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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 or bioreactors to abate CO₂ emissions from power plants.

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Introduction

The major 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 the biological, chemical, and physical conditions needed to optimize the native ability of coccolithophorid algae to sequester CO_2 in the form of CaCO_3 . This project will identify the parameters necessary to produce coccolithophorid blooms and the factors required to maximize calcification rates. 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 initial task of the research is to identify the species, cell strain and the specific growth conditions (e.g., temperature, light intensity, nutrient concentrations) that maximize population growth rates and rates of calcification. The second task of the project involves quantification of the amount of atmospheric CO_2 sequestered as CaCO_3 under conditions that maximize cell growth rates and calcification rates. During the first year of the project, we completed most of Task 1 and started work that is central to Task 2 and prerequisite for Tasks 3 and 4. In the second year of the project, we have completed Task 1 and made substantial progress in completing Task 2. In addition, we did preliminary work regarding Task 4.

Here we review the progress during the last year with emphasis on the last quarter's work, from June 30, 2003 through September 30, 2003.

Experimental

During the last year, we focused our experimental work on the coccolithophorid *Emiliana huxleyi*, cell strain CCMP 371 which we previously identified as the best candidate among available coccolithophorid cell strains for CO_2 sequestration. When we grew cell strain CCMP 371, in K media (Keller et al., 1987) and F/50 media (Guillard, 1975), we found that there was no difference in the growth rates, but the maximum cell density attained was somewhat higher (approximately 0.5 order of magnitude) in the K media than in the F/50. Because F/50 media is more economical to prepare than K media, however, we chose to first investigate F/50 media for potential use in coccolithophorid cultivation. The major differences in the compositions of F/50 and K media are listed in Table 1.

Table 1. Major differences in the compositions of F/50 media and K media.		
	F/50 Media	K Media
Nitrogen	883 μM Nitrate	883 μM Nitrate & 50 μM ammonia
Phosphate	36.3 μM Inorganic phosphate	10 μM Organic phosphate
EDTA	11.7 μM	111 μM
Selenium	None	10 μM

In the last year we advanced three different areas of the project: 1) determination of dissolved inorganic carbon (DIC) and total alkalinity (TA) in laboratory-made seawater and different growth media; 2) closed vessel experiments to investigate the impacts of coccolithophorid growth under different nitrogen conditions on the CO₂ system parameters; and 3) batch culture experiments to determine nutrient conditions that maximize cell growth and calcification. The experimental methods are briefly described below.

Dissolved Inorganic Carbon (DIC) and Total Alkalinity (TA) in Seawater

We developed a computer-controlled data acquisition program for the analyses of total alkalinity (TA) in seawater samples. This alkalinity titration program enables us to measure the TA of subsamples taken from the closed vessel experiments and other seawater samples. We can use the TA and the coulometrically-determined dissolved inorganic carbon (DIC) content of samples to calculate the other CO₂ system parameters. Measurements are checked by routine analyses of certified standards prepared by Dr. A. G. Dickson at the University of California San Diego Scripps Institution of Oceanography.

Closed Vessel Experiments

We conducted several closed-vessel experiments to investigate the impacts of coccolithophorid growth under different nitrogen conditions (i.e., source of nitrogen and total nitrogen concentration) on the CO₂ system parameters. In each experiment, we inoculated 7 liters of media with a small volume of *Emiliania huxleyi* cell strain CCMP 371. The culture was stirred continuously to keep cells uniformly suspended. The experimental vessel was placed in an environmental chamber maintained at 18.5°C and a light/dark cycle of 16 hours light/8 hours dark.

Throughout each experiment, 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 the following measurements:

- Cell density quantified by direct counts;
- Total alkalinity (TA) determined by potentiometric titration using software we developed; and
- Total dissolved inorganic carbon (DIC) determined by coulometry (Dickson and Goyet, 1995).

Subsamples for these measurements were taken without introducing additional atmospheric CO₂ into the experimental vessel. Each subsample volume removed for analyses was replaced with argon from a mylar reservoir.

