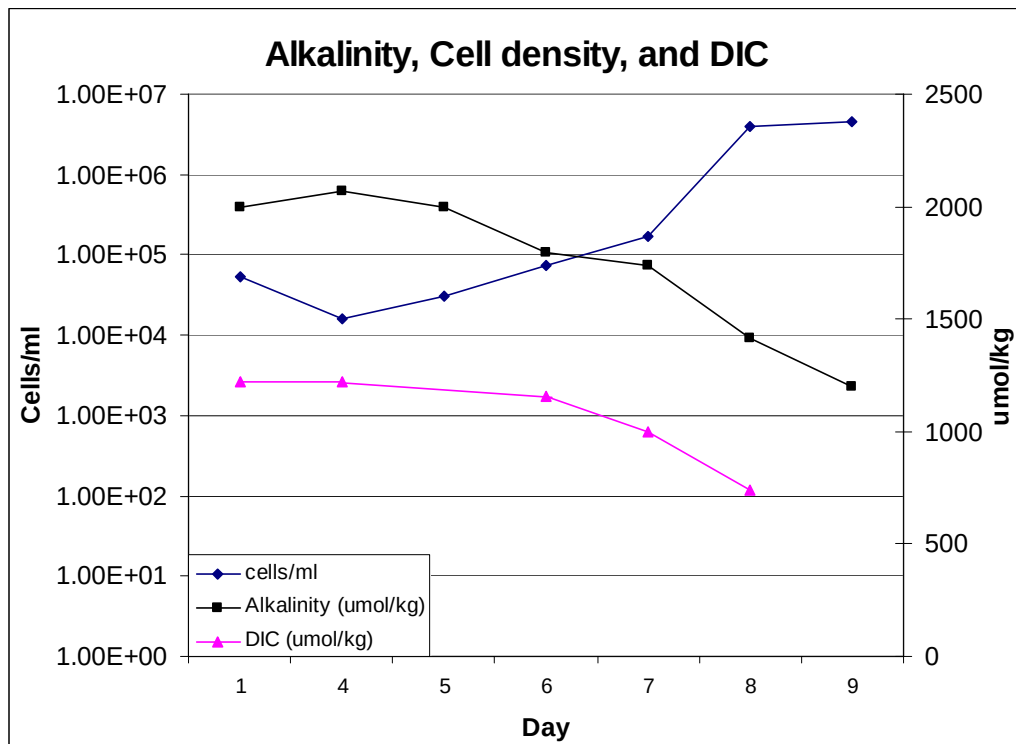


Figure 15. Changes in cell density (blue circles), total alkalinity (black squares), and total dissolved inorganic carbon (DIC; pink triangles) in closed vessel experiments with *Emiliana huxleyi* cell strain CCMP 371. (A) Cells grown in unmodified F/50 media with nitrogen content of 883μM nitrate; closed vessel experiment #1. (B) Cells grown in modified F/50 media with nitrogen content of 450μM nitrate; closed vessel experiment #3.

