^{13}C FRACTIONATION IN *EMILIANIA HUXLEYI*: STIRRED VERSUS UNSTIRRED DILUTE BATCH CULTURES

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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ABSTRACT

It has been shown that $\delta^{13}C$ of phytoplankton ($\delta^{13}C_P$) varies with $[CO_{2(aq)}]$ and rate of algal growth. It is possible that, by using $\delta^{13}C_P$ from sediments, $[CO_{2(aq)}]$ can be inferred. However, other factors can complicate the relationship between $\delta^{13}C_P$ and $[CO_{2(aq)}]$. Discrepancies in results acquired by different culturing methods (chemostat and dilute batch cultures) is an important issue in understanding carbon isotope fractionation in phytoplankton that must be resolved before paleo-CO_2 can be inferred from sediment cores. In this study, dilute batch cultures of *Emiliania huxleyi* were grown to observe possible differences in carbon isotopic fractionation (ε_P) in stirred and unstirred conditions. Three sets of experiments were performed and, in each set of cultures, stirred cultures exhibit greater fractionation than their unstirred counterparts. We postulate that phytoplankton in stirred cultures have greater ε_P because the boundary layer immediately surrounding the cells is constantly agitated and does not become enriched in ^{12}C over time as carbon dioxide is assimilated into the cell. Stirred cultures displayed ε_P values 1.73‰, 6.71‰, and 2.34‰ greater than unstirred cultures of the same sets (using mean values of ε_P when applicable). However, this does not completely account for the magnitude of differences in and trends of fractionation observed in the chemostat and dilute batch cultures. Factors that may contribute to these discrepancies are discussed.

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1. ε_P = carbon isotope fractionation in phytoplankton
2. μ = growth rate of phytoplankton
3. $[CO_{2(aq)}]$ = aqueous carbon dioxide concentration
4. CO_2 = carbon dioxide
5. $\delta^{13}C_X$ = carbon isotopic composition of specified material, X

1. INTRODUCTION

The quest to understand the breadth and depth of the consequences of human activity on the environment is a complex, ongoing study. Scientists from a wide range of disciplines struggle to characterize the many components of the Earth's dynamic system. A growing awareness of people's dependency on the well-being of the environment, as well as a consciousness of changes already affected by human activities, drives the movement to understand the environment. However, to predict future effects of anthropogenic activities on varying ecosystems and on the natural environment as a whole, we must go beyond studying the present day environment. A strong understanding of the forces within the environment requires knowledge of how it has changed and behaved over time.

The importance of one particular component in the environment is well agreed upon: the carbon cycle (e.g. Berner,1999; Pagani *et al.*, 1999). Given that humans are very dependent on burning fossil fuels for energy, it is of interest how anthropogenic additions of carbon dioxide to the atmosphere will affect radiative forcing. The uncertainties of predicting the effects of these activities on the carbon cycle can be alleviated by an improved understanding of past climate change and the associated behavior of atmospheric carbon dioxide concentration.

1.1 *Background: $\delta^{13}C$ of Phytoplankton*

Great strides in paleoclimatology have been made in recent decades. In recognition of the sensitivity displayed by many marine organisms to environmental conditions during growth and development, paleoceanographers have developed varying

methods of paleosea-surface temperature reconstruction (Brassell, 1993). For example, oxygen isotopic abundance in planktonic formainferal tests and the degree of unsaturation in alkenones are two methods that have been studied and refined (e.g. Muller, *et al*., 1998). Much work is required to reliably quantify and constrain factors involved in the relationship between biomarker and signal. However, once established, these relationships may be used to interpret the paleoclimate records embedded in sediments.

Past studies have linked the carbon isotopic composition of marine organic matter, $\delta^{13}C_{org}$, to aqueous CO_2 concentration (Arthur *et al*., 1985; Hayes *et al*., 1989; Popp *et al*., 1989, Rau *et al*., 1989). An offset in carbon isotopic composition between aqueous CO_2 and marine organic matter exists that could possibly relate ^{13}C abundances of sedimentary organic matter to the aqueous carbon dioxide concentration during the formation of that matter. Because sediment diagenesis does not appear to alter isotopic abundances in organic matter (e.g. Arthur *et al*., 1985), the sedimentary isotopic record remains intact. However, it has become apparent that $\delta^{13}C_{org}$ varies beyond the range of the $\delta^{13}C$ of dissolved inorganic carbon in ocean water (e.g., Rau *et al*., 1997). These variations have been linked to photosynthetic fractionation of ^{13}C and other possible sources of discrimination in or by the cell. Studies have shown that factors such as cell geometry, growth rate or cellular carbon demand, the ability to actively assimilate inorganic carbon, and light cause variations in carbon isotopic fractionation by marine microalgae or ε_P (Rau *et al*., 1992; Goericke *et al*., 1994; Rau *et al*., 1996; Laws *et al*., 1995 & 1997; Bidigare *et al*., 1997; Popp *et al*., 1998 & 1999; Riebesell *et al*., 2000; Rost *et al*., 2002).