We conducted three closed-vessel experiments. In the first experiment, we grew *Emiliania huxleyi* cell strain CCMP 371 in unmodified F/50 media and used this initial experiment to test our equipment, software, and analytic protocols. The second and third experiments were conducted to investigate the effects of nutrient concentration on coccolithophorid growth and the concomitant perturbation to the CO₂ system. We hypothesized that coccolithophorid calcification would increase under conditions of

reduced nitrogen. To test this hypothesis, we grew coccolithophorids in media with only half as much nitrogen as F/50 media normally contains. In experiment 2, this decreased amount of nitrogen was available in two sources — nitrate and ammonia. In experiment 3, nitrate was the sole source of nitrogen. The conditions of these three experiments are listed in Table 2.

Table 2. 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

In addition to the standard suite of measurements, in experiment 3, we quantified the particulate calcium in subsamples taken at various times during the experiment. Subsamples were filtered onto 0.4 μm polycarbonate filters, rinsed with buffered distilled water, and analyzed for calcium concentration using flame atomic absorption spectrometry (Varian AA, model FS220).

Batch Culture Experiments

a. Low Salinity

As part of our series of experiments investigating coccolithophorid cell growth as a function of selenium enrichment, we grew *Emiliana huxleyi* cell strains CCMP 371 and 374 in F/50 media at two different salinities, 20 ppt and 31 ppt, and with and without selenium. This experiment was the first one we conducted to investigate the response of coccolithophorid calcification to low salinity (20 ppt) media. Cell densities were determined by direct counts.

b. Nitrate vs. Nitrate plus Ammonia

We 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 which contains nitrogen only as nitrate at a concentration of 883 μM . The second treatment consisted of modified F/50 media in which we added two sources of nitrogen — 833 μM nitrate and 50 μM ammonia. Thus, the total nitrogen in each treatment was the same, however, the second treatment had less nitrate and added a second source of nitrogen in the form of ammonia. *Emiliana 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 particulate calcium was measured as previously described.

c. 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. Cell densities were determined by direct counts and particulate calcium was determined by flame atomic absorption as previously described.

Results and Discussion

Dissolved Inorganic Carbon (DIC) and Total Alkalinity (TA) in Seawater

The successful design and implementation of our software program and experimental protocols for total alkalinity (TA) and dissolved inorganic carbon (DIC) resulted in the unexpected finding that the composition of our laboratory-made seawater contained levels of TA and DIC that were substantially lower than what is typically found in natural seawater. Because our formula for artificial seawater is widely used to culture marine algae, this was a surprising discovery. We adjusted the composition to produce a laboratory-made seawater with TA and DIC concentrations of 2100 $\mu\text{mol kg}^{-1}$ and 1600 $\mu\text{mol kg}^{-1}$, respectively. We are currently working to increase the DIC within the range of 1800 to 2000 $\mu\text{mol kg}^{-1}$. DIC in laboratory-made seawater is lost due to outgassing when the seawater is autoclaved.

Closed Vessel Experiments

In the first experiment, we grew *Emiliana huxleyi* cell strain CCMP 371 in unmodified F/50 media and ran the experiment for 26 days. This initial experiment was a successful test of our equipment, software, and experimental methods, as described in Quarterly Reports 7 and 8, and led to important changes in the composition of our laboratory-made seawater (see above). Cell density, alkalinity, and DIC are plotted over the first 9 days of the experiment in Figure 1A.

In the second closed vessel experiment, we tested growth of *Emiliana huxleyi* cell strain CCMP 371 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 after 10 days still had not reached the logarithmic phase of population growth. Moreover, microscopic examination of the cells indicated an abnormal cell shape, with only weak calcification. We terminated the experiment at 10 days. We hypothesized that ammonia toxicity caused the abnormal cell shape and slow cell growth, however, subsequent experiments with batch cultures (see below) do not support this interpretation.

Thus, we do not know the cause of these results and may repeat the experiment in the future.

The third closed vessel experiment investigated growth of *Emiliania huxleyi* cell strain CCMP 371 at a reduced nitrogen level of 450 μM nitrate (approximately half that of F/50), with nitrate the sole source of nitrogen. Temporal changes in cell density, alkalinity and dissolved inorganic carbon (DIC) are graphed in Figure 1B. Comparison of the two graphs in Figure 1 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 (Fig. 1B) than when the nitrate concentration was twice as high (Fig. 1A). 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. 1B) may have had a stimulatory effect on cell growth. Although the initial content of DIC was lower in the first experiment (Fig. 1A), after 8 days, DIC was depleted to a lower value in the experiment with half the normal nitrate concentration (Fig. 1B).