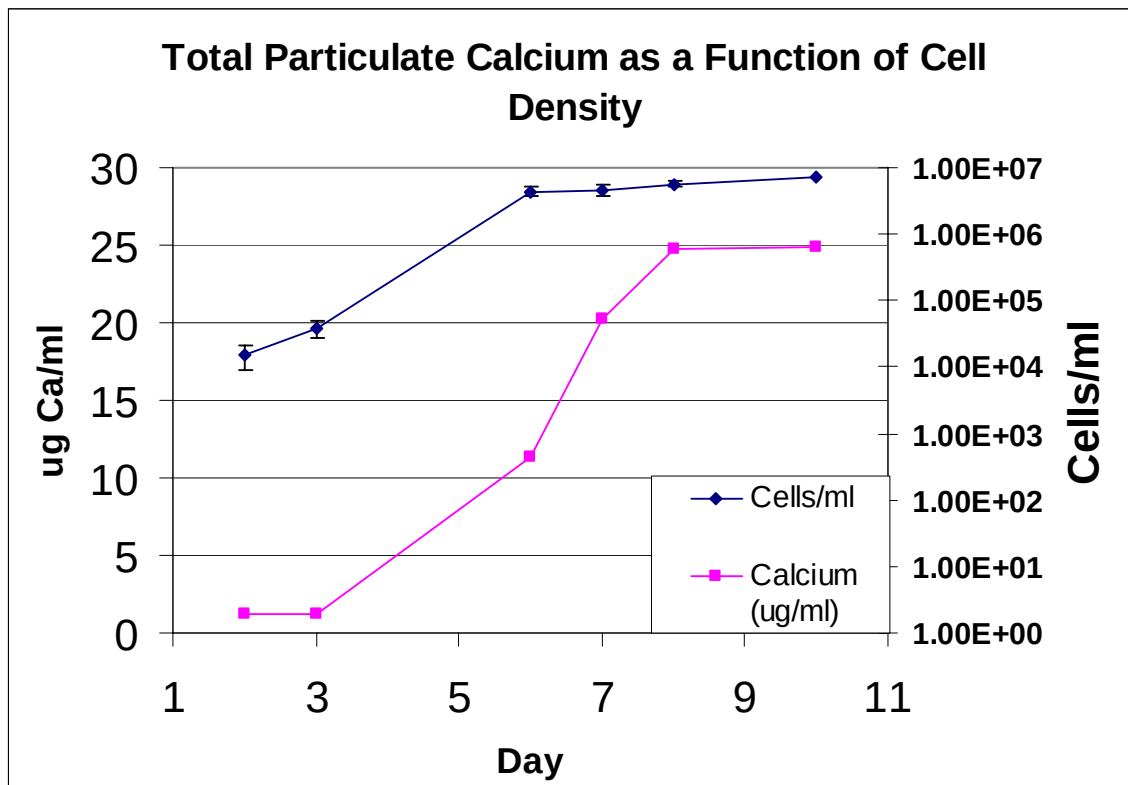


Figure 16. Changes in total particulate calcium ($\mu\text{g ml}^{-1}$; pink squares) and cell density (cells ml^{-1} ; blue diamonds) during closed vessel experiment #3.