Under the assumption that $CO_{2\,(aq)}$ passively diffuses through the cell membrane to support photosynthesis, a link between the $\delta^{13}C$ of phytoplankton ($\delta^{13}C_P$) and [$CO_{2\,(aq)}$] has been made with the possibility that sedimentary records of $\delta^{13}C_{org}$ may give insight to [$CO_{2\,(aq)}$] (Rau *et al*. 1997). Recent studies have shown that ^{13}C fractionation by phytoplankton is linearly related to growth rate (μ) and [$CO_{2(aq)}$] in natural conditions (Laws *et al*., 1995 & 1997; Bidigare *et al*., 1997). In addition, Popp *et al*. (1998) account for much of the variation found in ε_P by different species of phytoplankton by factoring cell geometry into the relationship. With many important strides being made in understanding ε_P, methods of relating the significant offset of $\delta^{13}C$ of phytoplankton to ambient conditions of [CO_2] have been proposed and are, at present, in the refinement stages. However, it is recognized that carbon isotope fractionation is complex and many factors exceed the scope of this paper. Thus, only a few variables of particular interest are discussed.

A mathematical model introduced by Rau *et al*. (1996) evaluates the effects of varying growth rate, cell radius, temperature, ambient [$CO_{2\,(aq)}$], cell wall permeability and enzymatic fractionation. The model shows that with increasing cell radius, ε_p decreases and that this effect is amplified with increasing growth rate. Naturally, ε_p mirrors the activity of the fractionation associated with the enzymes responsible for inorganic carbon fixation, ε_f, increasing or decreasing with it. ε_p increases with increasing ambient [$CO_{2(aq)}$] and cell wall permeability to CO_2. However, with increasing growth rate, fractionation and $\delta^{13}C$ are less sensitive to changes in ambient [$CO_{2(aq)}$] and cell wall permeability. With increasing growth rate and cellular carbon demand, there is a greater disequilibrium between extracellular and intracellular carbon

concentrations. Under these conditions, the importance of enzymatic fractionation decreases relative to diffusive fractionation in seawater (Rau *et al*., 1996). Lastly, as temperature increases, a slight increase in fractionation is observed, though it must be noted that this is probably a reflection of the dependence of growth rate or CO_2 solubility on temperature. The model by Rau *et al*. (1996) shows that while ^{13}C fractionation is not simple, it is not impossible to understand and hopefully constrain.

Furthermore, under certain conditions a "boundary layer" effect may contribute to variability in the isotopic composition of the cell. As CO_2 is drawn from the bulk media, concentrations decline in the immediate vicinity, or boundary layer, of the cell. This leaves more ^{13}C-enriched inorganic carbon in the boundary layer. Also, as ^{13}C is discriminated against during diffusive uptake and enzymatic fractionation, the boundary layer is further enriched in ^{13}C relative to the surrounding seawater. Goericke *et al*. (1994) state that CO_2 uptake can be diffusion limited with the CO_2 in the boundary layer enriched in ^{13}C relative to that in the bulk media. A correlation between turbulence and $\delta^{13}C$ has been seen, with low turbulence associated with high $\delta^{13}C$ (Goericke *et al*., 1994). However, this is not always the case and it may be a matter of the species of carbon utilized and whether or not active uptake of inorganic carbon is employed.

1.2 *Discrepencies Between Culturing Methods*

Besides the expected complications in constraining natural factors in ^{13}C fractionation, discrepancies exist between various culturing methods (see Laws *et al*., 2001). The applicability of these methods to the ocean environment is uncertain and direct field measurements to compare with laboratory measurements are often

problematic to obtain. Differences in $\delta^{13}C$ of the same species grown under similar conditions have been observed in experiments performed in different labs, which may be a result of varying culturing methods (Bidigare *et al.*, 1997; Riebesell *et al.*, 2000). For example, studies with similar [$CO_{2(aq)}$] and growth rates show variation in ε_p for the same algal species in chemostats and dilute batch cultures (Bidigare et al., 1997; Popp, et al., 1998; Riebesell et al., 2000). Maximum fractionation in continuous cultures (i.e. chemostats) approaches that of the consensus maximum fractionation caused by key enzymes in carbon fixation reactions, such as ribulose bisphosphate carboxylase-oxygenase (RUBISCO): ~25-27‰ (Laws *et al.*, 1995 & 1997; Bidigare *et al.*, 1997, Popp *et al.*, 1998; Rosenthal *et al.*, 2000). Much lower values are observed in dilute batch cultures: ~15‰ (Hinga *et al.*, 1994; Burkhardt *et al.*, 1999; Riebesell *et al.*, 2000). Also, there is a strong dependence of ε_p on μ/CO_2 in chemostats, while in dilute batch cultures, the dependence observed is weak.

Differences observed between culturing methods may be a product of different light cycles (continuous vs. light:dark cycles), nutrient saturation or limitation, differences in turbulence (stirring vs. occasional agitation), and phases of growth (steady state vs. exponential growth). While the debate of which culturing methods are most representative of natural systems is important, it is also necessary to understand why different methods cause variations in ^{13}C fractionation.

1.3 *This Study*

The goal of this study is to resolve the discrepencies in observations from continuous cultures (nutrient limited chemostats) and dilute batch cultures as reported by Bidigare *et al.*, 1997 and Riebesell *et al.*, 2000. While there are several differences

between the culturing methods of the two studies (see Table 1), this study examines the effects of media agitation specifically. We show that a difference in fractionation exists between stirred and unstirred cultures and speculate that this difference is influenced by a boundary layer effect that occurs in cultures that are only gently agitated 1-2 times a day. *Emiliania huxleyi* is used to best replicate studies in which differences in observations may be atrributed to culturing methods.

Table 1. Details of culturing methods as performed by Riebesell et al. (2000), Bidigare et al. (1997), and in this study.