The change in particulate calcium over the course of experiment 3 is shown in Figure 2. Particulate calcium ($\mu\text{g ml}^{-1}$) increased in concert with the cell density, however, when the cell density started to level off at day 6, the particulate calcium content ($\mu\text{g Ca ml}^{-1}$) continued to increase until day 8, at which time it leveled off to a concentration of 25 $\mu\text{g ml}^{-1}$. Moreover, marked changes in the ratio of particulate calcium to cell density occurred during the 8-day experiment (Fig. 3). The day after cells were inoculated, the ratio of particulate calcium to cell density was the greatest —81 pg Ca/cell. This ratio drastically decreased to 2.6 pg Ca/cell when the cell density reached $10^6 \text{ cells ml}^{-1}$ and remained <5 for the duration of the experiment. Because the ratio of particulate calcium to cell density provides an index of calcification, the data suggest that the calcification rates were highest during lag phase to early logarithmic phase of the population growth. When cells reached mid to late log phase and then stationary phase of population growth, calcification decreased.

The seemingly paradoxical circumstance wherein the total particulate calcium content of the media ($\mu\text{g Ca ml}^{-1}$) continued to increase while the ratio of particulate calcium to cell density (pg Ca/cell) decreased can be explained by the fact that growing cells discard coccoliths into the ambient water and these suspended, detached coccoliths persist throughout the experiment. These detached coccoliths are measured in the total particulate calcium analyses along with any newly precipitated coccoliths that are still attached to the cell as a surface covering.

The high rate of calcification during the lag to early log phase of the population growth compared to the marked decrease in calcification during later logarithmic and stationary growth phases is apparent in photomicrographs of cells (Fig. 4). Figure 4A shows a completely calcified coccolithophore from a lag phase to early log phase culture of *Emiliania huxleyi* cell strain CCMP 371. The photograph illustrates a coccosphere with 1-2 layers of coccoliths that completely surround the cell. In contrast, Figure 4B shows

cells in stationary phase that are primarily naked or almost entirely devoid of coccoliths, indicating that calcification is much reduced at this stage. A large aggregate of discarded coccoliths (30-40 μm in size) is also shown in Figure 4B. Such clumps of calcareous coccoliths are typical at this stage of population growth.

We hypothesize that the decrease in calcification when cell densities reach $\geq 10^6$ cells ml^{-1} is due to nutrient, DIC, or alkalinity limitation, rather than some other factor such as shading or toxic cell exudates that might inhibit calcification at high cell densities. Thus, if coccolithophores are grown in a bioreactor using a chemostat approach, wherein limitation by nutrients, DIC, and alkalinity would not occur, we predict that a high rate of calcification would persist indefinitely.

Batch Culture Experiments

a. Low 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 (see Fig. 1 of Quarterly Report #6).

An unexpected result of the low salinity experiments was the appearance of inordinately heavily calcified cells. While only 25 to 50% of the total number of cells were calcified, those cells which were calcified had multiple layers of coccoliths. In many cases, the extra layers of coccoliths resulted in a coccosphere (cell plus attached coccoliths) diameter that was twice the diameter of the cell itself (see Fig. 2 of Quarterly Report #6). We have tried to repeat this experiment but, thus far, have not been able to culture the extraordinarily calcified cells that resulted previously. We will continue our attempts to establish a monoculture of such heavily calcified cells, however.

b. Nitrate vs. Nitrate plus Ammonia

To investigate the effects of nitrogen source on cell growth and calcification, we grew *Emiliania huxleyi* CCMP cell strain 371 in batch cultures of unmodified F/50 media (which has a nitrogen content of 883 μM nitrate) and modified F/50 media with two sources of nitrogen: 833 μM nitrate and 50 μM ammonia. On day 6 and 7, the mean cell density in the unmodified F/50 media was 5.2×10^4 cells ml^{-1} and 1.4×10^5 cells ml^{-1} , respectively. The mean cell density of the modified F/50 media, which contained both nitrate and ammonia, was 2.5×10^4 cells ml^{-1} and 6.2×10^4 cells ml^{-1} on days 6 and 7, respectively. Thus, the cell densities were somewhat higher in the unmodified F/50 media containing nitrate only, but the difference in cell numbers between the two treatments was not significant on either day 6 or day 7 (t test, $P > 0.05$).