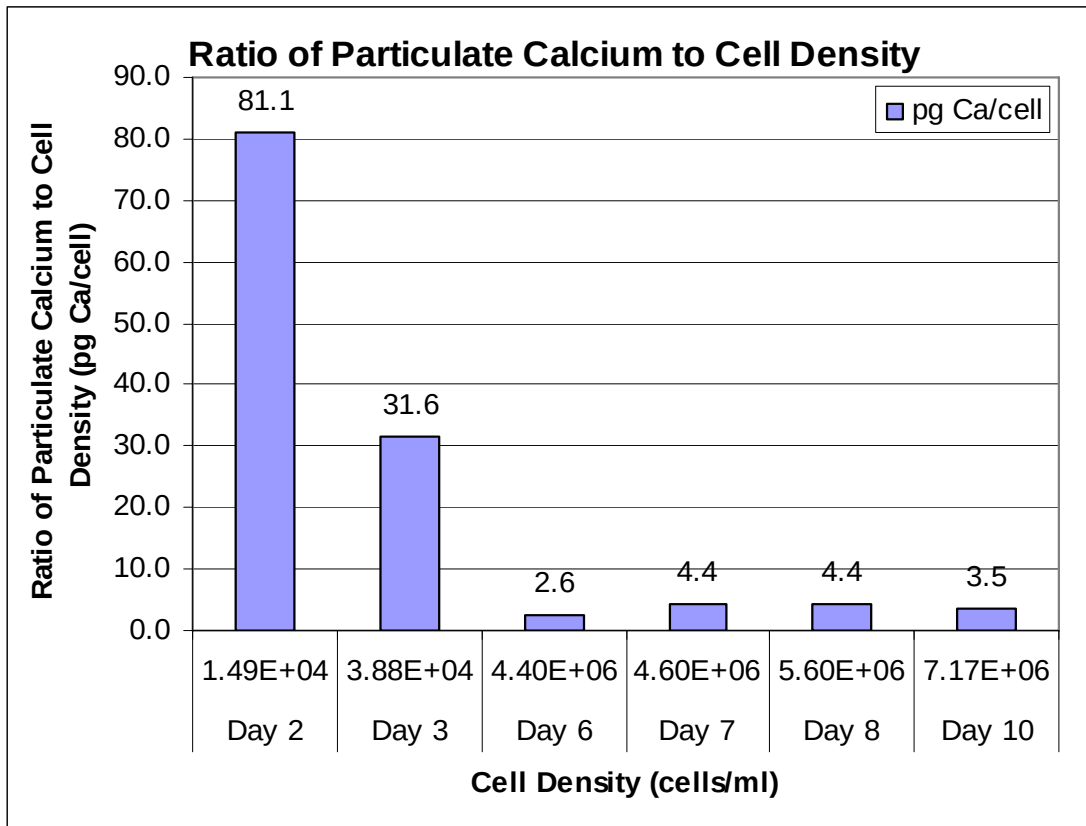


Figure 17. Changes in the ratio of particulate calcium to cell density (pg Ca/cell) as a function of cell density during closed vessel experiment #3.