	Riebesell *et al.*, 2000	Bidigare et al., 1997	This Work
Culturing Method	Dilute Batch Culture	Continuous Chemostat	Dilute Batch Culture
Algal Species	Emiliania huxleyi B92/11, calcifying strain	Emiliania huxleyi B92/11, calcifying strain; Emiliania huxleyi BT6, noncalcifying	Emiliania huxleyi BT6 and 55a, non-calcifying strains
Light Source	Light:Dark Cycle 16:8 hours	Continuous, Saturated	Continuous, Saturated
Nutrients	Nutrient Replete	Nitrate-Limited	Nutrient Replete
Agitation	Gently inverted 2-3 times a day	Continuously stirred by magnetic stir bar	Combination of cultures continuously stirred and inverted 2-3 times a day
Culture Density	Innoculated at <300 cells mL^{-1} Harvested at 30,000±10,000 cells mL^{-1}	Culture grown to desired density, often more than 500,000 cells mL^{-1}	Innoculated at ~300 cells mL^{-1} Harvested at 30,000±10,000 cells mL^{-1}
CO_2 system manipulations	Addition of HCl or NaOH to create varying CO_2 concentrations	Air mixture bubbled in at rates varying by desired growth rate	Air-equilibrated at manipulated and/or recorded temperatures

2. MATERIALS AND METHODS

2.1 *Algal Culturing*

The haptophyte *Emiliania huxleyi* (noncalcifying strains BT6 and 55a CCMP 1742 obtained from the Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine) was grown in dilute batch cultures under conditions of nutrient and light saturation at room temperature and also at a constant, controlled temperature. Dilute batch culturing, defined here as <30,000 cells mL^{-1}, allows several simultaneous experiments in a short time period. In one experiment, 8 cultures of BT6 were kept at room temperature, 4 of which were constantly agitated by stir bar and 4 were agitated 1-2 times a day by gentle inversion. In the other two experiments, three 4-liter polycarbonate bottles were immersed in a temperature-controlled water bath kept at 15° and 20°C. Of these, two cultures were constantly stirred and one culture was agitated 1-2 times a day. For the temperature-controlled experiments, culture temperature was logged (Onset Stow Away TidbiT temperature loggers) every two minutes for the entirety of the experiment. Temperature varied by less than 0.7°C. For all three experiments, light was supplied continuously by a bank of fluorescent lamps at an intensity of 318 μE m^{-2} s^{-1}.

Each dilute batch culture consisted of 3.8 liters of sterilized, 0.2 μm filtered seawater and nutrients according to f/2 media. Dissolved inorganic carbon (DIC) and alkalinity samples were taken before inoculation to determine initial concentrations of inorganic carbon in the media. The cultures were inoculated at 300 cells mL^{-1}. Growth rates, determined by daily change in cell density, varied from 0.35 d^{-1} to 1.23 d^{-1}. Cell density was determined using a Coulter Z1 dual threshold particle counter using a 50 μm aperature tube and a 3-8 μm threshold to optimize detection of these ~5 um diameter

cells. Cultures were sampled at a cell density of ~30,000 cells mL^{-1}, which was reached in approximately 6-7 doublings. Harvesting cells at this density had been found to have a minimal effect on seawater inorganic carbon chemistry (Goericke *et al.*, 1994).

2.2 *Determination of* ε_p

Total dissolved inorganic carbon (DIC) and the $\delta^{13}C$ of DIC ($\delta^{13}C_{DIC}$) were determined using a system modified after Kroopnick (1985). Samples for DIC (20 mL) were preserved with 200 μL of $HgCl_2$ and stored in precombusted glass crimp top vials at 4°C in the dark until analyzed. Briefly, 8.43 mL of 0.2 μm filtered seawater and approximately 3.4 mL of 30% H_3PO_4 were sparged with N_2 in a 13 mL column fitted at the base with a fritted glass disk. The sparged CO_2 from acidification of the DIC was trapped using liquid nitrogen on a multiloop trap and transferred to a vacuum distillation line where the quantity of CO_2 was determined manometrically (MKS Baratron model 122). The concentration of DIC based on the P-V calculation yielded an accuracy and precision of ~10 μM. Isotopic abundances were measured on cryogenically purified CO_2 using a Finnigan MAT 252 mass spectrometer (Santrock *et al.*, 1985). Analytical uncertainty for carbon isotopic analyses was less than 0.1‰. Carbon isotopic compositions are reported in δ-notation relative to Vienna PeeDee belemnite (VPDB).

Alkalinity samples (20 mL) were preserved with 20 μL of $HgCl_2$ and stored in the dark at 4°C in plastic scintillation vials with conical caps until analysis. Determinations of total alkalinity were made using the Gran method using computer-controlled titration. Precision and accuracy as determined by analyses of a certified seawater reference material for oceanic CO_2 measurements was less than, on average, 11 μeq kg^{-1}.

Nutrients remaining in the dilute batch culture at time of harvest were collected (25 mL), filtered (0.2 μm), and stored in an acid-washed plastic bottle and frozen until analysis. Phosphate, silicate, and nitrate concentrations were determined using the colorimetric techniques of Strickland and Parsons (1972) on a Technicon Auto Analyzer II. Minimal detectable limits for this system are 0.13 μm L^{-1} for nitrate, 0.1 μm L^{-1} for phosphates, and 0.5 μm L^{-1} for silicates.

The abundance of $CO_{2(aq)}$ was determined from concentrations of DIC, phosphate, silicate, and total alkalinity following Millero (1995). The dissociation constants for carbonic and boric acids used in this calculation were from Dickson (1990a,b) and Roy *et al*. (1993). The isotopic composition of $CO_{2(aq)}$ was determined from δ_{DIC}, the relative abundances of bicarbonate, carbonate, and CO_2 and the temperature-fractionation relationships of Deines *et al*. (1974) and Mook *et al*. (1974).