In contrast, the ratio of particulate calcium to cell density was significantly different between nitrogen treatments on both days (t test; $P < 0.05$). As illustrated in Figure 5, on day 6, the nitrate only cultures had an average ratio of particulate calcium to cell density of 44.2 pg Ca/cell, whereas the nitrate plus ammonia cultures had an average ratio of particulate calcium to cell density of 31.2 pg Ca/cell. On day 7, the nitrate only cultures

had an average ratio of particulate calcium to cell density of 39.9 pg Ca/cell and the nitrate plus ammonia cultures had a mean ratio of only 23.7 pg Ca/cell. These data suggest that calcification is greater when nitrogen is supplied solely as nitrate, as opposed to nitrate plus ammonia. Moreover, the decrease in the ratio of particulate calcium to cell density that was observed between days 6 and 7, when cultures were at early to mid log phase, is consistent with our results from the closed vessel experiment #3. This effect appears to be more pronounced in the nitrate plus ammonia cultures than in nitrate only treatment, but additional data would be needed to test this hypothesis.

c. Different Nitrate Loadings

In this experiment, we tested the effects of different nitrate concentrations on cell growth rates and calcification. We grew *Emiliana huxleyi* CCMP cell strain 371 in F/50 media prepared with 4 different nitrate concentrations: 0.88mM (=unmodified F/50 media), 1.5 mM, 2.0mM, and 2.5 mM. Cell growth rates did not significantly differ between nitrate loadings, as seen by the slopes of the growth curves in Figure 6. The shift in growth curves of the 2.0 and 2.5 mM nitrate cultures from those of the 0.88 mM and 1.5 mM reflects differences in the cell density of the initial inoculum, rather than differences in growth rates between treatments. We do not currently have a complete data set with which to ascertain any differences in the ratio of particulate calcium to cell density as a function of nitrate concentration, but plan to investigate this in the future.

Conclusion and Overview of Future Work

Using *Emiliana huxleyi* strain CCMP 371 (which we identified as the best candidate for CO₂ removal technology), we conducted closed vessel experiments and batch culture experiments to investigate the effects of nutrients on cell growth rates and calcification. Our results demonstrate that the total particulate calcium in the media ($\mu\text{g Ca ml}^{-1}$) increases with the population growth of cells, but the total particulate calcium concentration level off *after* cells have reached stationary phase of population growth. Our results also reveal that the ratio of particulate calcium to cell density (pg Ca/cell) is highest when the population is at the lag phase or early logarithmic phase of growth. Our results suggest that growing coccolithophorids in a bioreactor using a chemostat approach, in which nutrients, DIC, and alkalinity are continuously supplied, would yield a consistently high rate of calcification.

Additional experimental results indicate that coccolithophorid calcification was greater when nitrogen was supplied as nitrate only, than when nitrogen was supplied as nitrate plus ammonia. Increasing the nitrate concentration did not enhance the cell growth rates of coccolithophorids. In the near future, we will determine the impact of increased nitrate loadings on calcification.

Our workplan for the next year focuses on three research areas:

- Determination of optimal nitrogen level and source to maximize cell growth and calcification in *Emiliana huxleyi* cell strain CCMP 371;

- Determine if increased levels of dissolved CO₂ enhance calcification rates and empirically identify the highest concentration of dissolved CO₂ that can be used successfully in coccolithophorid CO₂ sequestration; and
- Continue investigation of coccolithophorid growth and CaCO₃ production in low salinity water.

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

Fig. 2. 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.

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

Fig. 4. Nomarski and polarized light micrographs of *Emiliana huxleyi* strain CCMP 371 at different stages of population growth. (A) Cell at lag phase to early logarithmic growth phase is completely covered by 1-2 layers of coccoliths. (B) Cells at late logarithmic growth phase to stationary phase are almost completely devoid of coccoliths. Note large aggregates of discarded calcareous coccoliths.

Fig. 5. 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 μ M nitrate and modified F/50 media (magenta bars) containing two sources of nitrogen, 833 μ M nitrate plus 50 μ M ammonia. (A) Day 6 of batch culture experiment in which cell densities were in early logarithmic growth phase. (B) Day 7 of batch culture experiment in which cell densities were in early to mid logarithmic growth phase.

Fig. 6. Growth of *Emiliana huxleyi* CCMP 371 in F/50 media with different nitrate concentrations: 0.88mM (dark blue diamonds); 1.5 mM (pink squares); 2.0mM (red triangles); and 2.5mM (light blue “x”).