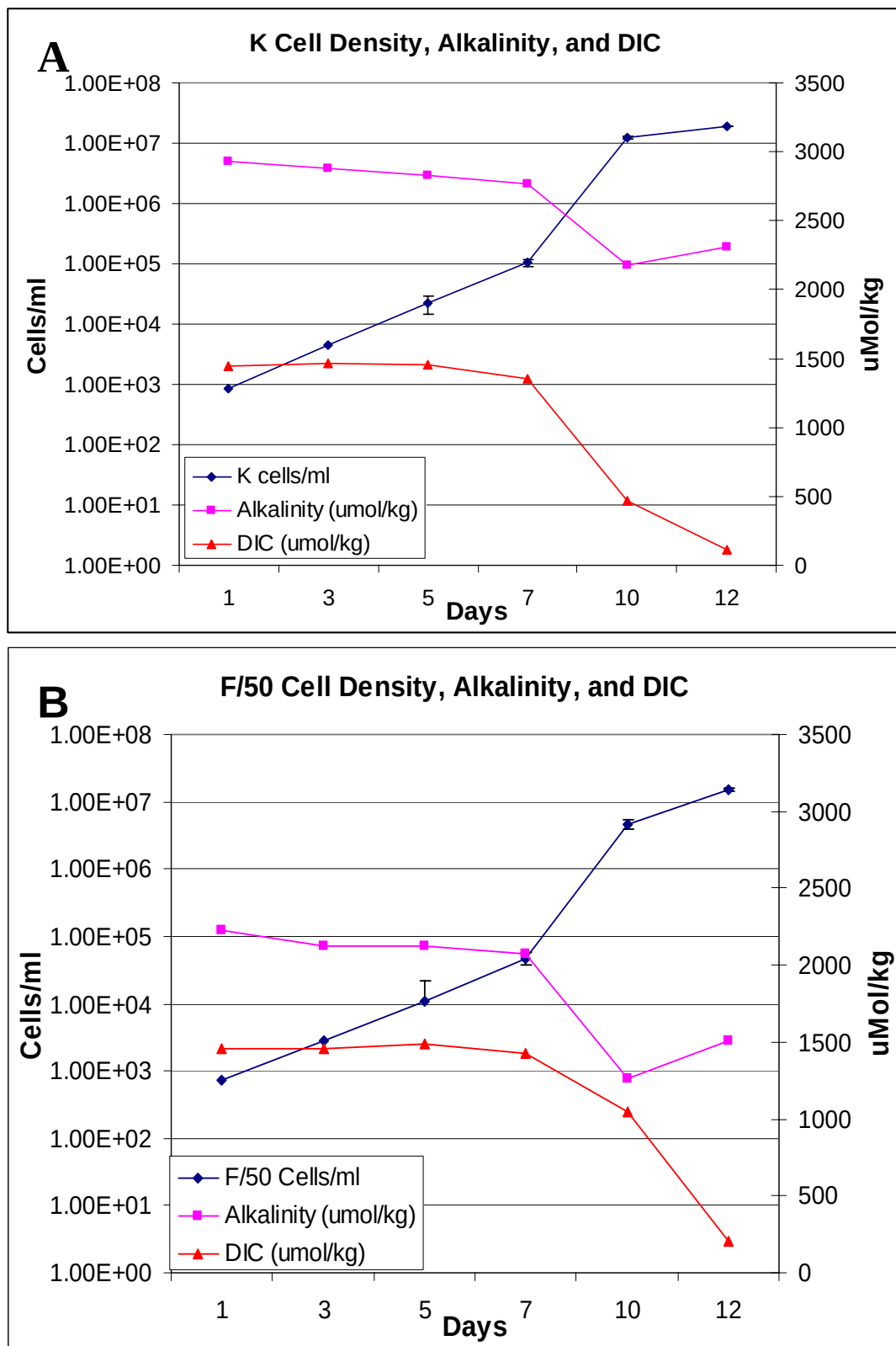


Figure 18. Changes in cell density (blue diamonds), total alkalinity (pink squares), and total dissolved inorganic carbon (DIC; red triangles) in closed vessel