Samples of *E. huxleyi* for carbon isotopic analysis were filtered onto a precombusted (500°C for a minimum of 4 hours) glass fiber filter (Whatman GF/F) from 250-300 mL of water. The filters were wrapped in precombusted aluminum foil, placed immediately in liquid nitrogen, and stored frozen until analysis. Particulate organic carbon (POC) contents and isotopic analyses were determined using a Carla Erba elemental analyzer coupled with a Finnigan Delta Plus mass spectrometer. Analytic uncertainty was less than 0.2‰.

Carbon-isotopic fractionation associated with photosynthesis (ε_p) is determined using the carbon isotopic compositions of $CO_{2(aq)}$ and POC (δ_{CO2} and δ_{POC}, respectively). The equation for ε_p by Freeman and Hayes (1992) is as follows:

$$\varepsilon_P \equiv \left(\frac{\delta_{\mathrm{CO2(aq)}} + 1000}{\delta_{\mathrm{POC}} + 1000} - 1 \right) 1000 .$$

3. RESULTS AND DISCUSSION

3.1 *Stirred vs. Unstirred Cultures in This Study*

The results of this study are summarized in Table 2. In the first set of cultures, eight dilute batch cultures with *Emiliania huxleyi* BT6 were grown at ambient room temperature. Four of these cultures were stirred continuously with a stir bar and four were gently agitated 2-3 times a day. In this experiment, the temperatures ranged from 22.5º - 24.4º C and the growth rates spanned 0.79 – 1.23 day^{-1}. $[CO_{2(aq)}]$ ranged from 3.90 – 9.53 μmol L^{-1}. ε_P ranged from 18.55‰ – 19.74‰ in the stirred cultures and 17.02‰ – 17.83‰ in the unstirred.

In the second set of cultures in this study, 1 stirred and 1 unstirred culture of *Emiliania huxleyi* 55a were grown at a controlled temperature of 15.32º C. The stirred culture had a growth rate of 0.23 day^{-1} and the unstirred had a growth rate of 0.21 day^{-1}. The stirred culture had a $[CO_{2(aq)}]$ of 4.72 μmol L^{-1} and ε_P was 26.46‰. The unstirred culture had 4.20 μmol L^{-1} of aqueous carbon dioxide and ε_P was 19.75 ‰.

The third set, also with strain 55a, was conducted at 19.49º C. The stirred cultures had 5.43 and 5.74 μmol L^{-1} of $[CO_{2(aq)}]$ while the unstirred had 5.33 μmol L^{-1}. The growth rates of the two stirred cultures were 0.44 and 0.47 day^{-1} and the unstirred culture grew at a rate of 0.66 day^{-1}. The stirred cultures exhibited fractionations of 22.35‰ and 21.51‰ while the unstirred had a lower ε_P of 19.59‰. Growth rates for each set of experiments are shown in figure 1. High R^2 values ranging from 0.86 – 0.99 indicate high confidence in the calculated growth rates as well as the linearity and fit of data in the calculation.

It is apparent from the three experiments performed that ^{13}C fractionation in

Table 2. Results of Emiliania huxleyi dilute batch culture studies. Also included are mean values of experiments with replicate cultures. Data not indicated were not available.

	Temp	$CO_{2(aq)}$	DIC	μ	μ/CO_2	ε_P	$\delta^{13}C_{DIC}$	$\delta^{13}C_{CO2}$	$\delta^{13}C_P$	C cell^{-1}	C/N
Experiment	ºC	(μmol L^{-1})	(mmol kg^{-1})	(d^{-1})	(L μmol $^{-1}$ d^{-1})	‰				(pg cell^{-1})	
Set 1: *Emiliania huxleyi* BT6											
Stirred 1	22.9	3.90	1.674	1.05	0.27	19.17	1.050	-7.43	-26.10	0.82	6.03
Stirred 2	23.0	4.41	1.709	1.23	0.28	19.74	0.590	-7.89	-27.10	1.15	5.96
Stirred 3	23.5	5.41	1.745	0.79	0.15	18.55	0.890	-7.59	-25.66	1.23	7.14
Stirred 4	24.4	4.82	1.662	0.81	0.17	19.12	0.933	-7.46	-26.08	1.47	6.83
Stirred (Mean of 4)	23.5	4.64	1.698	0.97	0.22	19.15	0.866	-7.59	-26.24	1.17	6.49
Unstirred 1	22.6	4.31	1.633	0.85	0.20	17.83	0.839	-7.69	-25.07	0.78	4.47
Unstirred 2	22.5	4.10	1.688	0.98	0.24	17.60	0.715	-7.80	-24.96	0.97	5.05
Unstirred 3	23.5	7.18	1.803	1.00	0.14	17.02	0.518	-7.99	-24.59	1.03	5.04
Unstirred 4	22.8	9.53	1.861	0.90	0.09	17.22	0.601	-8.00	-24.79	1.48	4.82
Unsitrred (mean of 4)	22.9	6.28	1.746	0.93	0.17	17.42	0.668	-7.87	-24.85	1.07	4.85
Set 2: *Emiliania huxleyi* 55a, 15ºC											
Stirred	15.32	4.72	1.773	0.23	0.05	26.46	0.410	-8.75	-34.30	5.25	13.71
Unstirred	15.32	4.20	1.732	0.21	0.05	19.75	0.505	-8.63	-27.83	4.11	7.98
Set 3: *Emiliania huxleyi* 55a, 20ºC											
Stirred 1	19.49	5.43	1.786	0.44	0.08	22.35	0.338	-8.47	-30.15	9.01	
Stirred 2	19.49	5.74	1.807	0.47	0.08	21.51	0.405	-8.41	-29.29	3.68	
Stirred (mean of 2)	19.49	5.59	1.797	0.46	0.08	21.93	0.372	-8.44	-29.72	6.34	
Unstirred 1	19.49	5.33	1.787	0.66	0.12	19.59	0.435	-8.37	-27.43	3.54	