experiments with *Emiliania huxleyi* cell strain CCMP 371. (A) Cells grown in unmodified K media. (B) Cells grown in F/50 media.

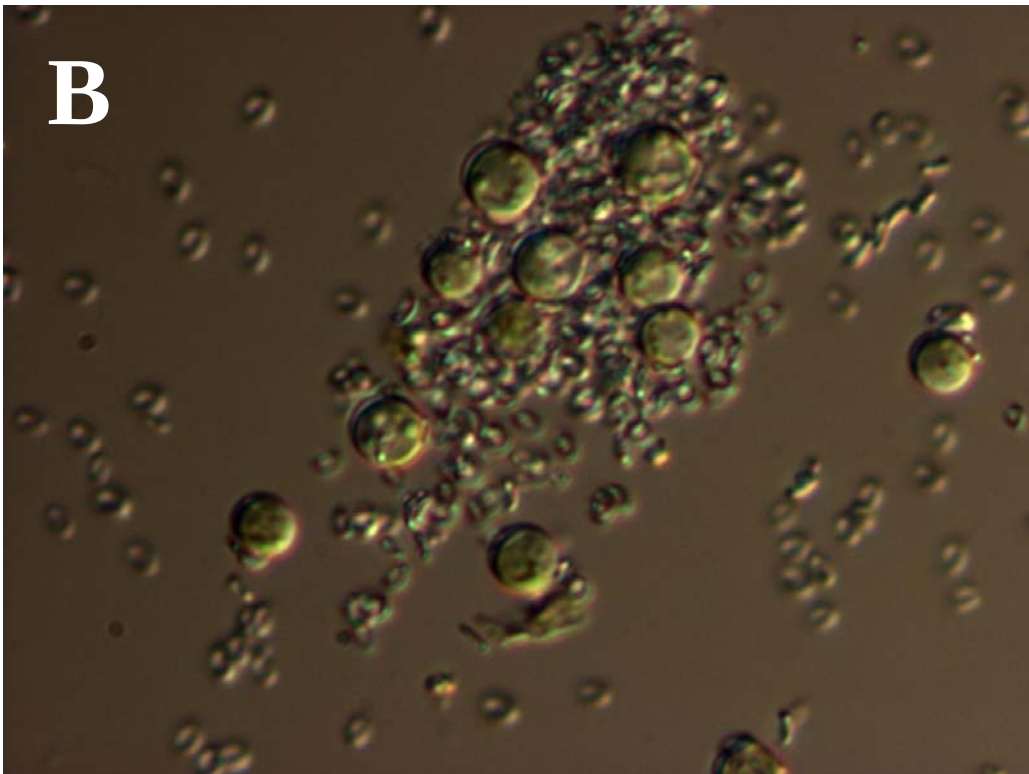
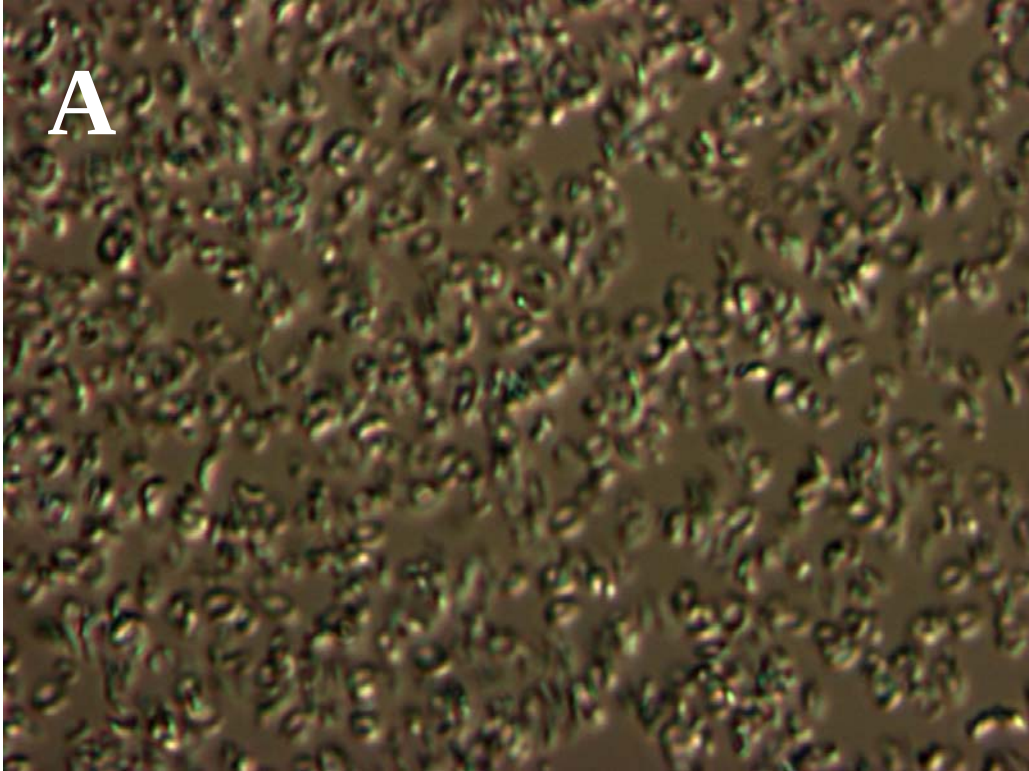