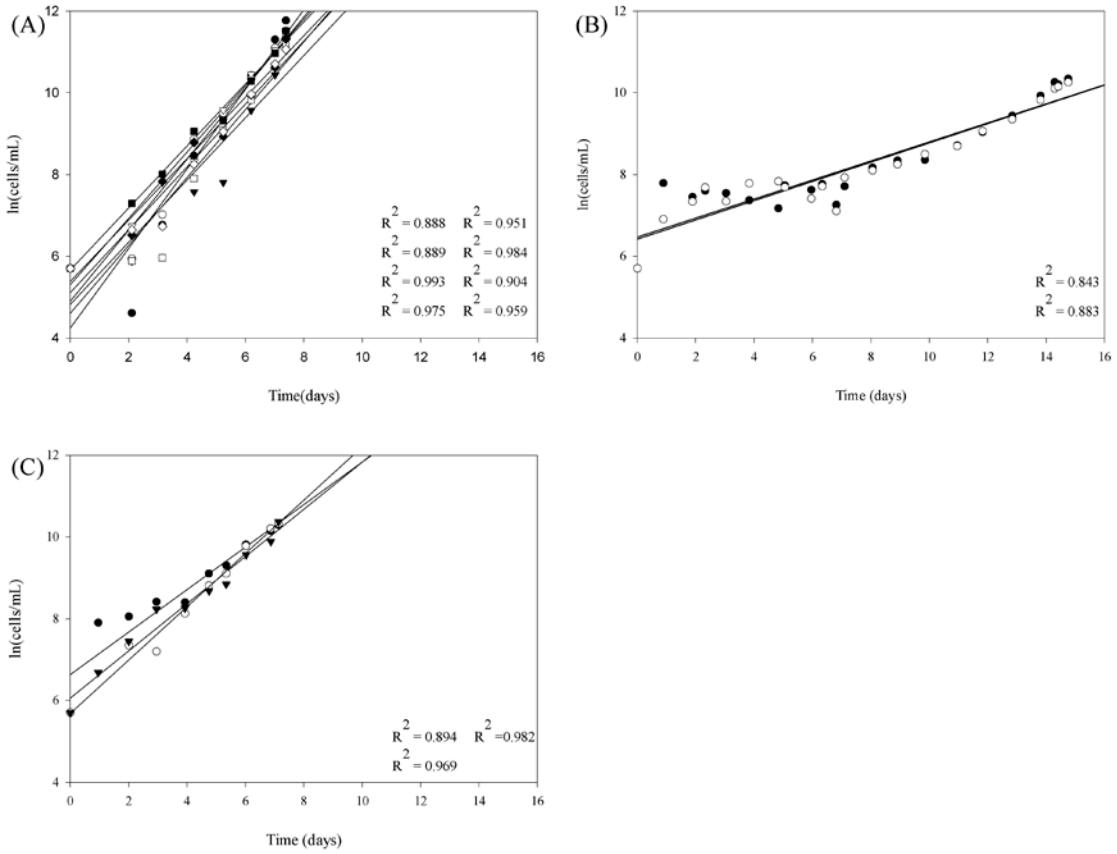

Figure 1. Plots of natural log of (cells/mL) versus time (days) of experiment sets 1-3 (a-c respectively). Growth rate is calculated by the slope of the linear regressions. R^2 values are shown on the bottom right of each plot with stirred cultures on the left and unstirred cultures on the right in descending order. (A) Growth rates for Set 1 with *E. huxleyi* BT6. The stirred cultures 1-4 are represented by filled-in circles, upside down triangles, squares, and diamonds respectively. Unstirred cultures 1-4 are depicted by open circles, upside down triangles, squares, and diamonds. (B) Growth rates for Set 2 with *E. huxleyi* 55a grown at 15ºC. The stirred culture is represented by a filled in circle, the unstirred with an open circle. (C) Growth rates for Set 3 with *E. huxleyi* 55a grown at 20ºC. Stirred cultures 1 and 2 are represented by filled in circles and upside down triangles; the unstirred culture is represented by an open circle.

stirred cultures is generally higher than that in unstirred cultures. This can be seen clearly in a plot of ε_P vs μ/CO_2 (Figure 2). Although culture conditions aren't ideally matched, stirred and unstirred cultures within each experiment are still well paired. The mean values of μ/CO_2 for stirred and unstirred cultures of the three sets were 0.22 and 0.17,