Figure 19. (A) Homogenous suspension of coccoliths resulting from digestion of *E. huxleyi* CCMP 317 with Triton X-100 detergent. Note the lack of any visible intact cells. (B) A sample of the same CCMP 317 culture not exposed to detergent.

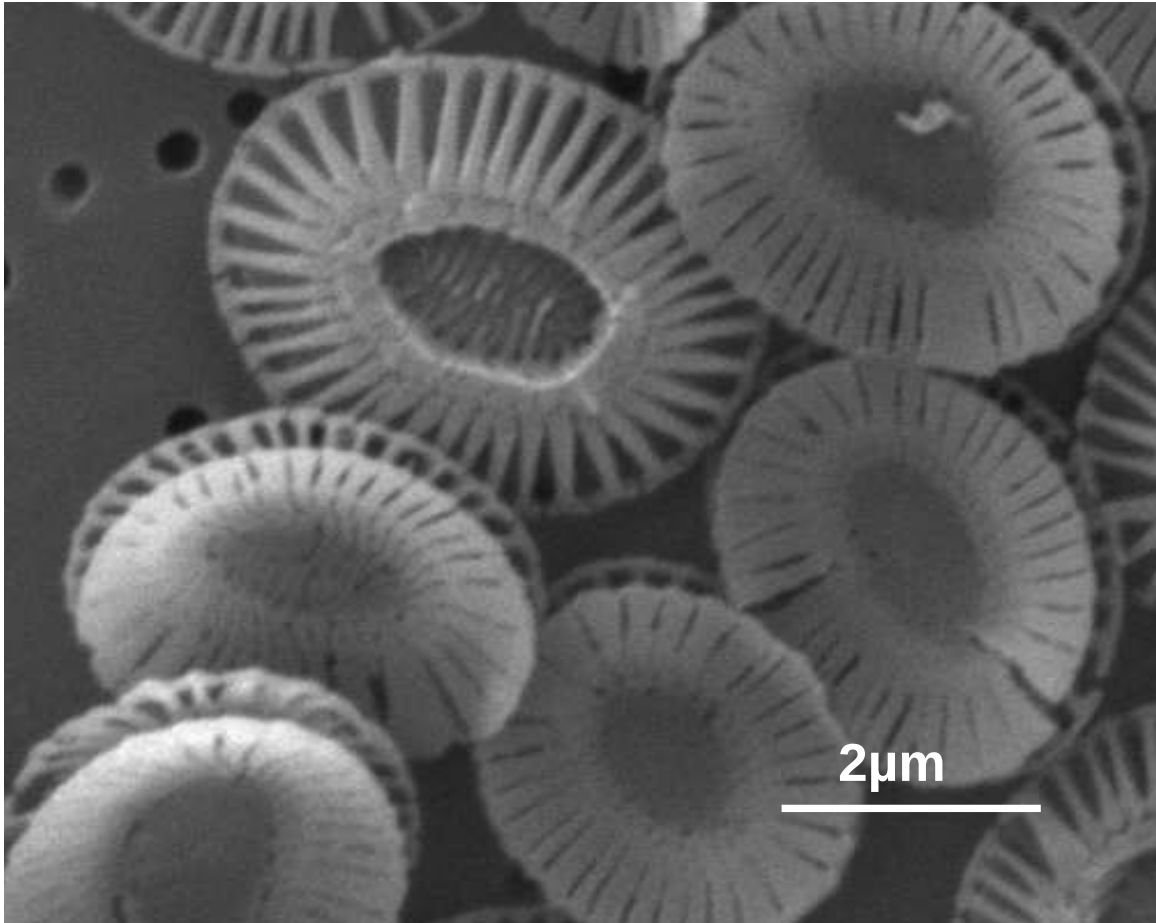


Figure 20. SEM micrographs of coccoliths of *Emiliana huxleyi* strain CCMP371 harvested using Triton X-100 detergent to solubilize the organic component of the material.

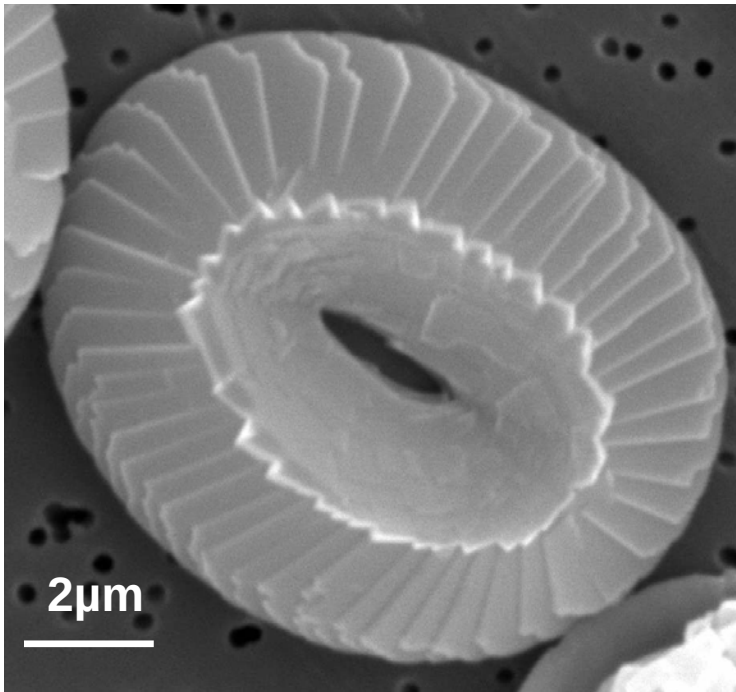
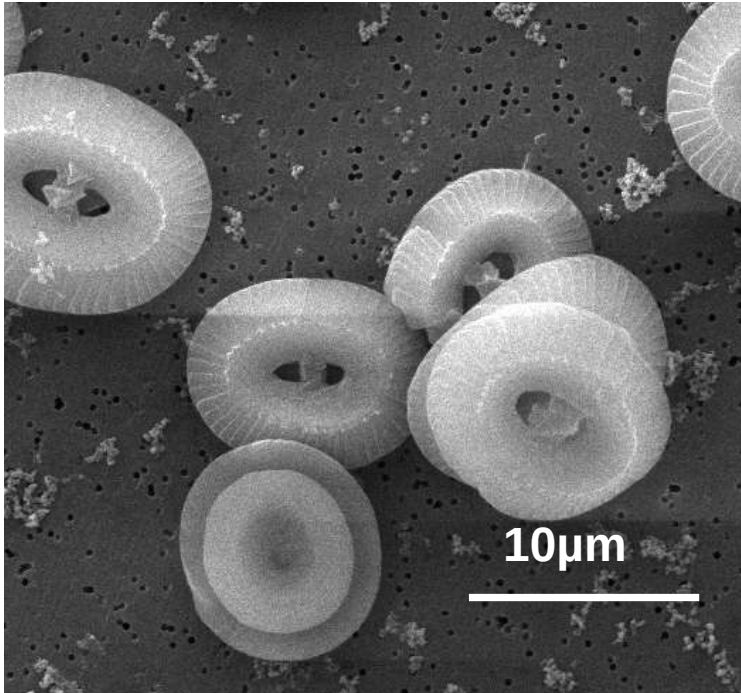