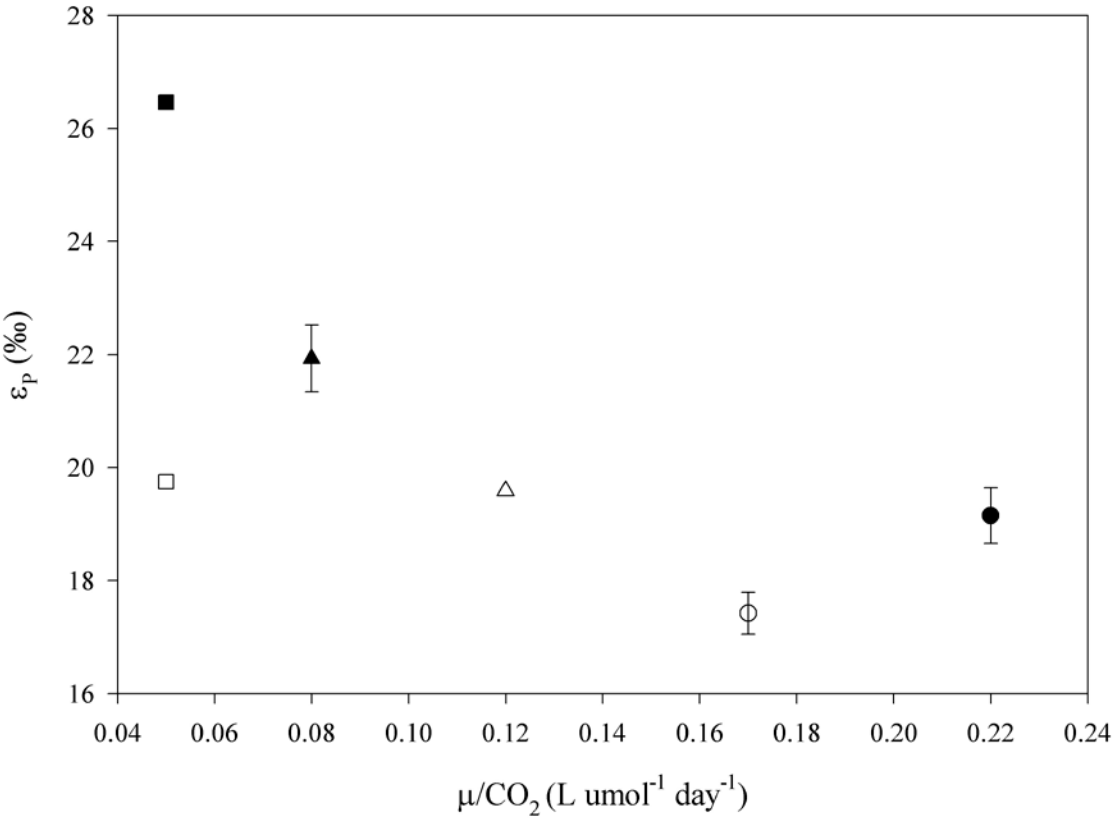

Figure 2. ^{13}C fractionation as a function of growth rate and the $[CO_{2(aq)}]$ in *E. huxleyi* for 3 sets of experiments comparing stirred and unstirred cultures. Filled in symbols represent stirred cultures, while open symbols represent unstirred cultures. Sets 1 (BT6), 2 (55a at 15ºC), and 3 (55a at 20ºC) are denoted by circles, squares, and triangles, respectively.

0.05 and 0.05, and 0.08 and 0.12 L μmol^{-1} day^{-1}. The set of cultures in which μ/CO_2 paired best, set 2 with 55a run at 15ºC, shows the most defined difference in ε_P. While μ/CO_2 values for this experiment in stirred and unstirred cultures are very close, ε_P is 6.91‰ greater in the stirred culture. This is in agreement with our hypothesis that ^{13}C fractionation will be greater in cultures that are continuously agitated and never develop a well-defined ^{13}C-enriched boundary layer.

3.2 *Comparing the Three Studies*

The three studies differ in several ways. First, the magnitude of fractionation varies between each study. Fractionation by the batch cultures of this study range from 17.42‰ – 26.46‰, which is much closer to values obtained in the chemostat (17.2‰ – 24.9‰; Bidigare *et al*., 1997). The range of ε_P displayed by the dilute batch cultures in the study by Riebesell *et al*. is far lower: 7.4‰ – 13.8‰.

Secondly, when examining the relationship between ε_P and μ/CO_2 for all three studies, it is apparent that the dilute batch cultures of this study are much more similar to