Figure 21. SEM micrographs of coccoliths of *Coccolithus pelagicus* strain AC400 harvested using triton X-100 detergent to digest cells. These coccoliths are about twice the size of those of *E. huxleyi* and are quite different in structure.

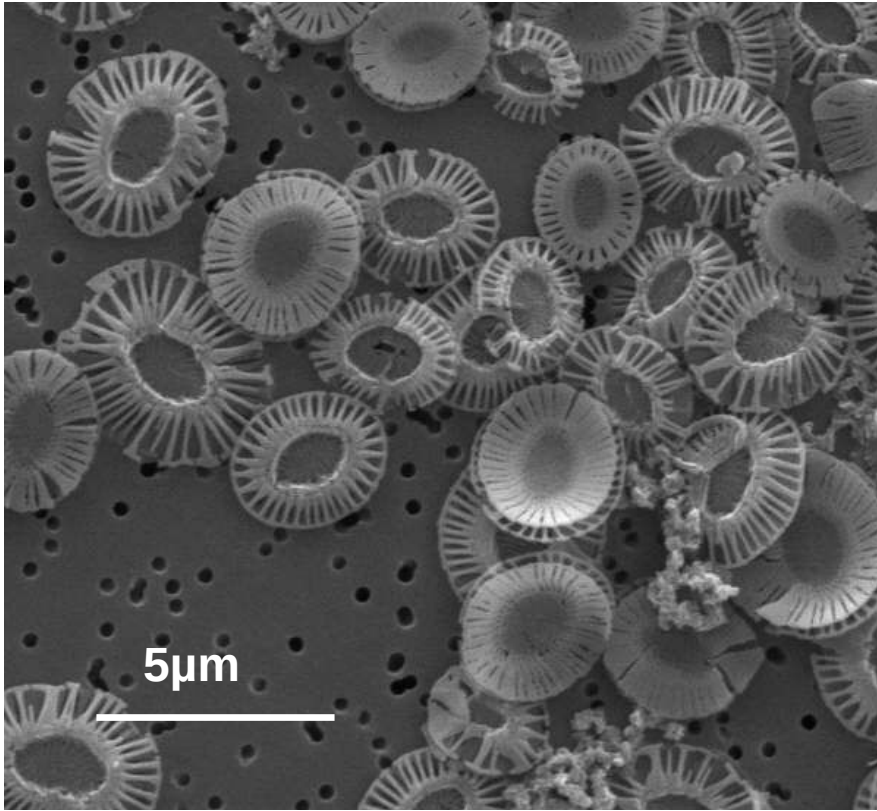


Figure 22. SEM micrograph of coccoliths of *Emiliana huxleyi* strain CCMP371 after harvesting using triton X-100 detergent to solubilize the organic fraction of the sample. This sample was part of a time series and exposed to the detergent solution for 24 hours. Note that the coccoliths have begun to exhibit dissolution and damage due to the prolonged exposure to the solubilizing agent.