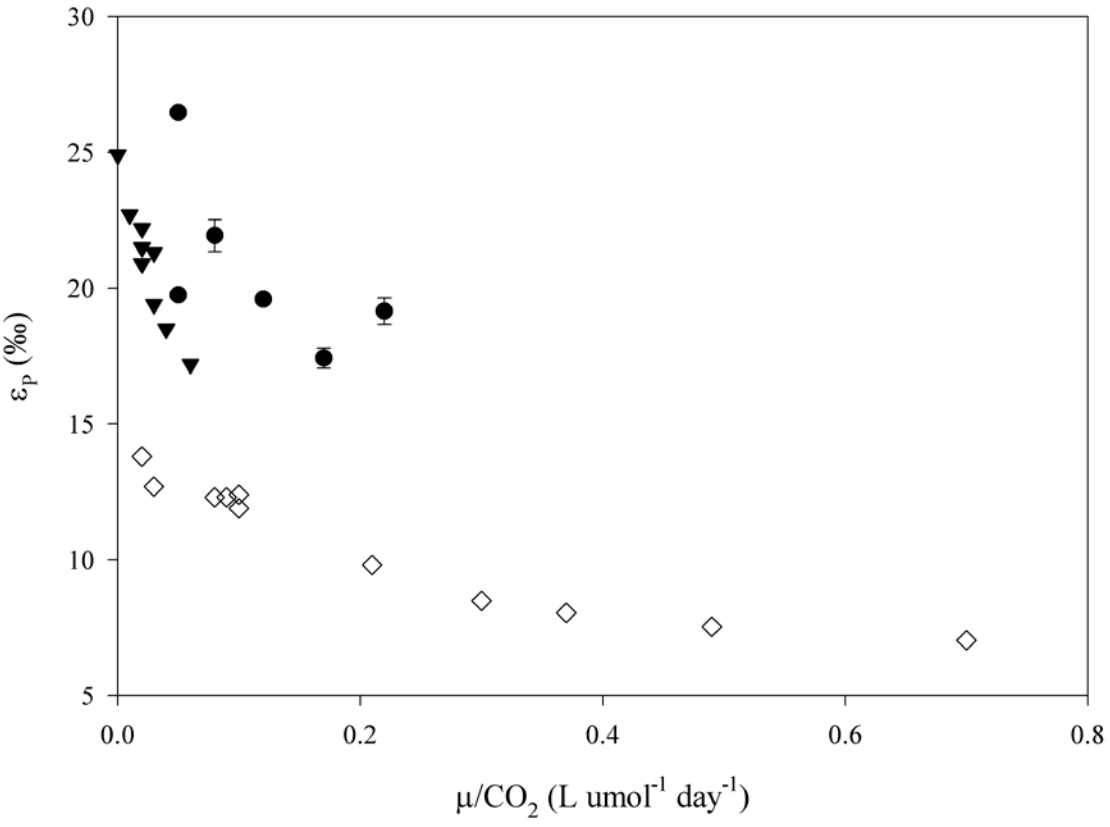

Figure 3. Comparison of ε_P vs. μ/CO_2. Studies are by Bidigare *et al*. (1997) (as denoted by filled upside-down triangles), Riebesell *et al*. (2000) (open diamonds), and this study (filled circles).

the chemostat work (Figure 3). Also, at comparable μ/CO_2 values, ε_P in the chemostat work is ~5‰ - 9‰ higher than in the dilute batch cultures by Riebesell *et al*. Dilute batch cultures from this study show fractionation 2‰ - 9‰ greater than even the chemostats, apparently regardless of the presence or absence of stirring. Both the chemostat work and this study show a linear, negative trend in the relationship between ε_P and μ/CO_2. This is very dissimilar to the trend observed in results from dilute batch cultures in the Riebesell study. These cultures show a non-linear trend that flattens out as μ/CO_2 increases. However, in considering the apparent trends of each study, it must be noted that the range of μ/CO_2 from this study and the chemostat study do not extend as far as the dilute batch cultures by Riebesell *et al*. Therefore, it cannot be stated with certainty how the trends seen in the chemostats and in this study may evolve over a larger range.

Although the belief that stirred cultures will exhibit greater fractionation has been enforced by results from the stirred and unstirred dilute batch cultures, the trends of these results are quite contrary to preliminary expectations. If the presence of agitation truly resolves the discrepancy between results from Bidigare's and Riebesell's studies, the stirred cultures should appear to be very similar to Bidigare's stirred chemostats and the unstirred cultures from this study should be similar to that of dilute batch culture work by Riebesell *et al*. (2000). As expected, the stirred dilute batch cultures show similar ε_P to the stirred chemostats. While the unstirred cultures do exhibit less fractionation than the stirred, the cultures don't exhibit the same trend or magnitude of fractionation as Riebesell's unstirred cultures. In addition, ε_P values and trends of this study's unstirred dilute cultures are much more similar to those of stirred chemostats. This is contrary to

the initial belief that the dilute batch cultures performed in this study would mimic the results of the study with the same stirring regime. It is clear that factors in addition to culture method can affect the carbon isotopic fractionation of *E. huxleyi*.

3.3 *Exploring Possiblities Beyond Stirring*

The results of the stirred and unstirred dilute batch cultures infer that something besides stirring is contributing to the discrepancy between chemostat and unstirred dilute batch culture results. Comparing elements in culturing methods, this time between the three studies, may provide some insight. One important difference between culturing methods is nutrient availability. However this does not appear to have a differentiating effect. The cultures of this study are grown under nutrient saturation, which is identical to Riebesell's cultures, though the results vary greatly. Instead, observed fractionation and behavior is more like the nutrient limited chemostats, indicating that nutrient availability is not causing the discrepancy.

Another factor is utilization of different strains of *Emiliania huxleyi*. A calcifying strain of *E. huxleyi* was used in the unstirred dilute batch cultures (Riebesell *et al*., 2000) while a non-calcifying strain was used in this study. However, in prior chemostat work, very little difference in ε_P was observed between calcifying and non-calcifying strains (Bidigare *et al*., 1997). Also, two strains are used in this study and do not display the striking difference in fractionation seen between the Bidigare *et al*. and Riebesell *et al*. work.

Possibly related to the use of different strains is the apparent difference in cellular carbon. Cellular carbon content for each experiment varies greatly (Table 3). The dilute

Table 3. Carbon to nitrogen ratios and cellular carbon contents of dilute batch cultures by Riebesell *et al*. (2000), this study, and chemostats by Bidigare *et al*. (1997). C:N data for Set 3 of this study and for the chemostats are not available.

Experiment	C:N (molar)	C Cell-1 (pg cell-1)
Riebesell *et al*., 2000		
1	7.5	5.3
1	7.4	6.0
1	8.2	9.2
1	8.4	10.2
1	8.2	9.0
1	9.4	10.4
1	8.6	9.4
2	8.0	11.4
2	7.5	10.6
2	7.5	10.0
2	7.6	11.7
This Study		
Set 1 stirred 1	6.0	0.8
Set 1 stirred 2	6.0	1.2
Set 1 stirred 3	7.1	1.2
Set 1 sitrred 4	6.8	1.5
Set 1 unstirred 1	4.5	0.8
Set 1 unstirred 2	5.1	1.0
Set 1 unstirred 3	5.0	1.0
Set 1 unstirred 4	4.8	1.5
Set 2 stirred	13.7	5.3
Set 2 unstirred	8.0	4.1
Set 3 stirred 1	-	9.0
Set 3 stirred 2	-	3.7
Set 3 unstirred	-	3.5
Bidigare *et al*., 1997		
an average, as stated in Popp et al., 1998	-	8.3

batch cultures of Riebesell have 5.3 – 11.7 pg of carbon per cell. This is much larger than cells in dilute batch cultures performed in this study, which have 0.8 – 9.0 pg of carbon per cell. The chemostats (Bidigare *et al*., 1997) have an average of 8.3 pg of carbon per cell, which is much more similar to Riebesell's batch cultures, but very different from that of the dilute batch cultures of this study. Since the fractionation displayed in the cultures of this study is more similar to that in the chemostats, variations in cellular carbon cannot be linked to the discrepancies in results between studies.

However, such drastic variations in cellular carbon prove to be another factor that is not kept constant between studies and methods, and thus its effects remain unknown.

Light cycles are another factor that may cause differences in results. The stirred and unstirred dilute batch cultures and the chemostats were performed under light saturation and continuous light. The dilute batch cultures by Riebesell *et al*., however, were grown under a 16:8 light:dark cycle. A dramatic difference in ε_P between cultures grown under continuous and 16:8 light:dark cycles was observed by Rost et al. (2002). Continuous light cultures exhibited ε_P 6-8‰ greater than cultures with a 16:8 cycle. This was also observed by Burkhardt *et al*. (1999b), though difference in ε_P was less (~6‰). This may account for the fact that dilute batch cultures performed in this study showed carbon isotope fractionation generally 10-13‰ greater than Riebesell's dilute batch cultures. However, it was also observed in chemostat cultures of *Phaeodactylum tricornutum* that there is no apparent difference in ε_P between cultures grown under continuous light or 12:12 light:dark cycles (Laws *et al*., 1995). In a plot of ε_P versus μ/CO_2, all of these chemostat cultures fall into a linear, negative trend, regardless of the light cycle.

While the differences in culturing methods must be addressed and considered, it should also be noted that the studies discussed do not have similar μ/CO_2 ranges. This is mostly due to the fact the chemostats are unable to have elevated $[CO_{2(aq)}]$ and higher growth rates without detriment to the culture itself. Also, $[CO_{2(aq)}]$ alteration was not the main focus of this study and was not performed. The ranges seen in this study and in Bidigare's chemostats do not exceed 0.22 μmol L^{-1} day^{-1}. The batch cultures by Riebesell ranged from 0.02 – 0.70 μmol L^{-1} day^{-1}. It is difficult to predict how the

chemostats and the dilute batch cultures of this study will behave at conditions of higher μ/CO_2. In Rost *et al*. (2002), a plot of ε_P versus $\mu/(CO_2 \times \text{Surface Area})$ shows that cultures by Bidigare *et al*., Riebesell *et al*., and Rost *et al*. actually tie in together, rather than disagree, as $\mu/(CO_2 \times \text{Surface Area})$ increases.

4. CONCLUSIONS

While results of this study do not completely explain the discrepancies seen between varying culturing methods, it can be concluded that stirring does have an effect on ^{13}C fractionation. The magnitude and trend of fractionation in both the stirred and unstirred cultures appears to be very similar to the results of chemostat experiments reported by Bidigare *et al*., though continuation of the trend over greater values of μ/CO_2 has not yet been studied and is uncertain. Since the unstirred dilute batch cultures performed in this study differ drastically from the unstirred dilute batch cultures of Riebesell, it cannot be concluded that agitation is the sole reason of the discrepancy between the studies by Riebesell *et al*. (2000) and Bidigare *et al*. (1997).

Many studies examine possible factors in ε_P and many have very different results. Better understanding requires studies that will examine factors one by one, rather than studies that differ in several ways. Future studies to better understand ^{13}C fractionation in phytoplankton may include the comparison of light:dark cycles in stirred and unstirred cultures. This may reveal why the unstirred cultures of this study display much greater fractionation than that of Riebesell, *et al*. Also, taking past studies and including surface area and volume to the $\varepsilon_P - \mu - [CO_{2(aq)}]$ relationship may provide new insights. A primary form of additional research would be studying stirred and unstirred dilute batch cultures over a greater range of μ/CO_2. The trend of the dilute batch cultures performed by this study only spans a range of 0.15 L μmol^{-1} day^{-1} and it is uncertain, as μ/CO_2 increases, whether they will take a negative direction like that of the chemostats or similar to Riebesell's dilute batch cultures. Additional batch culture studies that span a

wider range of [$CO_{2(aq)}$] could address this question and perhaps provide a better tie between the two culturing methods.

While understanding the mechanisms of carbon isotope fractionation in laboratory cultures is very important in the quest to reliably link the $\delta^{13}C$ of sedimentary organic matter to [$CO_{2(aq)}$], understanding the applicability of laboratory studies to natural systems is also very important. Pure lab cultures are not able to replicate species competition or grazing pressures. To understand what happens in the ocean, field data must be collected. Currently, projects are in progress that could further understanding of growth rate in the ocean and therefore be able to observe fractionation as a function of growth rate and carbon dioxide concentration in a natural setting (Laws *et al*., 2001). Perhaps once a better grasp on how ^{13}C fractionation in phytoplankton works in the ocean is obtained, it will be easier to choose and analyze laboratory data that best represent nature.

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