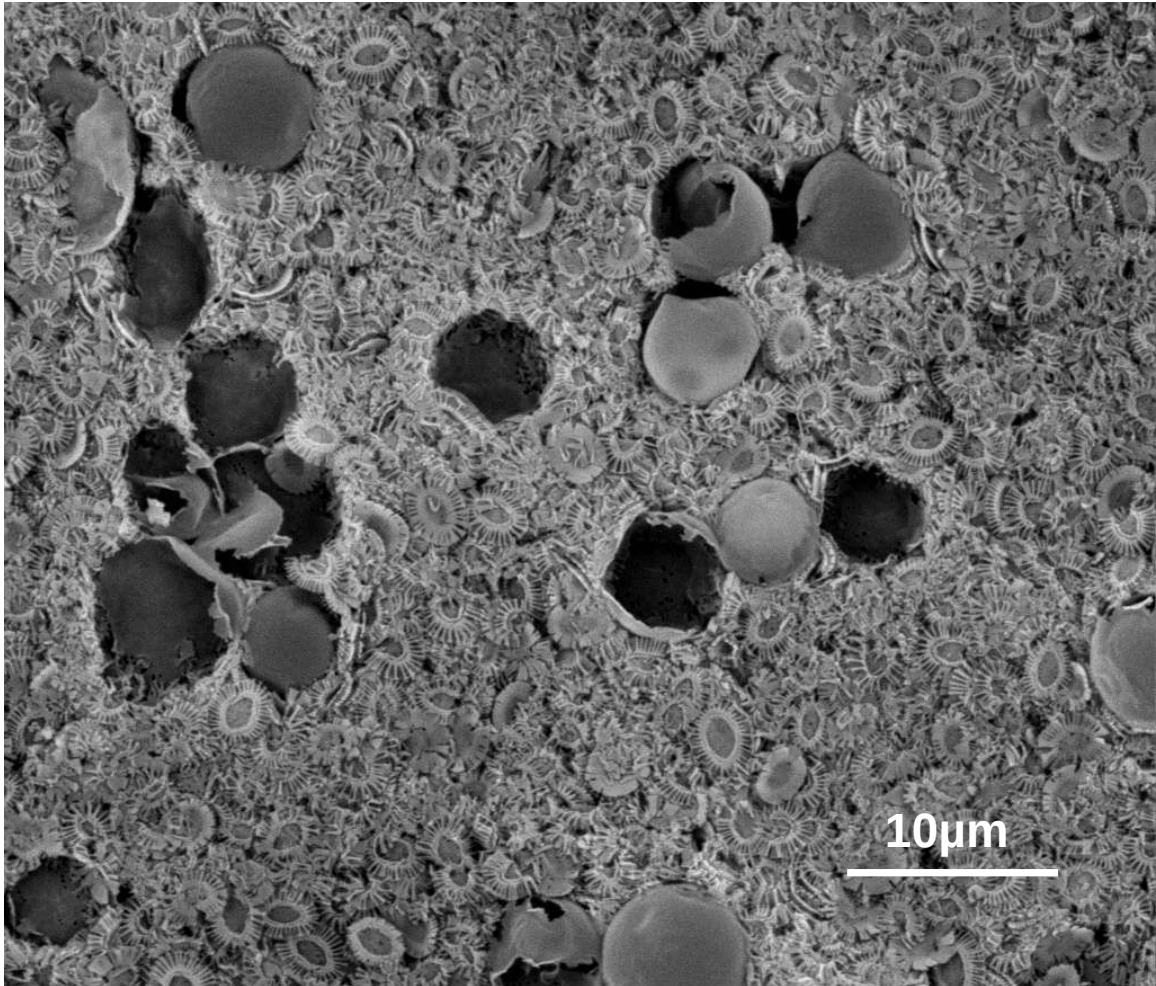


Figure 24. SEM micrograph of a cell suspension of *E. huxleyi* that was not treated with detergent to digest the organic component. Note the presence of many naked cells. It is unclear whether these cells lacked coccoliths in culture or they were mechanically removed during centrifugation.